



Phenolic removal in a model olive oil mill wastewater using *Pleurotus ostreatus* in bioreactor cultures and biological evaluation of the process

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Abstract

Pleurotus ostreatus grown in bioreactor batch cultures in a model phenolic wastewater (diluted and sterilized olive oil mill wastewater—OMW), caused significant phenolic removal. Laccase, the sole ligninolytic enzyme detected in the growth environment, was produced during primary metabolic growth. The bioprocess was simulated with the aid of a mathematical model and the parameters of growth were determined. When the fungal biomass was increased in the reactor (during repeated batch experiments) the rate of reducing sugars consumption progressively increased, but a phenolic fraction seemed of being strongly resistant to oxidation. The toxicity of OMW against the seeds of *Lepidium sativum* and the marine Branchiopoda *Artemia* sp. was significantly decreased after biotreatment. On the contrary, the toxicity against the freshwater Branchiopoda *Daphnia magna* was not affected by the treatment, whereas on the soil and freshwater sediments Ostracoda *Heterocypris incongruens* was slightly decreased. Both treated and untreated OMWs, used as water for irrigation of lettuce and tomato plants, did not significantly affect the uptake of several nutrients by the cultivated plants, but resulted in a decrease in the plant yields, which was minimized when high OMW dilutions were used. As a conclusion, *P. ostreatus* is able to reduce phenolic content and toxicity of sterilized OMW, in bioreactor cultures. However, high OMW dilutions should be used, and/or additional treatment should be applied before use of the OMW in the environment, e.g. as water for irrigation. Further research should be done in order to transfer this technology under industrial conditions (e.g. by using unsterilized OMW).

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Abbreviations: OMW—Olive mill wastewater; LiP—Lignin peroxidase; MnP—Manganese-dependent peroxidase; Lac—Laccase (Phenol oxidase); GI—Germination index; S (g l^{-1})—Reducing sugars concentration in OMW; Ph (g l^{-1})—Phenolic compounds concentration; Ph_r (g l^{-1})—Phenolic compounds resistant to oxidation; x (g l^{-1})—Biomass concentration; $Y_{x/s}$ (g g^{-1})—biomass yield on S ; r_{max} (h^{-1})—Maximum specific growth rate; m_s (h^{-1})—Specific rate of S consumption for biomass maintenance; $r_{\text{Lac(max)}}$ (h^{-1})—Maximum specific rate of phenolics oxidation by Lac; r_{DL} (h^{-1})—Specific rate of Lac destruction; K_m (g l^{-1})—Saturation constant of laccase; α (Units g^{-1}) and β (Units $(\text{g h})^{-1}$)—parameters that correlate Lac production rate to microbial growth.

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1. Introduction

Phenolic wastes are nowadays produced in significant quantities from several industrial processes (e.g. coal conversion, petroleum refining, etc.). Especially in Mediterranean countries olive oil mill wastewater (OMW), which is produced in quantities more than 30 million m³ per year, is an important phenolic waste. Its phenolic compounds are responsible for its black color and, along with its high content in soluble salts, for its toxic properties for the ecosystems [1,2].

The research on OMW valorization is presently focused on the degradation of the phenolic compounds, since their breakdown is considered as the limiting step in the biotreatment of OMW [2,3]. The degradation of these compounds by using *Phanerochaete* sp. and other molds and yeasts has been investigated by several research groups using sterilized OMWs and it has been demonstrated that phenolic compounds concentration and OMW toxicity significantly decreased, whereas the color changed from black to bright yellow [2–10]. It was suggested that in the case of *Ph. chrysosporium* lignin peroxidase (LiP, EC 1.11.1.14) was responsible for the degradation of phenolic compounds in OMW [6,8]. In *Ph. flavido-alba*, the enzymes involved in the OMW decolorization process were manganese-dependent peroxidase (MnP, EC 1.11.1.13) and phenol oxidase (Laccase, EC 1.10.3.2), whereas LiP was not detected in the growth environment [5]. In *Pleurotus ostreatus*, laccase (Lac) was considered of being responsible for the oxidation of phenolic compounds and aromatic amines, by reducing molecular oxygen to water [2,11,12]. However, MnP and Mn-independent peroxidase activities were, additionally to Lac, detected during treatment of effluents from green olive debittering process with *P. ostreatus* [13]. On the contrary, only Lac was detected in synthetic dyes media during treatment with *P. pulmonarius* [14].

