Olive mill wastewater biological treatment by fungi biomass

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Abstract Olive oil extraction is one of the most important traditional food industries in the Mediterranean region, especially in Italy. In addition to olive oil, this industry produces by-products, in particular olive mill wastewaters (OMWs) and olive husks, which represent a serious environmental problem. OMWs can be rarely treated in a municipal WWTP, using conventional wastewater treatments. A novel biological process has to be considered in order to treat OMWs. Literature data show that yeasts and different kinds of fungi are able to reduce both the organic and the phenolic content of the OMW. The present work is aimed at investigating the growth of a biomass rich in fungi in a batch reactor filled with OMW and its capacity to degrade the organic and phenolic load. The aerobic OMW degradation obtained using this biomass reached a COD and TP removal efficiency of 86 and 70%, respectively. Respirometric tests have been carried out in order to measure the biomass activity on different substrates: OMW and phenolic compounds (gallic and p-coumaric acids). The polyphenolic biodegradation efficiency of fungi biomass was higher than the one of a non-acclimated activated sludge biomass. Fungi biomass was able to completely degrade pure phenolic compounds.

Keywords Aerobic biodegradability; fungi biomass; olive mill wastewater; phenolic compounds; respirometry

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Introduction

Olive oil production, one of the main agro-industries in the Mediterranean countries, generates significant amounts of olive mill wastewaters (OMWs). Mediterranean countries account for approximately 95% of the world olive oil production, corresponding to about 11 millions tons of olives produced per year and 1.7 million tons of extracted oil (Aktas *et al.*, 2001). In addition to olive oil, this industry produces by-products, such as wastewaters and olive husks, which represent a serious environmental problem. Olive oil mill wastewaters are characterized by a high organic load (80–300 g/L of COD) and a low degree of biodegradability due to the acidic pH and, in particular, to the presence of phenolic and lipidic compounds, well known to be toxic to bacteria (Beccari *et al.*, 1999; Gernjak *et al.*, 2004). It is estimated that every ton of milled olives corresponds to about 0.80 ton of OMW (Aktas *et al.*, 2001). In the Mediterranean countries OMW total production reaches about 30 million m^3 per year (Dionisi *et al.*, 2005). Italy is one of the major olive oil-producing countries, totalling 530,000 tons per year, corresponding to 2,200,000 tons of OMW. Traditional disposal on the soil is still the typical solution adopted in Italy. The Italian law in force (L. 574/96) allows discharge of a maximum of 50 m^3/ha when OMW comes from a traditional mill and 80 m^3/ha when OMW comes from a continuous mill. The disposal on the soil costs about 20–25 €/m^3 of discharged OMW. Publiacqua S.p.A. manages the wastewater treatment plants and the water supply systems of Firenze, Prato and Pistoia (Tuscany). Table 1 shows the hydraulic load and

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Table 1 OMW production and PE estimation per year and during a milling period (90 days)

Provinces	OMW (m^3)	Oil/OMW (% w/w)	PE (per year)	PE (90 d)
Firenze	15,505	29.2	59,400	240,910
Pistoia	5,515	33.8	23,250	69,474
Prato	1,610	36.6	6,200	25,136
Total	22,630	–	88,850	360,336

the OMW organic load (based on 130 gCOD/d per population equivalent, (PE)) due to the olive oil production in this area.

Literature studies, carried out with specialised biomass, show that yeasts (Chigusa *et al.*, 1996) and different kinds of fungi (white-rot and brown-rot fungi) are able to degrade recalcitrant compounds which are present in OMW. Fungi biomass produces highly oxidative enzymes such as laccase, Mn-peroxidase, ligninase, so that they are able to degrade lignin-related compounds, such as polyphenols. The removal efficiency ranges between 40 and 70% as regards polyphenols and ranges from 60 to 70% as regards COD (Borja *et al.*, 1995; Garcia *et al.*, 2000; Fountoulakis *et al.*, 2002; Tsioulpas *et al.*, 2002; D'Annibale *et al.*, 2004). Literature data generally refer to laboratory pure fungi cultures applied to OMW pre-treated by means of sterilisation, dilution and thermal processes (Hamdi *et al.*, 1991; D'Annibale *et al.*, 1998; Aggelis *et al.*, 2003).

