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ORIGINAL PAPER

Evaluation of tertiary treatment by fungi, enzymatic and photo-Fenton oxidation on the removal of phenols from a kraft pulp mill effluent: a comparative study

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Abstract Pulp and paper mills generate pollutants associated to their effluents depending upon the type of process, type of the wood materials, process technology applied, management practices, internal recirculation of the effluent for recovery, the amount of water used in the industrial process and type of secondary treatment. This study is the first that reports a simultaneous evaluation of the effects of tertiary treatments by fungi (*Rhizopus oryzae* and *Pleurotus sajor caju*), by enzyme (laccase) and by an oxidation process (photo-Fenton) on individual phenols (vanillin, guaiacol, phloroglucinol, vanillic acid and syringic acid) of a *Eucalyptus globulus* bleached kraft pulp and paper mill final effluent after secondary treatment (BKPME). The tertiary treatments were applied on BKPME samples and in BKPME samples supplemented with extra concentration of each phenol. Tertiary treatments by *Rhizopus oryzae* and photo-Fenton oxidation were able of complete removal (100%) of phenols on BKPME samples whereas *P. sajor caju* and laccase were able of 60–85% removal. On BKPME samples with added concentration of each phenol, photo-Fenton was the only treatment capable of total phenols removal (100%), which suggests a great potential for its application.

Keywords *Eucalyptus globulus* bleached kraft pulp mill · Laccase · Phenolics · Photo-Fenton · *Pleurotus sajor caju* · *Rhizopus oryzae*

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Introduction

Bleached Kraft pulp mill effluent has a dark-brown colour and contains a considerable amount of pollutants such as chromophoric and polymeric lignin derivatives or organochlorine compounds which are toxic, mutagenic, persistent, bioaccumulating and considered as an environmental problem to receiving waters (Pokhrel and Viraraghavan 2004; Ragunathan and Swaminathan 2004; Río et al. 2001). Some of these contaminants are non-biodegradable and conventional methods of wastewater treatment such as activated sludge or aerated lagoons were not efficient (Catalkaya and Kargi 2007; Prabu and Udayasoorian 2005). The environmental impacts of pulp and paper mill effluents have been tentatively reduced through the development of tertiary treatments using bacteria, fungi, enzymes or oxidation process. In this way, studies have been carried out on the removal of colour and organic compounds from pulp mill effluents using, for example, PCP-degrading bacterial strains—

Bacillus cereus and *Serratia marcescens* (Chandra et al. 2009) and white-rot fungi (Bourbonnais and Paice 1999; Munari et al. 2007; Prabu and Udayasoorian 2005) as tertiary treatment. Nagarathnamma and Bajpai (1999) also described the ability of soft-rot fungi *R. oryzae* to remove colour and adsorbable organic halide of kraft pulp effluent. The bacterial degradation of chlorolignins and chlorophenols was showed in a pulp and paper mill effluent with reduction in colour, lignin and toxicity (Chandra et al. 2009). Recent and complete reviews discussed these and others methods to the decolourization and different types of treatment of pulp and paper mill effluents (Latorre et al. 2007; Rajesh et al. 2009).

It is known that white-rot as well as soft-rot fungi have extracellular enzymes which catalyze the degradation of lignin and chlorolignins, and oxidize persistent aromatic and halogenated compounds (Ikehata et al. 2004). *Pleurotus sajor-caju* secretes laccase and veratryl alcohol oxidase as ligninolytic enzymes (Bourbonnais and Paice 1999) and some phenol-oxidases that enable the use of recalcitrant compounds as substrates (Munari et al. 2007). According to Nagarathnamma and Bajpai (1999) *R. oryzae* has laccase and manganese peroxidase as ligninolytic enzymes.