In this work, the ability of selected *P. ostreatus* strains to produce ligninolytic enzymes and to remove phenolic compounds from a sterilized OMW, used as a model phenolic wastewater, was evaluated in bioreactor cultures. The toxicity of untreated and treated OMWs, against several freshwater and marine arthropods, as well as against *Lepidium sativum* seeds was determined. Finally, the effect of the treated effluent, considered as water for irrigation of lettuce and tomato plants grown in a greenhouse, on plant yields and soil properties was discussed.

2. Materials and methods

2.1. Biological material and culture conditions

P. ostreatus, strains LGAM P113 and P115 from the culture collection of the Laboratory of General and

Agricultural Microbiology, were used. They were isolated in central Greece forests from *Abies cephalonica* woods. The strains were maintained at $T=4\pm1^{\circ}\text{C}$ on Potato Dextrose Agar (PDA, Plasmatec laboratory products Ltd., Dorset, UK) slants, and routinely subcultivated every 3 months.

OMWs used were obtained from two three-phase decanter manufactures of the Prefectures of Fthiotida and East Attiki, in central Greece. They were immediately transported to the laboratory and frozen at -20°C .

Kinetics on solid media were conducted in duplicate, in plates containing 10 ml of OMW diluted in water, and 10% (w/v) agar (Plasmatec), sterilized at 121°C for 20 min. The plates were inoculated in the center with a 6-mm diameter dish, of earlier uniformly colonized PDA. Bioreactor cultures were conducted in a Bioengineering NG (Wald, Switzerland) Benchtop Fermenter (Type ALF, active volume 3 l). Agitation rate was 200 ± 2 rpm and aeration 1 VVM. Initial pH was 6.0 ± 0.3 . pH regulation and addition of antifoam was not required. Flask cultures, used as inocula, were conducted in 250-ml conical flasks containing 50 ml of OMW diluted 50% in water. They were inoculated with mycelia developed on PDA, and incubated for 7 days in an orbital incubator (Gallenkamp, England) at agitation rate of 110 rpm. Sterilization was done in an autoclave at 121°C for 45 min (in the case of reactor) or 20 min (in the case of flasks). All cultures were done at $T=26\pm1^{\circ}\text{C}$.

2.2. Analyses of OMW and enzyme activities

Reducing sugars were determined using the Somogyi method [15]. Determination of phenolics and protein was carried out in the culture-filtered supernatant as described elsewhere [12].

Lac activity was determined using syringaldazine as a chromogenic substrate [16]. The assay mixture contained 0.1 ml of 1 mM syringaldazine solution in 95% ethanol, 1.7 ml of 0.1 M phosphate buffer (pH 6.8) and 0.2 ml of filtered supernatant (crude enzyme), appropriately diluted in distilled water in order to obtain a linear with time activity. Syringaldazine oxidation was followed at 525 nm. LiP activity was determined using veratryl alcohol as substrate [17]. The assay mixture contained 2 mM veratryl alcohol, 0.4 mM H₂O₂ in 50 mM sodium tartrate buffer (pH 6.8) and 0.2 ml of filtered supernatant. Veratryl alcohol oxidation was followed at 310 nm. MnP activity was determined using MnSO₄ as substrate [18]. The assay mixture contained 0.5 mM MnSO₄, 0.5 mM H₂O₂ in 50 mM sodium malonate buffer (pH 4.5) and 0.2 ml of filtered supernatant. Oxidation of Mn²⁺ was followed at 270 nm. All enzyme activities were done in triplicate and expressed in Units l⁻¹ [μmol (min l)⁻¹]. Assays were monitored with a Hitachi (model U-2001) spectrophotometer (Hitachi

Instruments Inc., Danbury, USA), at $T=25^{\circ}\text{C}$ and atmospheric oxygen conditions.