In the present study a fungi biomass has been developed under controlled conditions (pH: 4.5, T: 35 °C) directly on OMW, pre-treated by means of centrifugation. The fungi biomass efficiency for COD and total polyphenols (TP) removal has been investigated both by respirometric trials carried out on pure phenolic acids and OMW samples, and by a fed-batch pilot aerobic bioreactor. In order to investigate the possible use of OMW as a fuel (Caputo *et al.*, 2003), its calorific value after a dewatering process has been evaluated.

Methods

The experimentation was carried out on wastewaters taken from one of the largest three-phase mills in Italy (2004/2005 olive oil campaign), located in Quarrata (Tuscany). The samples were taken from a 5 m^3 storage tank from February to September 2005. Raw OMW samples were centrifuged at 4000 r.p.m. for 10 minutes in order to separate the liquid phase from the solid phase. All the trials were carried out on the liquid phase, whose main characteristics are summarized in Table 2.

TP content has been determined by the Folin-Ciocalteau spectrophotometric method and HPLC analysis (high performance liquid chromatography) with a LiChrosorb RP-18 column. Both the analyses were carried out on OMW samples after filtration at 0.45 μm. Gallic and p-coumaric acids were used as standards to calibrate the Folin-Ciocalteau method. HPLC tests were displayed using both an UV and a mass spectrometry analysis. COD, TSS, VSS, TN (total nitrogen), P_{tot} (total phosphorus) and BOD were assessed

Table 2 Raw and centrifuged OMW characteristics

	Raw OMW	Centrifuged OMW
pH	4.4–4.8	4.6–5.12
COD (mg/L)	262,750–301,600	48,850–72,720
Total polyphenols, TP (mg/L)	9,600–10,600	2,360–2,930
Total suspended solids, TSS (g/L)	113.5–128.4	2.19–3.02
Volatile suspended solids, VSS (%)	91.63–94.5	95.5

according to *Standard Methods* (2005). The filtered COD (fCOD) was measured after 0.45 μm membrane filtration.

The aerobic treatment with fungi biomass was performed in a batch filled with centrifuged OMW without biomass inoculum. The completely mixed batch reactor used was composed of a 4 L glass vessel (Applikon) and a mechanical stirrer with variable speeds. The speed was controlled within the range of 200–500 rpm in order to ensure no limiting oxygen conditions. Oxygen, temperature (fixed at 30 °C) and pH (fixed at 4.5), control and monitoring of the laboratory-scale reactor were performed by a biocontroller (ADI 1010 Applikon). On-line pH control was ensured by NaOH (4 M) and H_2SO_4 (1 M) addition with peristaltic pumps. Nutrient solutions $(NH_4)_2SO_4$ and NaH_2PO_4 were added periodically to the batch reactor.

The pilot plant operated in batch mode or in fed-batch mode. The latter consists of four different phases: (1) centrifuged OMW load; (2) aeration (stopped when the OUR (oxygen uptake rate), value was similar to the endogenous respiration rate and the filtered COD value did not significantly change); (3) clarification; (4) supernatant extraction.

Respirometric tests were performed using two different instruments:

(1) A custom-made automated respirometer consisting of an aerated 2 L batch reactor (Applikon BV), a completely stirred airtight respiration chamber (125 mL), a peristaltic pump (Watson-Marlow 313 U), an oxygen probe and oximeter (WTW Oxi 3000), an acquisition board (National Instruments PCI MIO 16E 1) and an automation software program (National Instruments Labview 5.0) (Marsili-Libelli and Tabani, 2002). Temperature and pH are controlled respectively by a temperature control unit (Haake) and a Biocontroller 1030 unit (Applikon BV).
(2) MARTINA (multiple analysis programmable titration analyser, SPES, Italy). The respirometer is connected to a 1.5 L jacked batch reactor. Temperature, pH, O_2 are controlled directly by MARTINA.

In order to investigate pure polyphenols' aerobic degradability, respirometric trials were carried out using gallic acid, the most commonly used compound for the calibration of the polyphenol colorimetric analytical method, and p-coumaric acid which is one of the most recalcitrant compounds present in the OMW. Different respirometric techniques (Andreottola *et al.*, 2001) were used in order to evaluate biomass activity, specific growth rate (μ_{max}), affinity constant (K_s), yield factor (Y_H), endogenous decay coefficient (b_H), by considering different substrates: centrifuged OMW (injections of 3, 5, 50, 100 mL), gallic acid and p-coumaric acid (injections of 50–180 mg). The respirometric tests were used in order to compare, through short-term BOD (BOD_{st}) evaluation, the OMW degradation determined by the acclimated fungi biomass and a non-acclimated sludge taken from a municipal waste water treatment plant (WWTP). Biodegradable COD fraction (BCOD) was assessed as $BOD_{st}/(1\text{-}Y_H)$.