Ikehata et al. (2004) review the advances in the production of extracellular fungal peroxidases and laccases to the treatment of wastewater and discuss their applications on the removal of toxic phenolic compounds. The high efficiency, high selectivity, and environmentally benign reactions are some of the advantages of their utilization. In terms of treatment of bleached pulp mill effluents, the ligninolytic enzymes were already used (Bourbonnais et al. 1995; Camarero et al. 2007; Garg and Modi 1999). They are able to oxidize phenolic compounds creating phenoxy radicals while nonphenolic compounds are oxidized via cation radicals.

Besides the use of fungi and enzymes in treatments, the advanced oxidation processes revealed also to be very efficient on the removal of the wastewater pollutants such as pesticides, phenols and alkydic resins (Chen et al. 2007; Kušić et al. 2006; Oliveira et al. 2007). The Fenton oxidation is one of these processes and it was considered as possible method of clean and ecologically safe remedial treatment for degradation of organic compounds (Pignatello et al. 2006). None of the above mentioned treatments were studied in terms of the effects on the individual phenols from a *Eucalyptus globulus* kraft pulp mill effluent after secondary treatment (BKPME). For these reasons, three different tertiary treatment strategies have been proposed to remove phenols (vanillin, guaiacol, phloroglucinol, vanillic acid and syringic acid) present in BKPME. Tertiary treatment by two fungal species (*P. sajor caju* and *R. oryzae*), by one enzyme (laccase) and by one oxidation process (photo-Fenton) were the experimental approaches applied on BKPME samples and in BKPME samples supplemented with extra concentration of each phenol.

Materials and methods

E. globulus kraft pulp mill effluent after secondary treatment (BKPME) samples

Samples from a *E. globulus* kraft pulp mill effluent after secondary treatment (BKPME) were collected from an elemental chlorine free (ECF) kraft pulp mill. BKPME samples were collected in dark glass bottles, acidified with nitric acid (Panreac, Spain) until pH 2.0 (initial pH of 7.3) and kept at 4°C before analysis. BKPME was characterized for pH, chemical oxygen demand (COD), absorbance values (Table 1) and individual phenol quantification. Absorbance scan (200–500 nm) was obtained by spectrophotometry (GBC/Cintra10e). Before absorbance measurements, pH was adjusted to 4.0 ± 0.2, with sodium hydroxide (0.1–1 M) or hydrochloric acid (0.1–1 M), and filtered through GF/F (47 ∅mm, Whatman) glass-fibber filters and diluted 5 times with deionised water. COD was determined following standard method ASTM D 1252-88 (1994). Individual phenols were extracted from BKPME samples by solid-phase extraction and separated and quantified by gas chromatography

Table 1 Physicochemical characteristics of BKPME

Parameters	Value
pH	7.3 ± 0.2
COD (mg/l)	232 ± 12
Abs (at 325 nm)	1.09 ± 0.02
Abs (at 400 nm)	0.29 ± 0.01
Abs (at 460 nm)	0.135 ± 0.004

coupled to mass spectrometry (GC-MS). Tertiary treatment by fungi, enzyme and oxidation process were applied to BKPME.

Phenols

Analytical grade phenols (vanillin, guaiacol, phloroglucinol, vanillic acid and syringic acid) were obtained from Sigma-Aldrich, Spain.

Tertiary treatment of BKPME by fungi and enzyme

Fungi species and culture conditions applied have been described in detail elsewhere (Belém et al. 2008; Freitas et al. 2009). The tertiary treatment with the ligninolytic fungi *P. sajor caju* and *R. oryzae* were performed on batch reactors with 250 ml of BKPME sample enriched with 2 g/l of glucose at a pH 5.5. For the tertiary treatment with *R. oryzae*, some modifications were performed: BKPME was enriched with 1 g/l of glucose, 1.5 g/l of calcium chloride, 0.2 g/l of magnesium sulphate, 1.0 g/l of potassium dihydrogen phosphate and 0.15 g/l of ammonium chloride and the pH was adjust to 4.0. Batch reactors with BKPME sample (2 replicates per each phenol and fungus species as well as two batch reactors without any addition of phenol), were inoculated with the mycelia of each fungus species, individually and, covered up with hydrophobic cotton and sterilized gaze. Each four batch reactors were supplemented with 150 μM of phenol (22 mg/l of vanillin, 18 mg/l of guaiacol, 18 mg/l of phloroglucinol, 26 mg/l of vanillic acid or 30 mg/l of syringic acid) and incubated at 25°C (*P. sajor caju*) or at 30°C (*R. oryzae*), with 120 ± 10 rpm stirring, for a maximum of 7 days. This procedure was also applied on the biological treatment of an olive oil mill wastewater effluent (OOMW) by Justino et al. (2010).

