

BIOLOGICAL AND PHOTO-FENTON TREATMENT OF OLIVE OIL MILL WASTEWATER

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ABSTRACT

Olive oil wastewaters (OOMW) are dark effluents which pose serious environmental problems. The aim of this work was to apply biological treatment with *Pleurotus* spp., and chemical treatment, through oxidation by Photo-Fenton, to OOMW. The biological treatment was not very efficient in colour reduction. In 25% OOMW dilution treated with *P. sajor caju* or *P. ostreatus*, high percentages of colour reduction (460 nm) were recorded after 6 days (70.3 and 54.7%) but, thereafter, great oscillations were observed. In terms of pH, a similar profile was recorded in 25-50% dilutions treated either with *P. sajor caju* or *P. ostreatus*. Both species grew in OOMW and were responsible for COD reduction, which was greater in the OOMW treated with *P. sajor caju*. The Photo-Fenton reaction was very efficient (> 90%) in colour reduction. The best combination of H₂O₂/Fe concentrations was found to be 6 ml of H₂O₂ (70%) plus 1 ml of FeSO₄ (0,5M). In terms of toxicity reduction tested for *Daphnia longispina*, the biological and chemical treatments also had a different efficiency. Further research will be carried out to assess the combined application of both treatments, and its effectiveness in the improvement of the main concerning properties of this industrial effluent.

KEYWORDS: Olive oil mill wastewater, Photo-Fenton oxidation, biological treatment, Fungi, *Pleurotus* spp.

1. INTRODUCTION

The chemical composition of the olive oil mill wastewater (OOMW) depends on the extraction process. OOMW is an aqueous, dark, foul smelling and turbid liquid, which includes emulsified grease; it is easily fermentable and has a high organic content (Sierra *et al.*, 2001). The three-phase olive oil extraction process generates an effluent which is characterized by a high polluting load, mainly explained by the presence of chemical compounds, phenols in particular, with biostatic and phytotoxic activity (Casa *et al.*, 2003). Treatment and disposal of OOMW represents one of the main problems for olive oil producing countries of the Mediterranean basin. Every year, a large amount of this wastewater is stored in ponds to precipitate solid organic compounds, until further production. The disposal of this effluent is usually made in local rivers or agricultural soils, representing a major environmental problem because of its potential to compromise the quality of groundwater and surface freshwater resources and, soil functions as well. Thus, it poses a serious risk to aquatic and terrestrial biota, and subsequently to the health of corresponding ecosystems. Studies focusing the characterisation and evolution of a soil affected by OOMW disposal showed that, even after two years of sediment removal, residual contamination levels are still considerable, in the

upper 40 cm of soil (Sierra *et al.*, 2001). Hence, the great risk of producing irreversible environmental problems justifies the development of appropriate technologies to reduce toxic compounds, especially the phenolic content of this industrial residue. Phenolics present on OOMW at high concentrations ($1\text{--}10\text{ g l}^{-1}$) are considered as the major recalcitrant compounds, which have antimicrobial properties, and are difficult to be biologically degraded (Ahmadi *et al.*, 2005). Besides biological treatments with fungi have been pointed by several authors as the most effective and inexpensive (e.g. Yesilada *et al.*, 1999; Isidori *et al.*, 2004), it is also known that conventional biological processes for the purification of OOMW are sometimes inefficient and must be coupled with other processes. Oxidation processes that utilize H_2O_2 , O_3 or O_2 as oxidants, are very promising techniques for remediation of wastewaters having non-biodegradable organic pollutants (Scott and Ollis, 1995, Gogate and Pandit, 2004). These processes involve the generation of the hydroxyl radical that is a reactive intermediate and has a high oxidation potential (Sarria *et al.*, 2002).

Based on these assumptions, the aim of this work was to apply a biological treatment to OOMW, stored in a pond for about one year, with *Pleurotus spp.*, as well as a Photo-Fenton's oxidation, which is considered as an iron-catalyzed H_2O_2 decomposition reaction. Between the different fungi used in OOMW treatment, *Pleurotus* presents the advantage of being an edible mushroom.

