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AREA 6 • ENVIRONMENTAL ANALYSES AND MONITORING • RESEARCH ARTICLE

Degradation of phenols in olive oil mill wastewater by biological, enzymatic, and photo-Fenton oxidation

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Abstract
Background, aim, and scope Olive oil mill wastewater (OOMW) environmental impacts minimization have been attempted by developing more effective processes, but no chemical or biological treatments were found to be totally effective to mitigate their impact on receiving systems. This work is the first that reports simultaneously the efficiency of three different approaches: biological treatment by two fungal species (*Trametes versicolor* or *Pleurotus sajor caju*), enzymatic treatment by laccase, and chemical treatment by photo-Fenton oxidation on phenols removal.
Materials and methods Those treatments were performed on OOMW with or without phenol supplement (*p*-coumaric, vanillin, guaiacol, vanillic acid, or tyrosol). OOMW samples resulted from treatments were extracted for phenols using liquid–liquid extraction and analyzed by gas chromatography coupled to mass spectrometry.
Results Treatment with *T. versicolor* or *P. sajor caju* were able to remove between 22% and 74% and between 8% and 76% of phenols, respectively. Treatment by laccase was able to reduce 4% to 70% of phenols whereas treatment by photo-Fenton oxidation was responsible for 100% phenols reduction.
Discussion Range of phenol degradation was equivalent between *T. versicolor*, *P. sajor caju* and laccase for *p*-coumaric, guaiacol, caffeic acid, and tyrosol in supplemented OOMW, which enhances this enzyme role in the biological treatment promoted by these two species.
Conclusions Phenols were removed more efficiently by photo-Fenton treatment than by biological or enzymatic treatments.
Recommendations and perspectives Use of fungi, laccase, or photo-Fenton presents great potential for removing phenols from OOMW. This should be further assessed by increasing the application scale and the reactor configurations effect on the performance, besides a toxicity evaluation of treated wastewater in comparison to raw wastewater.

Keywords Olive oil mill wastewater · Phenols · Photo-Fenton oxidation · Laccase · *Pleurotus sajor caju* · *Trametes versicolor*

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1 Background, aim, and scope

Olive oil mill wastewater (OOMW) resulting from mechanical olive oil production causes environmental prob-

lems due to its high content of organic compounds, including sugars, tannins, polyphenols, polyalcohols, pectins, and lipids (Lesage-Meessen et al. 2001; Obied et al. 2005; Ramos-Comerzana and Monteoliva-Sánchez 2000). The physical and chemical properties of OOMW, namely its chemical oxygen demand (COD) and phenolic content, depend on several factors such as olive extraction process, olive maturation, and cultivar as well as climatic and agronomic conditions (Fiorentino et al. 2003; Lesage-Meessen et al. 2001; Obied et al. 2005; Piperidou et al. 2000). Besides, the phenolic and aromatic compounds have been considered as the important contributors to the toxicity of OOMW (Martínez et al. 2005; Yeşilada et al. 1999). Several studies were already performed in order to promote the degradation of this type of organic compounds (Aggelis et al. 2003; Aissam et al. 2007; Dias et al. 2004; Mantzavinos and Kalogerakis 2005). For instance, biological treatment and advanced oxidation processes (AOP) have been applied for OOMW treatment with different efficiencies in the phenol degradation, as extensively reviewed by Paraskeva and Diamadopoulos (2006), Mantzavinos and Kalogerakis (2005), and Azbar et al. (2004). The white-rot fungi *Phanerochaete chrysosporium* (Dhouib et al. 2006; Reddy and Gold 2000), *Trametes versicolor* (Bourbonnais et al. 1995; Limura et al. 1996), and *Pleurotus* spp. (Tsioulpas et al. 2002) are among the most studied for potential applications in the biological treatment of OOMW. According to Hatakka (1994)) and Leonowicz et al. (2001), the ligninolytic fungal enzymes are able to catalyze one-electron oxidations in phenolic compounds with formation of phenoxy radicals. These radicals react spontaneously with nucleophiles and molecular oxygen cleaving C–O and C–C bonds with opening of aromatic ring of polymer (Akhtar et al. 1997). Laccases seems capable of decreasing the toxicity of phenolic compounds through a polymerisation process (Bollag et al. 1988), and they have the advantage over the peroxidases which required the peroxides as cosubstrate (Dias et al. 2004).