2.3. Toxicity assays

OMW phytotoxicity assays, carried out in triplicate, were performed using *Lepidium sativum* seeds [12]. Toxicity assays on Arthropoda were performed using biological tests developed by the research team of Professor Persoone (University of Ghent, Belgium). Toxicity test for freshwater was performed using *Daphnia magna* (DAPHNOTOXKIT, Creasel Ltd., Deinze, Belgium), while *Heterocypris incongruens* was used to estimate the toxicity for freshwater sediments and soils (OSTRACODTOXKIT, MicroBioTests Inc., Deinze, Belgium). Estuarine/marine toxicity screening test was carried out using *Artemia* sp. (ARTOXKIT, Creasel Ltd.). OMW toxicity on *Artemia* sp. and *D. magna* was quantitatively estimated using parameters 24h-LC50 and 48h-LC50, respectively, which represent the wastewater concentration in which mortality reaches to 50% of the initial population. Mortality of *H. incongruens* was estimated after 6 days exposure of the animals to contaminated river sediments. Additionally, the inhibitory effect of OMW on the growth of *H. incongruens* was evaluated by measuring the length of the animals grown in the presence of various concentrations of OMW. The inhibition was estimated as

$$\% \text{ Growth Inhibition} = 100 - \frac{\Delta L_{\text{OMW}}}{\Delta L_{\text{saline water}}} \times 100,$$

where ΔL is the difference of the means of animals' lengths before and after incubation in the presence of OMW or saline water used as a blank.

All toxicity tests were carried out in triplicate and the results were corrected according to the arthropods' mortalities observed in the appropriate control.

The inhibitory effect of the treated OMW on the growth of *P. ostreatus* was estimated by measuring the radial growth of *Pleurotus* colonies grown in Petri dishes on a solid medium containing 1.5% agar, 50% untreated OMW and 50% treated OMW diluted in distilled water.

2.4. Plant and soil chemical analyses

The suitability of the treated OMW as water for irrigation was tested in a greenhouse pot experiment, using lettuce (*Lactuca sativa*) and tomato (*Lycopersicon esculentum*) plants. During the experiment, temperature varied between 15°C and 22°C and humidity 70–85%. When temperature decreased less than 15°C , the environment was kept warm using an electrical heater. Lettuce was grown for 86 and tomato for 105 days on two sandy clay loamy soils weighing 1.1 and 1.3 kg pot⁻¹, respectively. The plants received nutrients such as N (as NH_4NO_3) at 5 doses of 100 mg N kg⁻¹ of soil each,

P (as KH_2PO_4) at 1 dose of 160 mg P kg⁻¹ of soil at the beginning, K (as K_2SO_4) at 5 doses of 100 mg K kg⁻¹ of soil each, Ca (as $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$) at 5 doses of 50 mg Ca kg⁻¹ of soil each, Mg (as $\text{MgSO}_4 \cdot \text{H}_2\text{O}$) at 5 doses of 25 mg Mg kg⁻¹ of soil each and Zn (as $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) at 5 doses of 5 mg Zn kg⁻¹ of soil each. Plants were irrigated as needed with water (W), untreated (UTOW) or treated (TOW) OMW with *P. ostreatus*. Dry weight and concentration of nutrients in the above ground plant parts were determined at the end of the experiment, according to Chapman and Pratt [19]. Several soil parameters, such as electrical conductivity (EC), pH, organic matter and nutrient concentrations were determined at harvesting [20].

3. Results

3.1. Treatment of sterilized OMW with *P. ostreatus*

Biological treatment of OMWs (used at two initial phenolics concentration) was conducted in batch bioreactor cultures. In all cases, *P. ostreatus* grew well in sterilized and diluted OMW without any addition of nutrients and any specific pre-treatment, and caused significant phenolic removal during growth. However, in low phenolic content OMWs, the percentage of the phenolics removed was greater. This finding was confirmed by several experiments in flask cultures of both P113 and P115 strains in OMWs having various initial phenolics concentrations, ranging from 0.15 to 4.5 g l⁻¹. In these trials, phenolic removal ranged from 80% (when low phenolic content OMW was used) to around 50% (when high phenolic content OMW was used). Therefore, in all cases a phenolic fraction remained untreated in the OMW solution. Tsioulpas et al. [12] have reported that phenolic compounds remaining in solution were more toxic against *Lepidium sativum* seeds than the initial phenolics. Consequently, it was supposed that some of the oxidation products (e.g. phenoxy radicals) probably had an inhibitory effect on *P. ostreatus* growth. However, this assumption has to be rejected since as it was demonstrated in a separate experiment, the radial growth of *P. ostreatus* was not affected by the treated OMW, present in the growth environment at various concentrations (data not shown).