2. MATERIAL AND METHODS

2.1. Source of inoculum and maintenance

Two species of *Pleurotus*: *P. sajor caju* and *P. ostreatus* were obtained from Faculdade de Ciências Agrárias – UNESP, São Paulo Brasil and subcultured and maintained on Potato Dextrose Agar (Himédia®, India), at 4°C . Growth of *Pleurotus* mycelia was performed in sterilized media containing 2 g l^{-1} glucose (Riedel-Haen®, Germany), 2 g l^{-1} starch (Absolve®, Portugal), 0.1 g l^{-1} peptone (Hi-media®, India), during 7 days, at 25°C and 120 ± 10 rpm. After growth, mycelia was collected by filtration with sterilized gaze and kept in sterilized plastic containers at 4°C , for a maximum of 24h, until the biological treatment of the OOMW started.

2.2. Effluent source

Olive oil mill wastewater with about one year, from settlement ponds of a company that receives wastes from local oil presses (S. João da Pesqueira, North of Portugal) was collected and stored in glass flask, in the dark, at 4°C until biological and Photo-Fenton treatment.

2.3. Biological treatment of OOMW in batch reactor

In order to determine the maximum OOMW concentration, at which fungal growth of *Pleurotus spp.* was not inhibited and, if autochthonous microorganisms can promote decolourisation as well, cultures on solid media were conducted in duplicate, in plates containing sterile and non-sterile solutions. These solutions were composed by OOMW in different concentrations (25, 50 and 75%), chemical additives and 16% (w/v) agar (Himedia, India). Two different mixtures of chemical additives were used, namely: (i) 20 g l^{-1} of glucose, 20 g l^{-1} of starch and 1 g l^{-1} peptone or (ii) 1 g l^{-1} KH_2PO_4 (Sigma, USA), 0.25 g l^{-1} NH_4Cl (Sigma, USA) and 0.05 g l^{-1} yeast extract (Hi-media, India).

To perform the biological treatment, liquid cultures of two different concentrations of the original OOMW (25 and 50%) were obtained through dilution with water. Thereafter, several compounds were added to the diluted OOMW according to Jaouani *et al.* (2003) suggestions: KH_2PO_4 (1 g l^{-1}), NH_4Cl (0.25 g l^{-1}) and yeast extract (0.05 g l^{-1}). Two replicas of 190 ml of each dilution were inoculated with 5–6 g of *Pleurotus ostreatus* or *P. sajor-caju* mycelia. The pH of both dilutions was adjusted to 5.4–5.5 and incubation was performed at 25°C and 120 ± 10 rpm, for 27 days.

Several parameters were analysed to monitor the biological treatment of OOMW with *Pleurotus spp.* Samples were withdrawn after 0, 6, 12, 20, and 27 days of incubation for pH and colour control. Colour removal was followed by UV-VIS scan (200–600nm) of diluted samples at pH 5.5. Chemical oxygen demand (COD) was analyzed according to ASTM method (1994) after 0 and 27 days of incubation. Mycelia of both species of *Pleurotus* was harvested by filtration and weighed after 27 days of treatment for biomass control.

2.4. Photo-Fenton treatment

The Photo-Fenton treatment was performed with aliquots of 50 ml of OOMW, with pH adjusted to 4.2, in a 100 ml glass reactor maintained at 20 °C. The aqueous solution of reactants was homogenised by magnetic stirring to avoid concentration gradients. The influence of different concentrations of H₂O₂ as well of iron was studied. Hence, to assess the influence of different H₂O₂ concentrations, 3 mL of FeSO₄ (0.5 M) and different volumes of H₂O₂ (70%), 3, 6, 9, 12 and 15 ml were added to 50 ml of OOMW, in batch assays. Each volume of H₂O₂ was divided in equal aliquots, to be added each 10 minutes, within the period of 1 hour. The same procedure was followed to study the influence of iron concentrations i.e. after the addition of 6 mL of H₂O₂ to the OOMW, different volumes of FeSO₄ 0.5 M (0.05, 0.2, 1, 2 and 3 ml) were added. The UV light was switch on, after addition of the first aliquot and left for 2 hours. After 6 days, aliquots of treated effluent were collected, filtered through a glass fiber and submitted to UV/Vis analysis to quantify colour reduction.