The AOP have been suggested as an alternative to biological treatments with aerobic or anaerobic microorganisms (Mantzavinos and Kalogerakis 2005; Paraskeva and Diamadopoulos 2006) for promoting the mineralization of toxic and hazardous organic pollutants of industrial wastewaters such as OOMW (Benatti et al. 2006). These chemical processes are based on the generation of very reactive and unstable species such as hydroxyl radical which accelerate the nonselectively oxidation of a broad range of dissolved organic pollutants (Arslan and Balcioğlu 1999; Uğurlu and Kula 2007) decreasing the COD content and color (Uğurlu and Kula 2007) of the effluents. Among the AOP, one of the most used is the photo-Fenton oxidation performed by Fenton's reagent with the ultraviolet (UV)/H_2O_2. Photo-Fenton oxidation leads to the production of ferric ion and of hydroxyl radical which reacts with organic compounds starting a chain reaction (Chamarro et al. 2001; Torrades et al. 2003; Pignatello et al. 2006) which leads to their decomposition in OOMW or in other agroindustrial wastes (Lucas and Peres 2009; Paraskeva and Diamadopoulos 2006). According to Arnold et al. (1995) and Benatti et al. (2006), photo-Fenton oxidation has advantages over other oxidizing treatments including high efficiency without producing residues, stability to treat a wide range of substances without the need of special equipment.

OOMW production is one of the acutest environmental problems in Mediterranean countries, and there has been a search for a clean and ecologically safe treatment for the degradation of the pollutants in such effluents. However, due to its high content in toxic and recalcitrant organic compounds, neither chemical nor biological totally effective treatments are available to deal with these wastewaters and mitigate their impact on receiving systems.

The objective of this work was to assess the efficiency of the three different treatment strategies for removing phenols (*p*-coumaric acid, vanillin, guaiacol, caffeic acid, vanillic acid, and tyrosol) in OOMW, thus decreasing its potential toxicity since phytotoxic effects of olive residues can be attributed mainly to their content in phenolic compounds (D'Annibale et al. 2004). Biological treatment by two fungal species (*T. versicolor* and *Pleurotus sajor caju*), enzymatic treatment by purified laccase, and chemical treatment by photo-Fenton oxidation were the experimental approaches applied on OOMW and in OOMW supplemented with extra concentration of each phenol.

2 Material and methods

2.1 OOMW samples

OOMW samples resulted from a stepwise extraction procedure (three-phase olive oil extraction process) made in small scale production, in the north of Portugal and collected from an evaporation pond. In fact, OOMWs storage in settlement basins is common practice in the Mediterranean region (Kallel et al. 2009). OOMW samples were collected in screw-cap glass flasks, which were loosely capped and immediately transported to laboratory. There, they were stored at −20 C until treatments. OOMW samples were characterized for pH, COD, total phenolic content (Table 1), and phenols. COD was determined following standard method American Society for Testing Materials D 1252-88 (1994) whereas total phenolic content was determined by colorimetric method of Folin–Ciocalteu (Mulinacci et al. 2001).

Individual phenols were extracted from samples of OOMW by liquid–liquid extraction, separated and quanti-

Table 1 Physicochemical characteristics of nondiluted OOMW

Parameters	Value
pH	4.0±0.1
COD (g L^{-1})	33.6±0.6
Total phenolic content (mg L^{-1})	243±14

fied by gas chromatography coupled to mass spectrometry (GC-MS). The phenols under study (*p*-coumaric acid, vanillin, guaiacol, caffeic acid, vanillic acid, and tyrosol) were analytical grade and purchased from Sigma-Aldrich, Spain.

2.2 Biological treatment by fungi and enzymatic treatment by laccase

P. sajor caju, obtained from São Paulo State University, Brazil, and *T. versicolor* (Pilát 38412, BCCMTM/MUCL-Culture Collection, Belgium) were cultured at 25 C and 30 C, respectively, in a media containing 20 g L^{-1} of malt extract, 1 g L^{-1} of peptone, and 16 g $L^{-1}$of agar. After a growth period, all species were preserved at 4 C, in the culture medium. Previously to the biological treatment, mycelia of *P. sajor caju* has grown in a liquid medium containing 20 g L^{-1} of malt extract and 1 g L^{-1} of peptone for 11 days, at 25 C and 120±10 rpm. Mycelia of *T. versicolor* were obtained by incubation at same conditions but for 8 days at 30 C. Wheat straw (2.5±0.1 g L^{-1}) was added to the culture medium to induce synthesis of extracellular enzymes as suggested by Jaouani et al. (2006). After these growth periods, mycelia of different species were collected, filtering the culture medium, through a sterilized gaze, and kept in sterilized plastic containers at 4 C, until starting the biological treatment of the OOMW samples.