Results and discussion

OMWs were pre-treated by centrifugation. This process was able to separate the liquid, which represents about 40% of the total volume, from the solid fraction. The centrifugation reduces the TSS content in the liquid phase of about 98%. Centrifuged OMW is also characterised by the following values: BOD_5: 25,500–27,015 mg/L, BOD_{20}: 40,000–43,000 mg/L, TN: 286–438 mg/L, P_{tot}: 275–463 mg/L. Nutrient deficiency must be considered during biological aerobic treatment.

The solid phase, whose humidity is about 80%, has been used for the gross calorific value (GCV) and the lower calorific value (LCV) assessment. Mean values of such characteristics are: GCV: 31,000 kJ/kgtotalsolids, LCV: 7,300 kJ/kgtotalsolids. The

aforementioned data show that the OMW solid phase could be properly used with olive husks in an incineration plant.

At the start-up, the aerobic reactor was filled with centrifuged OMW, continuously aerated and maintained at a constant temperature (30 °C) and pH value (4.5–4.7). In the pilot-batch reactor the biomass growth was assessed by the total COD and the TSS mass balance. During the first 20 days the reactor operated in batch mode, in order to allow fungi development and no other organic substrates were added. Afterwards, pure polyphenol compounds were added in order to enhance the fungi activity. Microscope observations (Figure 1), respirometric tests and experimental measures of COD and TP removal efficiency highlighted the development of a fungi biomass after 30 days (end of the first experimental period). During the second experimental period (90 days) the aerobic reactor was conducted in a fed-batch mode. The duration of the clarification phase decreased from 1 day to 3 hours when structured sludge flocs formed. The fed OMW volumes increased from 0.3 to 1.8 L. During the last 20 days of the second experimental period, mixed liquor was extracted from the reactor instead of the clarified supernatant, in order to maintain the TSS concentration around 15 g/L without biomass retention.

The results did not show any variation throughout the experiment for either the TP or the filtered COD removal efficiency. Moreover, the fungi biomass did not show any inhibiting effect due to phenolic compounds when the loaded volumes were increased. OMWs present an organic fraction very slowly biodegradable (10–20% of the fCOD). TP and fCOD trends obtained during the entire experimental period are shown in Figure 2 while the removal efficiency reached by fungi is summarized in Table 3.

TP contents measured by HPLC mass analysis were in agreement with the Folin-Ciocalteau values for the centrifuged OMW, while the two methods greatly differ for the fungi-treated samples. The differences could be explained by considering possible interferences in, the spectrophotometric method due to the intermediates, which come from the phenolic compounds degradation operated by fungi enzymes. Similar differences were found on analysing OMW Fenton's treated samples (Bettazzi *et al.*, 2006, Caffaz *et al.*, 2006). Table 4 summarizes the mean values measured using the two aforementioned methods.

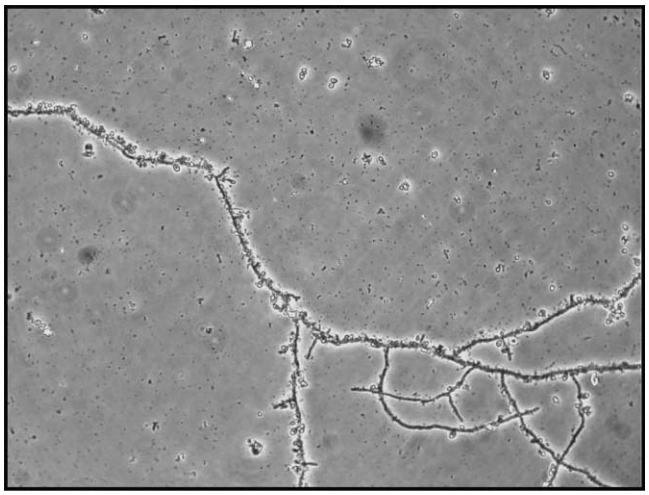

Figure 1 Fungi biomass observed by microscope after 30 days since the bioreactor start-up