2.5. Acute toxicity assays

Monoclonal cultures of *D. longispina* (clone EM7, *sensu* Antunes *et al.* 2003) were reared under a 16^L:8^Dh cycle and a temperature of 20±2°C. ASTM (1980) synthetic hard water medium was used as culture medium and rearing procedures followed the recommendations of standard protocols (e.g. ISO, 1996 and 2000; ASTM, 1997; OECD, 1998 and 2000). A standard organic additive was added to the culture to provide essential microelements to daphnids (Baird *et al.*, 1989). Animals were fed with *Pseudokirchneriella subcapitata*, which was cultured in Woods Hole MBL medium, in a semi continuous 8L batch culture at a 24^L:0^D h regime (at 20±2 °C). Algal ration was determined spectrophotometrically and daily supplied to the cladocerans (1.5×10⁵ cell/ml/day for *D. longispina*).

Sub-samples of OOMW, after have been treated by fungi or by Photo-Fenton reaction, and with pH adjusted to 7.5, were taken for acute toxicity evaluation with *D. longispina*. All assays were initiated with neonates (<24-h-old), born between the 3rd and 5th broods, which were obtained from bulk group cultures. Assays were performed in accordance with standard protocols (ISO, 1996; ASTM, 1997; OECD, 2000), under the same temperature and photoperiod regimes as described for rearing procedures. A static design was employed, using 20 animals (randomly divided into four groups of five animals) per control and per OOMW concentration (%). Test vessels (four per each concentration) consisted of glass beakers containing 100 ml of each effluent concentration. All experimental treatments were checked for immobilised individuals at 24 and 48h, which were counted for posterior determination of EC₅₀ values. EC₅₀ values for immobilisation data were determined using probit analysis (Finney, 1971).

3. RESULTS

Both species of *Pleurotus* spp. only were able to growth in 25% OOMW (sterilized and non-sterilized) solid cultures, where some decolourisation of the medium progressively occurred compared to the corresponding control (non-inoculated plates). In plates with 50 and 75% dilutions of OOMW, no growth was observed. In plates with the non-sterilized 25% dilution of OOMW and without the inoculation of fungi species, the growth of autochthonous microorganisms was recorded, but no decolourisation occurred, which indicates the inability of the OOMW autochthonous microflora to degrade complex organic compounds.

In liquid cultures, both species grew in the 25 and 50% OOMW dilutions, especially *P. ostreatus* whose growth rate was higher (1.6) in the 25% OOMW dilution (Table 1). According to Tsioulpas *et al.* (2002), OOMW contains all essential elements for microbial growth, but also several growth inhibitors, such as organic acids and phenolic compounds whose composition depends on maturity and variety of olive fruit and mainly on the oil extraction technology used.

OOMW is usually characterized by a high degree of organic pollution presenting values of COD up to 220 g l⁻¹ (Tsagaraki *et al.*, 2007). In our study, levels of COD reduction were greater in the OOMW inoculated with *P. sajor caju*. A 50 and 89% reduction in COD was attained in the 50% and 25% OOMW dilutions, respectively, after 27 days of incubation with *P. sajor caju*. *P. ostreatus* seemed to have more difficulties in promoting degradation of organic compounds responsible for COD levels in this type of effluent. As Jaouani *et al.*

(2003) observed, COD removal is correlated with high levels of decolourisation, especially for species such as *C. polyzona* and *P. sajor caju*.

Table 1. Biomass and COD values obtained in treated OOMW with *P. sajor caju* or *P. ostreatus*, throughout 27 days of incubation at 25°C and 120±10 rpm

Effluent composition	Fungi	Biomass (g l ⁻¹)			COD (g l ⁻¹)	
		0 d	27 d	GR ¹	0 d	14 d
25%	<i>P. sajor caju</i>	5.02	6.22	1.2	6.47	0.71
25%	<i>P. ostreatus</i>	5.32	8.34	1.6	6.47	4.41
50%	<i>P. sajor caju</i>	5.41	6.20	1.1	12.03	6.01

¹Growth rate = total biomass (g l⁻¹) / initial biomass (g l⁻¹)

The biological treatment, in liquid cultures, was not very efficient in terms of colour reduction, since throughout incubation time high oscillations in absorbance, at 250, 275, 325, 400 and 460 nm, were recorded (Table 2).

Table 2. Absorbance reduction (%)¹ for several wave lengths in treated 25 and 50% OOMW dilutions treated with *P. sajor caju* or *P. ostreatus*, throughout 27 days of incubation at 25°C and 120±10 rpm.