Lac was the responsible enzyme for phenolics removal in OMW, since activities of other enzymes related to phenolics degradation (LiP, MnP) were not detected during treatment. Additionally, it was found that the absorption of phenolics on killed fungal mycelia (at $121^{\circ}\text{C}/30\text{min}$) was only 105 mg g⁻¹ of dry mycelia, corresponding to around 8% of the initial phenolics.

According to the above-mentioned findings a mathematical model, which is focused on the consumption of

Table 1

Equations used to model biotreatment of OMW using *P. ostreatus*

Microbial growth rate	$\frac{dx}{dt} = rx, \quad r = r_{\max} \left(1 - \frac{x}{x_{\max}} \right)$
Reducing sugars consumption rate	$-\frac{dS}{dt} = \frac{dx}{dt} \frac{1}{Y_{x/S}} + m_S x$
Laccase synthesis rate	$\frac{dLac}{dt} = (a + b)x - r_{DL} Lac$
Phenolics removal rate	$-\frac{dPh}{dt} = r_{Lac(max)} \frac{Ph - Ph_r}{K_m + Ph - Ph_r} Lac$

reducing sugars by the fungus, as well as on the phenolics removal (oxidation), was developed (Table 1). As the limiting growth factor was unknown, a Verhulst-type equation, in which microbial specific growth rate was governed by cell density, was used. It was assumed that Lac synthesis occurred during both primary and secondary metabolic growth. However, parameter b (that is referred to the secondary growth) converged, during parameter optimization process, to very low values. Concerning Lac activity, several expressions of competitive and non-competitive inhibition (by the phenolic compounds) were used to model this activity in OMW, but they were not appropriate to correctly describe the removal of phenolics. Finally, a modified Michaelis–Menten equation, containing an empirical parameter named Ph_r , was introduced in order to reduce the available substrate of Lac.

The model was integrated by the Runge–Kutta method, whereas parameter values were optimized by the least-squares method. The Marquardt iterative search algorithm (initial $\lambda = 0.001$) was used to determine the parameter values that minimized the residual sum of squares. Optimized parameter values of the bioprocess were: $Y_{x/S} = 0.063 \text{ g g}^{-1}$; $x_{\max} = 1.07 \text{ g l}^{-1}$; $a = 143.4 \text{ U g}^{-1}$; $r_{\max} = 0.02 \text{ h}^{-1}$; $r_{Lac(max)} = 0.0027 \text{ h}^{-1}$; $K_m = 1.256 \text{ g l}^{-1}$; $Ph_r = 1.408 \text{ g l}^{-1}$. Since biomass concentration was precisely measured only during the 5–7 first days of culture (due to the fungal growth heterogeneities), parameter x_{\max} , may have been underestimated. The model was fitted well on the experimental data (e.g. Fig. 1a), having a ratio $\chi^2/\text{degree of freedom} < 24$. However, the predictive ability of the model was restricted to similar initial phenolics concentration runs, since initial phenolics concentration affected the efficiency of the biotreatment (e.g. phenolics removal).

In order to increase the fungal biomass concentration in the bioreactor, repeated batch fermentations were carried out with the fungal mycelia remaining in the reactor when the treated OMW was discharged. Accordingly, biomass concentration increased from