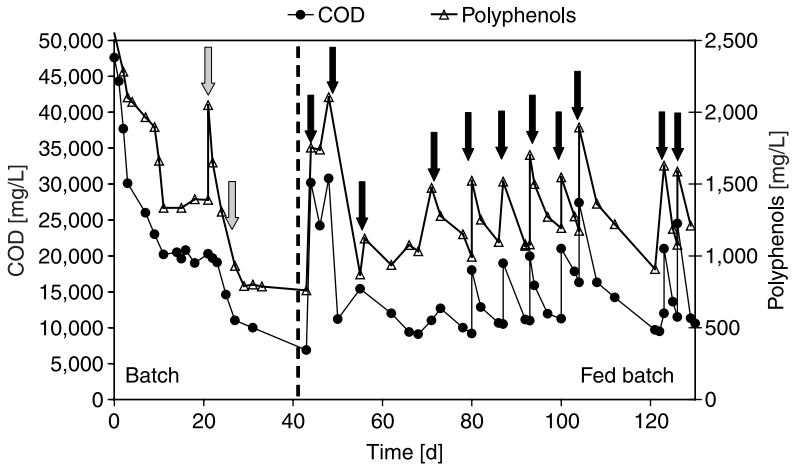

Figure 2 COD and TP concentration trends in the aerobic batch reactor. The grey arrows indicate pure phenolic compounds injections in the reactor, the black arrows indicate OMW injections

BOD_{10-20} tests, respirometric trials and anaerobic batch tests, performed on the aerobic supernatant using biomasses taken from a municipal WWTP, confirmed the refractory nature of the residual COD. In order to enhance the biodegradability of the residual organic and phenolic content after the fungi treatment, and according to literature studies (Beltran-Heredia *et al.*, 2000), ozone post-treatment has been tested. Ozonation trials were carried out in a 400 mL batch reactor (glass Mariotte vessel) with a O_3 mass flow rate set at 7.5 mg/min and contact time varying from 1 to 4 hours. COD and TP removal percentages mean values during ozone oxidation are shown in Table 5.

The results highlighted that the removal efficiency was only slightly affected by the ozone dosage, reaching a maximum value of 24% for COD and 92% for TP. Moreover, it was assessed by respirometric and BOD_{20} tests, carried out both with fungi biomass and activated sludge, that ozone treatment did not increase the biodegradability of the residual COD.

Concerning the respirometric tests, Figure 3 shows the OUR curve during a fungi degradation of a 100 mL OMW sample. This kind of test was performed with a high S_0/X_0 ratio (considered as the initial ratio, after the wastewater injection, between the filtered COD of wastewater, S_0, and the sludge COD, X_0). The curve shows the complexity of the OMW sample due to the presence of several organic substrates.

Table 3 COD and TP (Folin-Ciocalteau method) removal efficiency

	Centrifuged OMW (mg/L)	Supernatant mean value (mg/L)	Supernatant minimum value (mg/L)	Maximum removal (%)
fCOD	50,940	10,907	6,881	86
Total polyphenols	2,550	1,060	760	70

Table 4 TP evaluation by HPLC and Folin-Ciocalteau methods

	Centrifuged OMW (mg/L)	OMW after fungi degradation (mg/L)	Removal efficiency (%)
HPLC	2790.05	278	91.2
Folin-Ciocalteau	2,550	760	69.6

Table 5 Aerobic bioreactor effluent COD values during ozone oxidation

Ozone dosage (gO_3/gCOD)	0	0.125	0.25	0.375	0.5
Contact time (h)	0	1	2	3	4
COD (mg/L)	9,033	7,233	7,050	6,866	6,860
COD removal efficiency (%)	–	20	21.9	24	24
TP (mg/L)	1635.3	288	207	142	128
TP removal efficiency (%)	–	82.3	87.3	91.3	92.1