Effluent composition	Fungi	λ (nm)	Time (d)			
			6	12	20	27
25%	<i>P. sajor caju</i>	250	31.5	5.7	16.0	23.4
		275	39.1	3.7	13.7	18.4
		325	33.2	²	²	²
		400	49.8	²	21.4	23.2
		460	70.3	²	42.6	44.7
	<i>P. ostreatus</i>	250	10.0	2.0	1.5	2.5
		275	14.8	4.2	²	²
		325	5.9	²	²	²
		400	28.0	5.5	²	²
		460	54.7	32.5	23.8	18.4
50%	<i>P. sajor caju</i>	250	²	²	24.6	²
		275	16.1	21.5	36.2	10.9
		325	²	²	2.6	²
		400	²	2.3	21.5	²
		460	10.5	30.2	40.7	17.5

¹Absorbance reduction percentage = (Final Abs – Initial Abs)/Initial Abs) x 100;

²No absorbance reduction.

In the 25% OOMW dilution, treated with *P. sajor caju* and *P. ostreatus*, interesting percentages of colour reduction were recorded after 6 days of incubation at 460 nm (70.3 and 54.7%, for each species, respectively). Thereafter, great oscillations were observed and the percentages of colour reduction, at 460 nm, after 27 days of incubation, decreased for 44.7 and 18.4% in effluent incubated with *P. sajor caju* and *P. ostreatus*, respectively. The lowest values of colour reduction were observed in the 50% OOMW dilution. Jaouani *et al.* (2003) reported a transient colour increase phase during the first days of incubation of OOMW with fungi before effective decolourisation started. Other authors have reported a progressive decolourisation by *Pleurotus* spp. throughout incubation time (Tsioulpas *et al.*, 2002; Fountoulakis *et al.*, 2002). However, the colour variation observed could somewhat have been related with the inhibition of the fungal enzyme laccase, since in *Pleurotus ostreatus* this is the enzyme responsible for the oxidation of phenolic compounds and aromatic amines and

it is usually induced by OMWW or other substrates (Martinari *et al.*, 1986; Hublick and Schinner, 2000).

According to Piperidou *et al.* (2000), the OMWW contains some phenolic compounds, which are able to induce laccase synthesis. However, as reported by Tsajarakı *et al.* (2007), the majority of phenolic compounds removed are simple monomers, whereas polymerized molecules such as tannins are more resistant to degradation because these compounds absorb strongly to mycelia and intracellular enzymes, causing inactivation, impairing their own biodegradation. In fact, more than 30 different phenolic compounds have been detected which can be included in two categories: (i) phenolic monomers, flavenoids, not autoxidated tannins, and other compounds with MW \leq 10KDA and (ii) medium and high molecular weight (MW) dark coloured polymers resulting from the polymerization and autoxidation of phenolic compounds (Tsajarakı *et al.*, 2007).

In terms of pH, a similar profile was recorded in the 25 and 50% OMWW dilutions, inoculated either with *P. sajor caju* or *P. ostreatus* (Figure 1), throughout 27 days of incubation, at 25 °C and 120 \pm 10 rpm. In all the assays the highest values were recorded after 6 days of incubation (7.1-7.7), decreasing afterwards until values around 4 after 27 days of treatment. The highest values of pH are in agreement with higher values of absorbance reduction in 25 % OMWW dilution, recorded after 6 days of incubation.

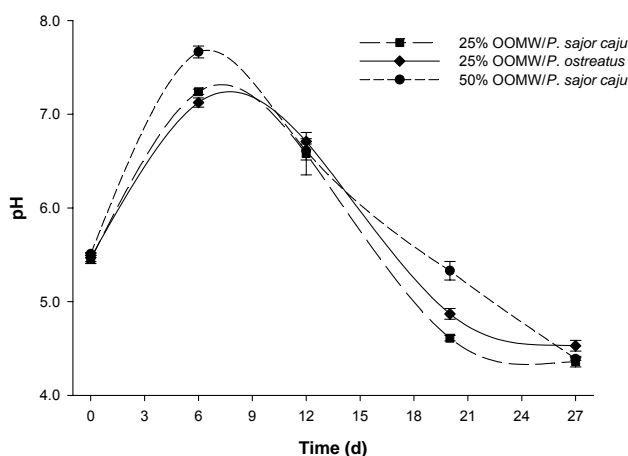


Figure 1. pH variation throughout time of 25% to 50% OOMW dilutions inoculated with *P. sajor caju* or *P. ostreatus* at 25 °C and 120 \pm 10 rpm