For the tertiary treatment by enzyme laccase, batch reactors of BKPME (2 replicas per phenol) were prepared as described for the tertiary treatment by fungi without addition of additives and incubated at 30°C with 120 ± 10 rpm stirring for 7 days. To each batch reactor it was added 96 mg of *Trametes versicolor* laccase (E.C.1.10.3.2; Fluka Analytical, Switzerland) enzyme (22.4 U/mg) based on work published by Jaouani et al. (2005).

In order to obtain more information about the degradation of each phenol via tertiary treatment by fungi or enzyme, the assay performed with BKPME samples was totally repeated in batch reactors with sodium tartrate buffer 100 mM (pH 4.0) or sodium acetate–acetic acid buffer (pH 5.5) instead of BKPME. Samples (5 ml) of BKPME or sodium tartarate buffer or sodium acetate–acetic acid buffer with vanillin or guaiacol, were withdrawn before (0 days) and after (7 days) tertiary treatments by fungi or enzyme in order to monitor the absorbance variation. Absorbance scan (200–500 nm) was obtained by spectrophotometry (GBC/Cintra10e). Before measurements, each sample pH was adjusted to 4.0 or 5.5, with sodium hydroxide (0.1–1 M) or hydrochloric acid (0.1–1 M); samples were filtered through GF/F (47 ∅mm, Whatman) glass-fibber filters and diluted 5 times with deionised water.

All samples resulting from tertiary treatments by fungi and enzyme were evaluated for individual phenols.

Tertiary treatment of BKPME samples by oxidation process (photo-Fenton)

The photo-Fenton treatment was carried out in 500 ml glass beakers containing 250 ml of BKPME samples with 150 μM of phenol. Two replicas per each phenol, and two replicas without phenol, were submitted to photo-Fenton oxidation at room temperature (20°C), with continuous magnetic stirring (100 rpm). A volume of 1.0 ml of iron sulphate heptahydrate (0.5 M) was added at each beaker and left mixing for 10 min, allowing salt dissolution. The pH of the mixture was then adjusted to 4.0 (with sulphuric acid, 1.25 M or sodium hydroxide, 2.5 M), and 5 ml aliquots of H_2O_2 (30% v/v) were added, every 10 min, within the period of 1 h, totalizing 35 ml per beaker. During this period, a UV lamp (Vilbert-Loumart VL-6-LC, 245–365 nm) was switched on after the first H_2O_2 aliquot has been added, to start the oxidation reaction in the beakers, and switched off after 2 h. Beakers were left for 7 days, during which the oxidation reaction proceeded with carbon dioxide release and production of a precipitate. This procedure was repeated in batch reactors with sodium tartarate buffer or sodium acetate–acetic acid buffer instead of BKPME. All samples resulting from tertiary treatment by photo-Fenton were evaluated for phenols. The

described principle of oxidation process is the same that was applied in the photo-Fenton treatment on an OOMW by Justino et al. (2010), except in terms of added concentrations of reactants (iron salt and hydrogen peroxide) which are in accordance to Pereira et al. (2009) and previously optimized by our research group.

Recovery tests

Recovery tests were performed to ensure the effectiveness of the extraction procedure of phenols from the complex matrix associated to the effluents from pulp mills. Matrix spikes were prepared by adding vanillin, guaiacol, phloroglucinol, vanillic acid and syringic acid.