The ligninolytic fungi *P. sajor caju and T. versicolor* were added to 250 mL of OOMW in batch reactors. According to previous work (Ferreira et al. 2008), the efficiency of biological treatment depends on composition and dilution of the OOMW. Hence, in this work, biological treatment was carried out with 25% of OOMW sample. Dilutions were made with deionized water. According to recommendations from Jaouani et al. (2003), the following additives were added to diluted OOMW: 1.0 g L^{-1} of potassium dihydrogen phosphate, 0.405 g L^{-1} of diammonium tartrate dibasic, and 0.05 g L^{-1} of yeast extract. pH was adjusted to 4.02±0.05 before starting treatment with different species of fungus. Batch reactors with diluted OOMW sample added with each phenol (two 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 (24 mg L^{-1} of *p*-coumaric acid, 22 mg L^{-1} of vanillin, 18 mg L^{-1} of guaiacol, 27 mg L^{-1} of caffeic acid, 26 mg L^{-1} of vanillic acid, or 20 mg L^{-1} of tyrosol) and incubated at 25 C (*P. sajor caju*) or at 30 C (*T. versicolor*), with 120±10 rpm stirring, for a maximum of 7 days. Batch reactors of diluted OOMW for enzymatic treatment (two replicates per phenol) were prepared as described for the biological treatment but without addition of any other additives and incubated at 30 C with 120±10 rpm stirring for 7 days. To each batch reactor, 96 mg of *T. versicolor* laccase (E.C.1.10.3.2; Fluka Analytical, Switzerland) enzyme (22.4 U mg^{-1}) was added. One unit of laccase activity was defined as the enzyme amount which converts 1 μmol substrate per minute. In order to obtain more information about the degradation of each phenol via biological or enzymatic, one essay was performed as described for diluted OOMW but in batch reactors with sodium tartrate buffer 100 mmol L^{-1} instead of diluted OOMW.

2.3 Chemical treatment by photo-Fenton oxidation

The photo-Fenton treatment was carried out in 500-mL glass beakers containing 250 mL of nondiluted OOMW spiked with 150 μmol L^{-1} of one of the selected phenols. Two replicates per each phenol and two replicates without phenol were submitted to photo-Fenton oxidation at room temperature (20 C), with continuous magnetic stirring (100 rpm). Iron sulfate heptahydrate (7.5 mL; 0.5 mol L^{-1}) were progressively added at each beaker and left mixing for 10 min, allowing salt dissolution. After, the mixture pH was adjusted to 4.0 (with sulfuric acid, 1.25 mol L^{-1} or sodium hydroxide, 2.5 mol L^{-1}). Then, 5 mL aliquots of H_2O_2 (30% *v*/*v*) were added every 10 min within the period of 1 h, totalizing 24 mL per beaker. During this period, a UV lamp (VL-6-LC, 245–365 nm, Vilbert–Loumart, France) 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 6 days, during which the oxidation reaction proceeded with carbon dioxide release and production of a precipitate. This procedure was repeated in batch reactors with deionized water instead of diluted OOMW.

2.4 Recovery tests

Recovery tests were performed in order to ensure analytical effectiveness of the liquid–liquid extraction procedure of phenols from the OOMW. Matrix spikes were prepared by adding *p*-coumaric acid, vanillin, guaiacol, caffeic acid,

vanillic acid, and tyrosol in concentrations of 24.00, 22.00, 18.00, 27.00, 26.00, and 20.00 mg L^{-1}, respectively.

2.5 Liquid–liquid extraction procedure

OOMW samples as well as OOMW samples resulted from biological, enzymatic, or chemical treatments and matrix spiked samples (200 mL per replicate) were submitted to liquid–liquid extraction. Thus, 200 mL of each sample and 50 mL of diethyl ether were transferred and mixed in a separation funnel. Thereafter, the upper layer was collected by aspiration. Extraction was repeated twice with 50 mL of diethyl ether and 50 mL of acetate ethyl, respectively. All organic extracts were then filtered through sodium sulfate, collected in 100-mL pear-shaped flask and placed 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 microtube. Samples derivatization was performed by addition of 250 μL of pyridine (Fluka, Spain), 250 μL of bis(trimethylsilyl)trifluoracetamide (Fluka, Spain), and 50 μL de chlorotrimethylsilan (Sigma-Aldrich, Germany) and then, holding the mixture at 70 C, in a sand bath, for 30 min.