The Photo-Fenton reaction revealed to be very efficient in terms of colour removal. Levels above 90% in colour reduction were found in several combinations of H₂O₂/iron concentrations. The best combination of H₂O₂/iron concentrations was found to be 6 ml of H₂O₂ (70%) plus 1 ml of FeSO₄ (0,5M). With this combination 85.7, 91.9 and 93.4% of color reduction were obtained at 400, 460 and 500 nm, respectively (Figure 2). The Fe²⁺/H₂O₂ system is efficient to treat OMWW due to OH radical peroxidation and ferrous /ferric coagulation (Khoufi *et al.*, 2006). The non-biodegradable organics and toxic pollutants present in the wastewater such as polyphenols are usually destroyed by oxidation via production of oxidants such as hydroxyl radicals and complex coagulants that promote the flocculation of the matter present in the OOMW (Panizza *et al.*, 2000; Chen *et al.*, 2002).

A high OOMW effluent acute toxicity was observed for *D. longispina* [(with pH adjusted to 7.5) – EC₅₀ = 6.02% (Cl₉₅: 5.48% - 6.37%)]. *P. sajor caju* was more efficient in the reduction of toxicity of the OOMW to *Daphnia longispina*. The 48h-EC₅₀ increased 4.6 and 3.3 folds after the treatment with *P. sajor caju* and *P. ostreatus*, respectively. Regarding the effluent treated with Photo-Fenton reaction any improvement was recorded in its toxic properties. Although, it was not possible to determine an EC₅₀, immobilization was observed for daphnids in all the concentrations equal or above 7%. Further research will follow in order to study the combination of both, chemical oxidation and biological treatment, applied to larger volumes of OOMW, in increasing the removal of toxic compounds as products from a partial chemical degradation are more easily biodegradable.

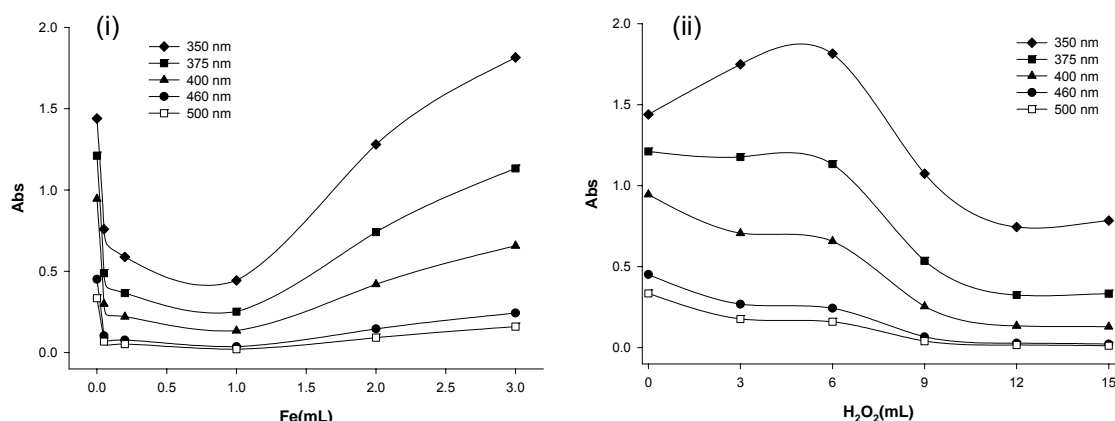


Figure 2. Absorbance values obtained from Photo-Fenton treatment of OOMW with (i) 6 ml of H₂O₂ varying the volume of Fe (0.05M) and (ii) 3 ml of Fe (0.05 M) varying the volume of H₂O₂ (37%)

4. CONCLUSIONS

Pleurotus spp. species appear to be able to grow on OMWW with levels of 25 g l⁻¹ of COD. However, and in contrast to results reported by several authors, the decolourisation of the effluent was not progressive. High oscillations of absorbance at several wave lengths was recorded which could be related with polymerisation and other reactions of several compounds namely phenolic monomers. In further research enzymatic activity, phenolic compounds and other organic molecules will be measured in order to follow the biological treatment of OMWW.

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