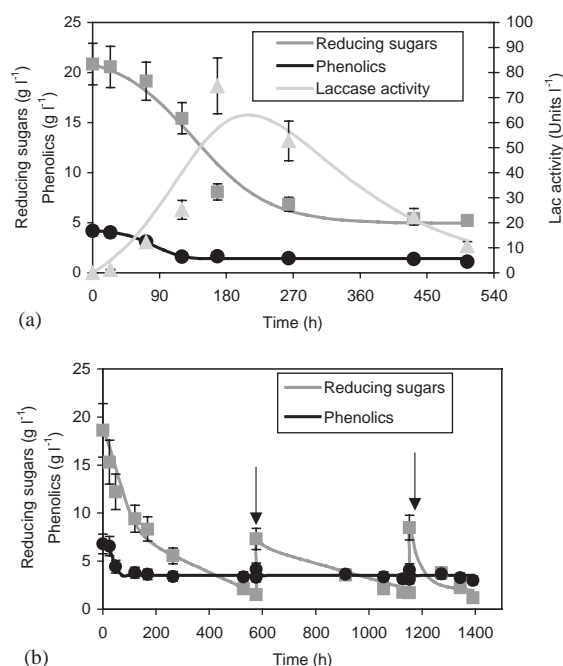


Fig. 1. Treatment of sterilized OMW in batch and repeated batch bioreactor modes. (a): Model fitted on experimental data derived from a bioreactor run of *P. ostreatus* 113 growing in OMW with initial concentration of phenolics = 4.18 g l^{-1} . Bars represent similar standard errors to those experimentally observed ($< 8\%$). (b): Repeated bioreactor runs of *P. ostreatus* 113 growing in OMW with initial concentration of phenolics = 6.57 g l^{-1} (arrows indicate time in which the waste in the reactor was renovated).

1 g l^{-1} (first batch) to 1.8 g l^{-1} (third batch), resulting in an increase of the reducing sugars consumption rate; however, a fraction of phenolics seemed of being strongly resistant to oxidation (Fig. 1b).

Additional experiments using non-sterile conditions were done in both flask and bioreactor cultures. However, in these conditions poor fungal growth was observed and reduction of phenolics was less than 20% of the initial phenolics (data not shown).

3.2. Toxicity of OMW treated by *P. ostreatus*

Toxicity of OMW on the seeds of *L. sativum* was significantly decreased after treatment with the strains LGAM P113 and P115, especially when high dilutions of OMW were used (Table 2). On the contrary, the OMW toxicity on the Branchiopoda (Diplostraca) *D. magna* was not affected by the treatment with *Pleurotus* strains (Fig. 2a). In this case, the parameter 48h-LC50 remained constant, equal to 2.5, while mortality of the population reached zero at very low OMWs concentration ($< 2\%$). The Branchiopoda (Anostraca) *Artemia* sp.

Table 2

Toxicity of untreated and treated OMWs against *Lepidium sativum* seeds estimated using the parameter of “Germination Index” (GI)^a

Dilution (%) in water	Phenolics content (g l ⁻¹)	G.I.
<i>Untreated OMW</i>		
50	4.42±0.36	0.0
37.5	3.32±0.30	0.0
25	2.21±0.10	0.0
<i>OMW treated with P. ostreatus 113</i>		
50	0.66±0.02	8.7±2.2
37.5	0.50±0.02	27.2±5.3
25	0.33±0.01	50.5±3.9
<i>OMW treated with P. ostreatus 115</i>		
50	0.61±0.02	16.0±1.8
37.5	0.46±0.00	37.5±9.7
25	0.31±0.05	62.1±13.2

$$^a \text{GI} = \frac{\text{rootlet's length in OMW}}{\text{rootlet's length in water}} \frac{\text{Germination in OMW}}{\text{Germination in water}} 100.$$

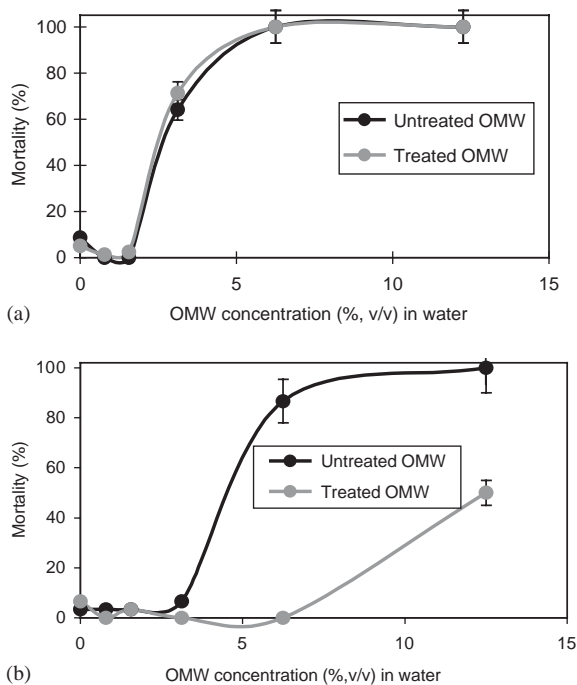


Fig. 2. Mortality (%) of *Daphnia magna* (a) and *Artemia* sp. (b) growing in the presence of untreated (concentration of phenolics = 4.18 g l⁻¹) and treated (concentration of phenolics = 1.13 g l⁻¹) with *P. ostreatus* OMWs.