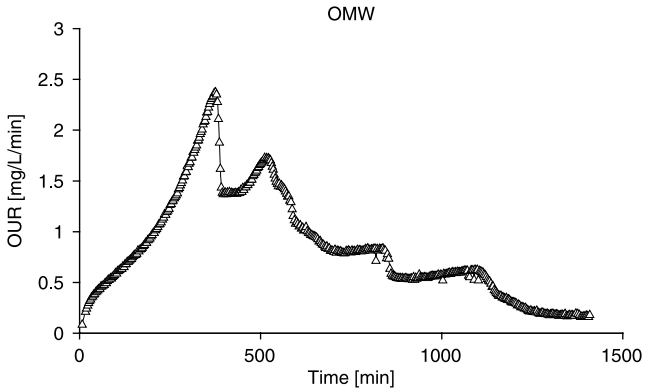

Figure 3 Respirometric test obtained adding 100 mL of OMW to the fungi biomass

As regards the specific growth rate of fungi biomass on rbCOD (readily biodegradable COD) contained in OMW, a μ_{max} value of $8.57\,d^{-1} \pm 0.5$ has been found, estimated by considering the initial exponential trend of the respirometric curve. An endogenous decay coefficient b_H of fungi biomass equal to $0.81\,d^{-1} \pm 0.08$ at 30 °C was evaluated by the exponential OUR decreasing trends measured during aeration without any substrate addition. The BOD_{st} (obtained with a low S_0/X_0 ratio ranging between 1:40 and 1:30) of centrifuged OMW evaluated on fungi biomass (about 18,000 mg/L) was twice as much as the BOD_{st} obtained with non-acclimated activated sludge (about 90,00 mg/L). The experimental results are shown in Figure 4 and Table 6. As regards the activated sludge biomass, Y_H value has been calculated by measuring the filtered COD degraded during a respirometric test. Referring to the fungi biomass, Y_H value has been set equal to the mean value of the results obtained by using the pure phenolic compounds.

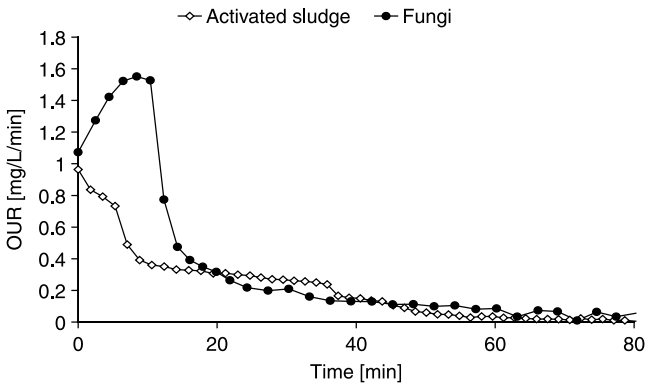

Figure 4 OMW respirometric tests with fungi and activated sludge biomass

Table 6 Respirometric test results

Sample (0.45 μm filtered)	Biomass	COD (mg/L)	BOD_{st} (mg/L)	Y_H (COD/COD)	BCOD/COD (%)
Centrifuged OMW	Activated sludge	51,250	8,879	0.65	49.5
Centrifuged OMW	Fungi	50,940	17,928	0.53	74.8

Figure 5 shows the respirograms obtained adding p-coumaric acid and gallic acid to the fungi biomass. These respirograms highlight the fungi capacity to completely degrade the organic acids. No accumulation of filtered COD and TP was observed at the end of these respirometric tests. In order to assess the complete biodegradability of pure compounds, the fungi biomass was centrifuged and resuspended in high quality drinking water (without chlorine residual) before the respirometric tests. Macro-nutrients (ammonium and phosphate) were added. Kinetic and stoichiometric parameters relative to phenolic compound degradation have been determined by appropriate respirometric tests (T = 30 °C) and are summarised in Table 7.

Moreover, respirometric tests have been carried out in order to compare the centrifuged OMW biodegradability using the fungi biomass and an activated sludge biomass taken from a municipal WWTP. Figure 6 shows two respirograms obtained with repeated injections of gallic acid (60–180 mg) on activated sludge and on fungi biomass. As far as municipal activated sludge is concerned, the residual COD was about 53% of the total

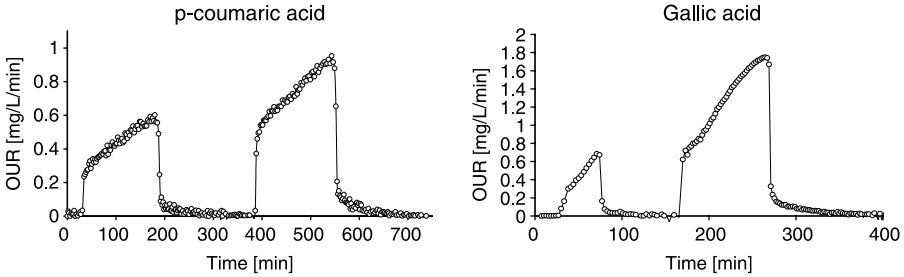

Figure 5 Respirometric tests obtained adding p-coumaric acid (left) and gallic acid (right) to the fungi biomass

Table 7 Kinetic and stoichiometric parameters of fungi biomass

Polyphenols	μ_{max} (d^{-1})	K_S (mgCOD/l)	Y_H (COD/COD)
Gallic acid	5.3 ± 0.5	5.2 ± 1.1	0.58 ± 0.05
p-coumaric acid	5.9 ± 0.7	1 ± 0.3	0.48 ± 0.05