Solid-phase extraction (SPE)

The extraction of samples from BKPME, BKPME after tertiary treatments by fungi, enzyme or by photo-Fenton, buffers after treatments by fungi or enzyme, blanks and matrix spiked for recovery tests (500 ml) were added in turn with 5 ml of methanol (Fluka, Spain) and extracted for phenols using an extraction apparatus (manifold) described in EPA method 3535 (2007) for SPE with ENVI-18 disks (Supelco, Spain).

Before use, 5 ml of dichloromethane (Labscan, Ireland) was added to the disk, do not allowing the disk to soak without vacuum, followed with the addition of 5 ml of methanol (Labscan, Ireland) and 5 ml of ultrapure water. After these solvents elution, the sample was added directly in the apparatus reservoir, under vacuum. Elution from the disk was performed by adding twice 10 ml of ethyl acetate (Labscan, Ireland) and 10 ml of diethylic ether (Labscan, Ireland). After each solvent addition, the volume of solvent was drawn through the disk under vacuum. After extraction, the samples were dried in a rotavapor, at 45°C, until a volume of 0.5 ml. The 0.5 ml extract was kept under N_2 until dryness. Dried samples were dissolved again with 3 ml diethyl ether and transferred to a microreaction vessel. Samples were derivatized by addition of 250 µl of pyridine (Fluka, Spain), 250 µl of bis(trimethylsilyl)trifluoracetamide, BSTFA (Fluka, Spain) and 50 µl de chlorotrimethylsilan, TMSCl (Sigma-Aldrich, Germany) and then, holding the mixture at 70°C for 30 min.

GC-MS methodology

For the identification and quantification of phenols, the extracts from BKPME, BKPME after tertiary treatments by fungi, enzyme, or by photo-Fenton, buffers after treatments by fungi or enzyme, blanks and matrix spiked for recovery tests were analyzed by GC-MS. The GC-MS analysis were performed on a Shimadzu QP 5000 (Japan) equipped with a capillary column (SPB-5; 30 m × 0.32 mm; 0.25 µm film thickness; Supelco, Spain). The temperature program was: 80°C during 5 min and 80–285°C at 4°C/min. The carrier gas used was helium at a rate of 35 ml/min. The injector and detector temperatures were maintained at 290°C. The GC-MS was operated in SCAN mode for phenols identification by comparing the mass spectra with mass spectra database in Wiley 229 (1999), NIST 27 (1999) and NIST 147 (1999) libraries, by mass fragmentography and by comparison with standards. On the other hand, GC-MS was operated in SIM mode to obtain the quantification of added phenols. Calibration curves were obtained by injection of 1 µl of different standard solutions whose interval concentrations were between 0.01 and 0.13 ng/l. The concentration of each compound was determined by direct interpolation in the standard curve within their linear dynamic range. The detection limit was calculated through formula: $y = y_B + 3\ S_B$, where S_B is the standard deviation of the blank signal estimated as $s_{y/x}$, the residual standard deviation taken from the calibration line, and y_B is the blank signal estimated from the intercept taken also from the calibration line (Miller and Miller 2005).

Results

Table 2 shows the individual phenols identified and quantified in *E. globulus* bleached kraft pulp mill effluent (BKMPE) as average and standard deviation in three BKPME samples. The detection limits were in a range of 0.001 and 0.021 ng/l and the concentration values ranged from 45.6 ng/l to vanillic acid and 53.8 ng/l to vanillin. Recoveries for 3 replicate samples of BKPME can also be found in Table 2 and were almost 100%.