2.6 GC-MS methodology

For identification and quantification of phenols, the extracts from OOMW samples; the samples resulting from biological, enzymatic, and photo-Fenton treatments; and the matrix spiked samples for recovery tests were analyzed by GC-MS 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 following oven temperature program was used: 80 C to 220 C at 3 C min^{-1}, then 220 C to 290 C at 6 C min^{-1}, and maintained at 290 C for 3.09 min. The carrier gas used was helium with an inlet pressure of 50 kPa (1 min) to 90 kPa (1.5 kPa min^{-1}) and then to 110 kPa (0.7 kPa min^{-1}). The GC-MS was operated in scan mode for phenols identification by comparing the mass spectra with mass spectra in Wiley 229 (1999), National Institute of Standards and Technology (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 selected ion monitoring mode to obtain the quantification of added phenols. According to Miller and Miller (2005), the detection limit was calculated through formula: $y=y_B+3\cdot S_B$, 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. Each phenol concentration was estimated by interpolation in the standard curve.

3 Results

Table 2 displays the phenols identified and quantified in OOMW. Vanillin, *p*-coumaric acid, caffeic acid, and tyrosol were the phenols present at higher concentration (from 15 to 19 μg L^{-1}) in OOMW whereas guaiacol and vanillic acid were at lower concentration (from 2 to 5 μg L^{-1}). Seven different free fatty acid molecules were also identified (data not shown) being palmitic, linoleic, and oleic acids the major free fatty acids present in the OOMW. It can also be found in Table 2 the recovery values for three replicate samples of OOMW, which were about 100%.

Table 3 shows the percentage of reduction in the concentration of phenols after 7 days of biological or enzymatic treatments and after 6 days of photo-Fenton oxidation in OOMW and sodium tartrate buffer supplemented with phenols. Reduction values ranged between 22% and 74% for *T. versicolor*, 8% and 76% for *P. sajor caju*, and 4% and 70% for laccase enzyme in both samples. Regarding biological treatment, *T. Versicolor*, of both supplemented diluted OOMW or buffer with phenols, was responsible for higher levels of degradation of tyrosol and guaiacol (66–72%), being able of lower values for *p*-coumaric, vanillin, caffeic acid, and vanillic acid (22–25%). Similar pattern of degradation for guaiacol and tyrosol (64–76%) as well for *p*-coumaric and caffeic acid (22–28%) was obtained with biological treatment by *P. sajor caju* as well by laccase; lower percentages of removal was observed for vanillic acid and vanillin (4–9%). Photo-Fenton oxidation, in turn, was responsible for 100% of reduction of phenols in both supplemented nondiluted OOMW and buffer since all phenols were detected at concentrations below detection limit (<0.02 ng L^{-1}).

In diluted OOMW samples (without phenol supplement), 23.9% and 29.9% of reduction were obtained after 7 days of biological treatment with *T. versicolor* and *P. sajor caju*, for all phenols in study, respectively. Laccase and photo-Fenton oxidation were able to remove completely all phenols determined in the OOMW.

4 Discussion

The individual phenols detected and quantified in OOMW samples are shown in Table 2. This list of phenols does not coincide with that reported by De Marco et al. (2007); only tyrosol, caffeic acid, and vanillic acid are common to both studies. It is noticeable the absence of hydroxytyrosol which is one of the most abundant phenolic compound present in OOMW (De Marco et al. 2007; Servili et al. 1999). However, according to Annesini and Gironi (1991), organic compounds and their corresponding concentrations in OOMW depend, among others factors, on the extraction

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

Phenol	OOMW[a] (μgL^{-1})	Added (mgL^{-1})	Recovery[a] (%)
3-(4-Hydroxyphenyl)-2-propenoic acid (*p*-coumaric acid)	18.0±0.9	24.00	100.1
4-Hydroxy-3-methoxybenzaldehyde (vanillin)	15.7±0.7	22.00	100.6
2-Methoxyphenol (guaiacol)	2.3±0.2	18.00	100.2
3-(3,4-Dihydroxyphenyl 2-propenoic acid (caffeic acid)	19.2±0.7	27.00	100.1
4-Hydroxy-3-methoxybenzoic acid (vanillic acid)	5.4±0.3	26.00	100.2
4-(2-Hydroxyethyl)phenol (tyrosol)	17.2±0.5	20.00	100.2

[a] Mean of three determinations

processes, olive ripening, and olives storage duration before milling, as well as the storage of the effluent in evaporation ponds. Recovery tests suggested a high extraction efficiency of the liquid–liquid extraction method used for isolation of phenols from OOMW samples, and no significant effects could be assigned to the complexity of the samples.