was more resistant against treated OMW, as the parameter 24h-LC50 significantly increased from 4.5 to 12.5 (Fig. 2b). The OMW concentration for zero mortality was increased from 3% (in untreated

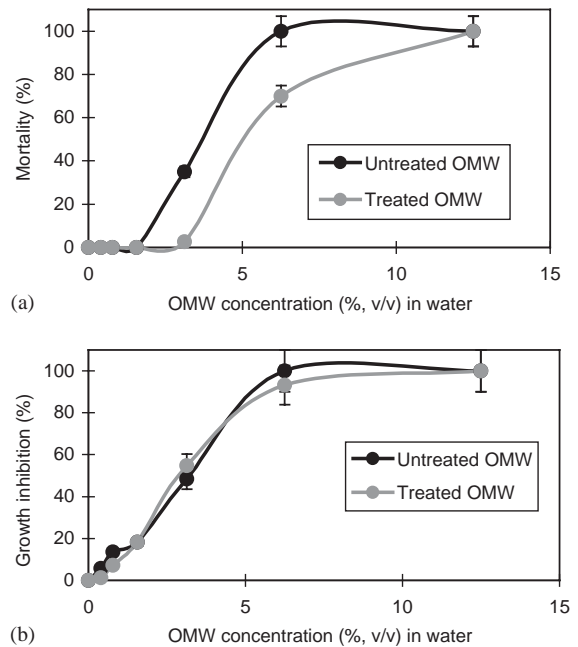


Fig. 3. Mortality (%) (a) and growth inhibition (%) (b) of *Heterocypris incongruens* growing in the presence of untreated (concentration of phenolics = 4.18 g l⁻¹) and treated (concentration of phenolics = 1.13 g l⁻¹) with *P. ostreatus* OMWs.

OMW), to 6% (in the treated OMW), respectively. Toxicity on the Ostracoda *H. incongruens* was slightly decreased after treatment (Fig. 3a). The concentration of OMW that caused 50% mortality was increased from 3.7% (in the untreated OMW) to 5.0% (in the treated OMW), while the OMW concentration in which the mortality of *Heterocypris* population reached to zero was increased from 2% (in untreated OMW) to 3% (in the treated OMW). However, the inhibitory effect of OMW on the growth of *H. incongruens* was not affected by the treatment (Fig. 3b).

3.3. Agricultural evaluation of OMW used as water for irrigation of lettuce and tomato plants

The untreated and treated OMWs (both diluted 50% in water), used in this experiment, contained phenolics 1.51 and 0.48 g l⁻¹, respectively and had EC = 4.51 ± 0.33 mS cm⁻¹ (EC value was not affected by the biotreatment). This EC value was very high to permit the use of OMWs as water for irrigation without dilution (EC < 2.5 mS cm⁻¹ is often recommended for crops irrigation). Both treated and untreated OMWs, used in dilutions 50%, resulted in a decrease in the plant yields (compared with the water + fertilization treatment) (Table 3). Indeed, the treated OMW had a more negative effect than the untreated OMW in both crops,

Table 3

Dry weight yield and nutrient concentration of lettuce and tomato plants irrigated with untreated and treated OMW (with *P. ostreatus*) diluted in water, and soil EC and pH at harvesting