Table 3 shows the reduction in concentration of phenols after tertiary treatment by fungi (*R. oryzae and P. sajor caju*), enzyme (laccase) and oxidation

Table 2 Phenol concentration in BKPME samples and recovery assay for phenols in spiked samples

Phenol	BKPME[a] (ng/l)	Added (mg/l)	Recovery[a] (%)
Vanillin	53.8 ± 0.5	22.00	100.2
Guaiacol	50.6 ± 0.3	18.00	100.1
Phloroglucinol	51.2 ± 0.4	18.00	100.2
Vanillic acid	45.6 ± 0.7	26.00	100.1
Syringic acid	47.7 ± 0.2	30.00	100.4

[a] Mean of three determinations

process (photo-Fenton) of BKPME samples without and with supplement of phenols. Reduction values for BKPME supplemented with phenols oscillated between 55 and 70% after treatment with *R. oryzae*, between 19 and 65% after treatment with *P. sajor caju*, between 25 and 60% after treatment with laccase and 100% after photo-Fenton oxidation. These values are, as expected, inferior than the ones obtained for BKPME samples without the supplement of phenols since their concentration were in ng/l in comparison to mg/l in BKPME supplemented with phenols.

Figure 1 shows the absorbance values between 250 and 500 nm of BKPME samples with or without vanillin and guaiacol supplement as well of buffer also with or without phenols supplement, before and after tertiary treatment by fungi and enzyme. In this figure, is clearly observable a wavelength zone related to absorbance of phenols in BKPME without phenols or with vanillin and guaiacol being detectable absorbance reductions due to decrease of concentration of phenols in special in BKPME with vanillin treated with *R. oryzae* (52%), *P. sajor caju* (39%) and laccase (36%), at 325 nm respectively. These percentage values are equivalent to those obtained with *R. oryzae* in Table 3 but lower in comparison to those obtained with *P. sajor caju* or laccase. In Fig. 1 it can also be observed that an absorbance increase was observed between 250 and 275 nm in buffer samples supplemented with or without phenols submitted to tertiary treatment by fungi.

Concerning photo-Fenton treatment, it was the process capable of removing completely the phenols present in the both BKPME samples and buffers (Table 3).

Discussion

The individual phenols detected and quantified in BKPME samples are shown in Table 2. The presence of phenols was also found by Rio et al. (1998), Río et al. (2001) and Ibarra et al. (2005) as a degradation product of *E. globulus* wood in studies of organic deposits and residual lignin markers in kraft pulps and paper pulps. A comparison, other than Rocha-Santos et al. (2010) in terms of phenol individual composition specially concerning *E. globulus* bleached kraft pulp mill effluents after secondary treatment, was not possible since such a specific study was not found in the available literature and, on the other hand, phenols concentration depends among other factors, on the type of process, type of wood materials, process technology, process bleaching and type of secondary treatment of the effluent. The very small range of concentrations of phenols (Table 2), between 45.6 and 53.8 ng/l, may be due to the fact that the secondary treatment of effluents by

Table 3 Removal percentage of phenols concentration by tertiary treatment with *R. oryzae* or *P. sajor caju* or laccase after 7 days of incubation at 25–30°C/120 ± 10 rpm, and by tertiary treatment with photo-Fenton treatment after 6 days

Phenols	Removal (%) on BKPME				Removal (%) on BKPME supplemented with phenols				Removal (%) on buffer supplemented with phenols			
	R. oryzae	*P. sajor caju*	Laccase	Photo-Fenton	*R. oryzae*	*P. sajor caju*	Laccase	Photo-Fenton	*R. oryzae*	*P. sajor caju*	Laccase	Photo-Fenton
Vanillin	100	75	75	100	55	56	56	100	52	55	44	100
Guaiacol	100	60	62	100	64	19	25	100	62	24	23	100
Phloroglucinol	100	80	70	100	69	64	58	100	63	64	61	100
Vanillic acid	100	78	62	100	55	56	47	100	52	55	44	100
Syringic acid	100	85	80	100	70	65	60	100	70	64	61	100