Several authors (Dias et al. 2004; Limura et al. 1996) reported that the biological treatment of OOMW represents a challenge due to the presence of resistant compounds such as tannins, polyphenols, polyalcohols, pectins, and lipids in OOMW. In this study, the biological treatment of diluted OOMW samples for 7 days was responsible for 24% to 30% removal for all phenols present in OOMW (Table 2). This scenario changed for diluted OOMW supplemented with phenols. Both *T. versicolor* and *P. sajor caju* as well as laccase were able of higher levels of removal for guaiacol and tyrosol (64% to 76%) and slightly lower levels of removal for *p*-coumaric and caffeic acid (22% to 29%). Similar results were obtained in buffer supplemented with phenols. Due to the complex OOMW matrix, it was expected higher levels of phenol degradation in buffer in comparison with OOMW; however, the magnitude of phenol increment may be overcomed the possible effect of OOMW matrix. Since the range of phenol degradation was equivalent between *T. versicolor*, *P. sajor caju*, and laccase for *p*-coumaric, guaiacol, caffeic acid, and tyrosol in both supplemented diluted OOMW and buffer with phenols, this enhances the possible role of the enzyme laccase in the biological treatment promoted by *T. versicolor* and *P. sajor caju*. It is well documented that, for *Pleurotus ostreatus*, the enzyme responsible for phenolic compounds and aromatic amines oxidation is the laccase induced by OOMW or other substrates (Martirani et al. 1996; Hublik and Schinner 2000). Laccases, which are the most studied enzymes produced by *T. versicolor* (Bourbonnais et al. 1995), are nonspecific and extracellular multicopper enzymes that use molecular oxygen as an electron acceptor (Robles et al. 2000). Different levels of removal efficiency for vanillin and vanillic acid were observed for both fungi species and laccase. *T. versicolor* was responsible for 22–23% removal in both supplemented diluted OOMW and buffer whereas *P. sajor caju* and laccase were only able to remove 4% to 9% in these samples.

In terms of efficiency, photo-Fenton was the best process, capable of removing completely the phenols present in the samples under study. With this treatment, and after 6 days, all phenols were totally removed from the nondiluted OOMW effluent. Uğurlu and Kula (2007) reported 99.5% phenol removal after 7 days of OOMW

Table 3 Removal percentage of phenols concentration by biological treatment with *T. versicolor* and *P. sajor caju* or enzymatic treatment with laccase after 7 days of incubation at 25–30 C and 120±10 rpm and by photo-Fenton treatment after 6 days

Phenols	Removal (%) on OOMW[a] supplemented with phenols				Removal (%) on buffer supplemented with phenols			
	Trametes versicolor	*Pleurotus sajor caju*	Laccase	Photo-Fenton	*Trametes versicolor*	*Pleurotus sajor caju*	Laccase	Photo-Fenton
p-Coumaric acid	22	25	26	100	22	25	20	100
Vanillin	23	9	9	100	23	9	5	100
Guaiacol	72	76	70	100	74	76	70	100
Caffeic acid	25	28	29	100	25	28	29	100
Vanillic acid	22	8	8	100	22	8	4	100
Tyrosol	66	69	64	100	66	68	61	100

[a] Biological and enzymatic treatments were performed on diluted OOMW. Photo-Fenton oxidation was performed on OOMW without dilution

by UV/H_2O_2 treatment. Oxidation processes that utilize H_2O_2, O_3, or O_2 as oxidants are very promising techniques for remediation of wastewaters having nonbiodegradable organic pollutants (Scott and Ollis 1995; Gogate and Pandit 2004). But according to some authors, this process alone is not effective enough for detoxification of OOMW, especially if it results in partial oxidation of organic compounds, leading to potentially harmful chemicals generation (Lathasree et al. 2004). This tendency is coincident with those reported by Pereira et al. (2009), describing an increment in toxicity of bleached kraft pulp mill effluent, for a battery of aquatic species, after photo-Fenton oxidation.

5 Conclusions

Biological and enzymatic treatments showed interesting potential especially for guaiacol and tyrosol removal in diluted OOMW. Photo-Fenton showed potential to complete phenols removal in nondiluted OOMW. Comparing the three treatments processes, it can be concluded that the photo-Fenton oxidation shows the most promising potential taking into consideration the obtained removal efficiencies for phenol.

6 Recommendations and perspectives

The reduction of the environmental impact caused by OOMW has been attempted through the development of more effective biological, enzymatic, and chemical treatments. This study is a first assessment of the potential of three completely different treatment systems for removal of phenols contained in OOMW: fungi for biological treatment, laccase for enzymatic treatment, and photo-Fenton oxidation for chemical treatment. In face of the results, at least, the treatment processes based on laccase and photo-Fenton oxidation could be researched further at a larger scale and with different reactor configurations. Furthermore, an evaluation of the compared toxicity between raw and treated effluent should complement this preliminary assessment.

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