Irrigation treatment ^a	Total DW of tops (g) ^b	EC (mS cm ⁻¹)	pH	Nutrient concentration (% of D.W.) ^b				
				N	P	K	Ca	Mg
<i>LETTUCE</i>								
W–Fertilization	4.88 a	0.97 a	7.6 b	2.3 a	0.397 a	5.1 a	1.92 c	0.47 b
W + Fertilization	9.53 c	2.66 b	7.2 a	4.4 c	0.562 b	6.7 b	1.79 c	0.47 b
UTOW/50% + Fertilization	6.15 ab	6.44 e	7.3 a	4.1 bc	0.536 b	9.2 c	0.97 a	0.37 a
TOW/50% + Fertilization	5.55 a	4.94 d	7.9 c	3.9 b	0.525 b	9.1 c	1.42 b	0.38 a
TOW/25% + Fertilization	6.14 ab	4.11 c	7.9 c	3.9 b	0.671 c	8.9 c	2.01 c	0.46 b
TOW/12,5% + Fertilization	7.59 b	3.43 c	7.7 b	4.1 bc	0.620 bc	8.4 c	1.85 c	0.45 b
<i>TOMATO</i>								
W–Fertilization	6.88 b	1.88 a	7.8 c	2.6 a	0.180 a	4.9 a	2.33 a	0.36 a
W + Fertilization	10.97 c	5.11 b	7.3 a	3.6 c	0.320 bc	5.8 ab	3.43 b	0.43 ab
UTOW/50% + Fertilization	7.48 b	12.90 d	7.6 b	3.0 ab	0.263 ab	8.2 d	1.97 a	0.40 ab
TOW/50% + Fertilization	3.98 a	8.02 c	8.3 e	3.4 bc	0.410 cd	6.7 bc	1.95 a	0.40 ab
TOW/25% + Fertilization	4.91 a	6.72 c	8.1 d	3.3 bc	0.441 cd	7.6 cd	1.86 a	0.44 ab
TOW/12,5% + Fertilization	7.85 b	7.41 c	7.9 c	3.3 bc	0.384 c	7.4 cd	3.41 b	0.50 b

^a Untreated OMW (UTOW) (50% in water) contained phenolics 1.51 g l⁻¹; treated OMW (TOW) (50% in water) contained phenolics: 0.48 g l⁻¹. EC values of both UTOW and TOW were 4.51 ± 0.33 mS cm⁻¹. Plain water (W) with (+) or without (–) fertilization was used as control.

^b The values represent the mean of four replications (SD < 15%). Differences of means followed by different letters are statistically significant at *P* = 0.05. The experimental data were treated according to Duncan's test [29].

especially on tomato plants where the positive effect of the fertilization was minimized. This negative effect was minimized when high OMW dilutions were used.

Plant analysis showed that the untreated OMW (50% in water) did not significantly affect the concentration of N and P in lettuce and P and Mg in tomato plants, but negatively affected Ca and Mg concentration in lettuce plants and N and Ca concentration in tomato plants (in comparison with the water + fertilization treatment) (Table 3). On the contrary, the untreated OMW (50% in water) increased the concentration of K in both plants significantly. When treated OMW (diluted 50% in water) was used, the concentration of Ca in lettuce plants and P in tomato plants increased, and K concentration in tomato plants decreased, whereas the concentration of the other nutrients was not affected (in comparison with the untreated OMW treatment).

Soils, analyzed at harvesting, were enriched in several nutrients such as N, P, K and Mg but also in Na due to the irrigation with treated and untreated OMWs, while organic matter (%) was not significantly affected by treatments (data not shown). EC was significantly increased, especially when untreated OMW was used, while pH was increased only when treated OMW was used (Table 3). The EC values found in the soil used for growth of tomato plants were high, even for salt-resistant plants. However, it seemed that the high EC values, found in the soil, did not dramatically decrease

the plant yield (e.g. comparison of treatments TOW 50% in water with UTOW 50% in water).

4. Discussion

Although OMW is a toxic substrate for many microorganisms, *P. ostreatus* was able to grow on sterilized OMW and to reduce its phenolic content in bioreactor cultures. Specific growth rate and biomass yield were satisfactory, as compared with the respective parameters, estimated from data given in the literature for various white-rot fungi growing in flask [3,7,10,12] or bioreactor [21] culture modes. However, it is noted that sterilized OMW should be considered only as a model phenolic wastewater since sterilization may cause important physicochemical alterations on several compounds such as oxidation of phenolics and quinoid compounds followed by precipitation (e.g. around 25% of phenolics precipitated while the dissolved COD was slightly decreased after sterilization [3]). Consequently, when non-sterilized OMW was used, the reduction of phenolics was not satisfactory. Similar results have been reported by other authors, working under non-sterile conditions, with *P. ostreatus* [3] or *P. chrysosporium* [10].

From the three essential ligninolytic enzymes secreted from white-rot fungi [22], only Lac was detected during growth of *P. ostreatus* in OMW. Accordingly, Zilly et al.