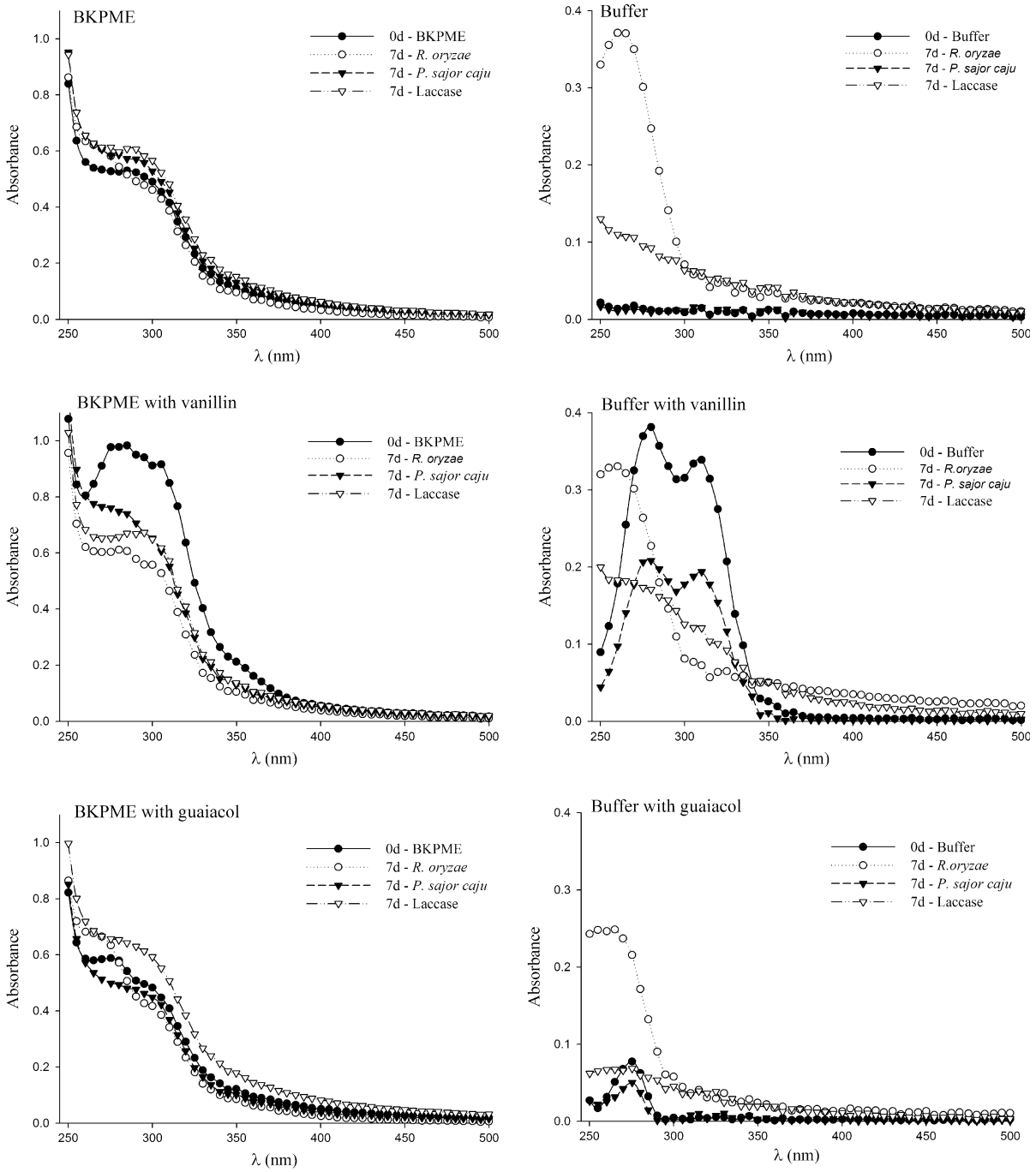

Fig. 1 Absorbance in a range of wavelengths between 250 and 500 nm for BKPME samples or buffer with or without addition of vanillin or guaiacol

activated sludge process could lead to low concentrations of phenols as reported by Dignac et al. (2000) in a study of domestic wastewater. The recovery tests showed a high extraction efficiency of the SPE procedure on the extraction of phenols from BKPME samples, suggesting that the low concentrations of phenols could not be attributed to an analytical artefact.