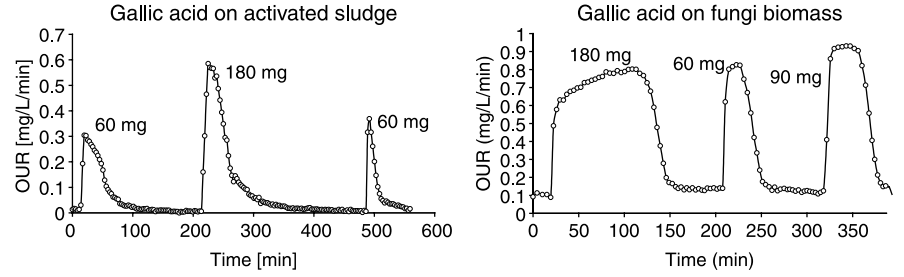

Figure 6 Repeated injections of gallic acid on activated sludge (left) and on fungi biomass (right)

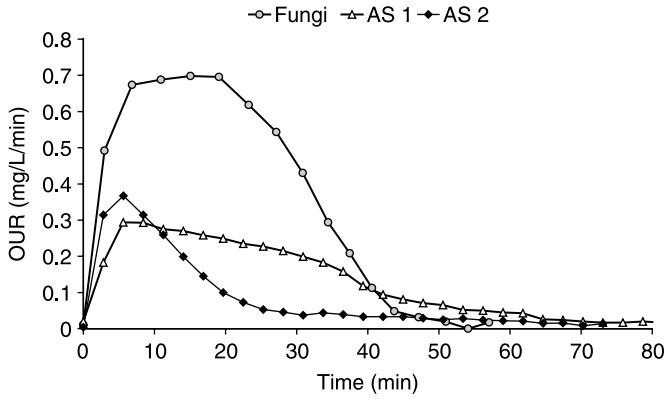

Figure 7 Respirograms obtained with 60 mg gallic acid injections on activated sludge and fungi biomass

COD added, confirming the recalcitrant nature of phenolic compounds. Moreover, after repeated gallic acid injections, a blue hue was observed in the activated sludge supernatant after a 0.45 μm filtration.

Figure 7, which shows the respirograms of two sequential injections (AS1 and AS2) of gallic acid (60 mg) on a municipal activated sludge sample, compared to an equal addition on the fungi biomass, also evidences the different biodegradability and the inhibiting effect determined by phenolic substances. These graphs show that the BOD_{st} of the gallic acid solution (6 g/L), evaluated using activated sludge, decreases after repeated additions from 1.6 to 1.1 g/L, while the fungi biomass shows a constant BOD_{st} value equal to 2.8 g/L.

Conclusions

OMW traditional disposal on the soil, even if allowed by the law in force, could constitute a serious environmental problem, causing phytotoxicity and reduction of permeability. As conventional processes are not suitable to properly treat OMW, the present research was aimed at investigating the use of a specialised aerobic biomass, composed by yeasts and fungi, grown on centrifuged OMW. Keeping OMW under controlled conditions (at 35 °C and pH 4.5) it was possible to develop a fungi biomass not subjected to inhibiting and toxic effects due to the phenolic content. The aerobic OMW degradation obtained using this biomass reached a COD and TP removal efficiency of 86 and 70%, respectively, according to the optimal literature results. The significant residual organic content (7–10 gCOD/L) still present after the fungi treatment showed a very low degree of degradability. Ozone oxidation was very efficient at degrading the residual TP content, whereas it did not improve the COD biodegradability.

Respirometric and BOD_{10-20} tests using activated sludge taken from a municipal WWTP showed a COD removal efficiency lower than the one due to the fungi biomass. The BCOD value of OMW obtained by using a non-acclimated activated sludge was about 25–35% lower than the one obtained with the fungi biomass. Non-acclimated activated sludge is able to only partially degrade the phenolic compounds, while a rapid and complete removal was obtained with the fungi biomass.

Possible future developments of the present research could be identified in the following issues: (1) the individuation of the selected fungi biomass; (2) the degradation of other phenolic compounds by the fungi biomass; (3) the analysis of the intermediates coming from the polyphenol degradation, which could cause interferences in the analytic methods; (4) the investigation of the soluble non-biodegradable residual COD after the fungi treatment.

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