[14] reported that Lac was the sole phenol-oxidizing enzyme in *P. pulmonarius*. Indeed, in the present report it was found that the synthesis of this enzyme occurred during primary metabolic growth, in agreement with the hypothesis that polyphenoloxidases are involved in the early modifications, preparing better conditions for the growing organisms [23]. Inversely, *Ph. chrysosporium*, a well-studied white-rot fungus, produced LiP and MnP, and degraded lignin during secondary growth [24]. However, Sayadi and Ellouz [6] suggested that phenolic removal by a strain of *Ph. chrysosporium* occurred either during primary or secondary metabolism, depending on the carbon source added in OMW.

The single-stage treatment of sterilized OMW using *P. ostreatus* reduced toxicity on seeds of *L. sativum* and on marine arthropods (*Artemia*), while toxicity on freshwater and soil arthropods was not significantly affected. However, in all cases the decrease of toxicity was not proportional to the phenolic removal in the OMW. Similar results were reported by Martirani et al. [2] when purified Lac was used, and by Tsioulpas et al. [12] when several strains of *P. ostreatus* were used. Accordingly, it seems that some of the oxidation products of the Lac reaction (e.g. quinonoids, phenoxy radicals) did not precipitate, probably due to the action of veratryl alcohol oxidase [25,26], and were more toxic than the original phenolics, since toxicity of some phenolics increases after oxidation. Additionally, in agreement with the results reported in the present study, elimination of phenolic compounds from the fermentation medium was higher at low phenolic content OMWs as compared with high phenolic content ones, during treatment of OMWs by various molds [3,10,12]. Recently, Kissi et al. [10] reported an excellent reduction of the OMW toxicity on *Bacillus cereus*, after treatment with *Ph. chrysosporium*.

The recycling of the OMWs and their use as water for irrigation in the agriculture is an attractive perspective for the Mediterranean countries in which water resources have been severely decreased in the last years. Additionally, the OMWs could be used, after treatment, as a natural plant fertilizer, since they contain many essential nutrients and microelements indispensable for plant growth. Although sterilization caused physico-chemical changes in several organic compounds, it did not affect, however, the soluble salts concentration in OMWs. Therefore, use of sterilized OMW (treated and untreated) as water for irrigation gives ideas about the existing possibilities to recycle this waste in agriculture. In the present report it was demonstrated that both untreated and treated model OMWs increased the uptake of many nutrients by lettuce and tomato plants. However, the high concentration of soluble salts in OMW has to be taken into account for preventing soil degradation, since soluble salts concentration was not affected by the biotreatment. Especially, the increase in

the available K in the soil and in K concentration in crops should be stressed. Similarly, Gallardo-Lara et al. [27] reported that OMW exhibited a considerable capacity for supplying K to ryegrass. The concentration of the exchangeable Ca in the soil was decreased probably due to the complementary-ion effect in the soil, as Na is released more readily in the soil solution than Ca does [28]. Consequently, the presence of increased amounts of exchangeable Na and Mg resulted in a decrease of exchangeable Ca and Ca concentration in both plants. Additionally, lettuce and tomato yields were decreased when plants were irrigated with OMW. This decrease was probably due to the high EC values found in the soils, which caused difficulties in water and nutrient uptake by the plants [28]. Therefore, high OMW dilutions should be used, and/or additional treatment of OMW should be applied (e.g. Fountoulakis et al. [3] have recently proposed an additional anaerobic treatment of OMW, previously treated with *P. ostreatus*).

5. Conclusions

P. ostreatus presented satisfactory growth and reduced the phenolic content of a model waste (sterilized OMW) in bioreactor cultures. This treatment resulted in a decrease of the OMW toxicity against the seeds of *Lepidium sativum* and the Arthropoda *Artemia* sp. In contrast, OMW toxicity against the Arthropoda *Daphnia magna* and *Heterocypris incognuens* was unaffected by the treatment. Treated and untreated OMWs used as water for irrigation of plants growing in pots did not significantly affect the uptake of various nutrients, but plant yields were decreased, probably due to the high OMWs salinity. Additional research should be done in order to transfer this technology in industrial conditions (e.g. by using unsterilized OMW).

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