In both BKPME samples as well in buffers supplemented with phenols, *R. oryzae* was generally responsible, for higher percentage of phenol removal than *P. sajor caju* or laccase (Table 3). According to Freitas et al. (2009) and Rocha-Santos et al. (2010) *R. oryzae* was the most effective in the biodegradation of organic compounds present in the BKPME, being responsible for the higher reduction of relative absorbance and of COD levels after 10 days of incubation at 30°C. The slightly lower values of phenol removal obtained with laccase treatment in comparison to fungi treatment could be explained on the fact that *R. oryzae* and *P. sajor caju* are able to produce other extracellular enzymes than laccase with phenol degradation potential (Garg and Modi 1999; Munari et al. 2007) especially for guaiacol. According to Chandra et al. (2009), chlorophenols are other class of organic compounds, present in pulp and paper mills, that were prejudicial and toxicant to environment and then, they applied bacterial strains (*B. cereus* and *S. marcescens*) for the dechlorination of a pulp and paper mill effluent. In optimum growth conditions (1.0% glucose, 0.5% peptone at 30 ± 1°C at 120 rpm for 14 days of incubation) these two bacterial strains showed reduction of colour (45–52%) and total phenol (32–40%) and high reduction of pentachlorophenol (85–90%).

According to the reported data in Table 3 the tertiary treatment with *R. oryzae* was able to remove phenols in the following order: vanillin = vanillic acid < guaiacol < pholoroglucinol < siringic acid, while the order changed for the tertiary treatment with *P. sajor caju* or laccase: guaiacol < vanillic acid ≤ vanillin < pholoroglucinol ≤ siringic acid, in both BKPME and buffer supplemented with phenols. This order was not observed in BKPME samples treated with *R. oryzae* since 100% of removal was achieved after 7 days of treatment but with *P. sajor caju* treatment or laccase, in general the same order of percentage removal was achieved. According to Salis et al. (2008), laccase is the enzyme responsible for phenolic compounds oxidation in *P. sajor caju*.

In this study and in terms of efficiency, photo-Fenton was demonstrated to be the best process, with the capacity to remove all phenols from BKPME samples. Justino et al. (2010) also reported for the treatment of an OOMW the total reduction of phenols with photo-Fenton oxidation in 6 days, comparatively with their results by biological (8–76%) and enzymatic processes (4–70%).

In order to check if some useful relation could be observable between percentages of removal of each phenol, measured by GC-MS after SPE, and the decrease of absorbance in a range of wavelengths, studies were conducted on some selected samples as described above. The lower affinity of laccase and *P. sajor caju* enzymes for degradation of guaiacol in BKPME was again demonstrated by the lower values of absorbance decrease depicted in Fig. 1, especially at 275–325 nm range. In fact, these results indicate that absorbance scan could be a simply way to observe the level of phenol reduction. In buffer samples supplemented with or without phenols submitted to tertiary treatment by fungi, the observed absorbance increase between 250 and 275 nm was probably resulting from metabolic activity of both fungi species; this phenomena is especially evident in the buffer sample with vanillin, where its degradation was overcome by the organic compounds responsible for absorbance increments in 250–275 nm range. Without BKPME matrix interference, the presence of vanillin was responsible for an absorbance area in 250–350 nm range whereas the presence of guaiacol was responsible for an absorbance area in 250–300 nm range.

As a tertiary treatment of BKPME, *R. oryzae* revealed also to be an efficient process to remove all phenols present in this type of effluent. This achievement is important because according to Pereira et al. (2009) the tertiary treatment with *R. oryzae* proved to be the most promising on in reducing the toxicity in comparison to the photo-Fenton tertiary treatment, which has been reported as highly effective in terms of degradation of complex organic compounds but gives rise to a highly toxic final effluent to several species: *Vibrio fisheri*, *Pseudokirchneriella subcapitata*, *Daphnia longispina* and *D. magna*.

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