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ScienceDirect
Ecotoxicology and Environmental Safety 70 (2008) 266–275
Ecotoxicology and Environmental Safety
www.elsevier.com/locate/ecoenv

Evaluation of olive oil mill wastewater toxicity on the mitochondrial bioenergetics after treatment with *Candida oleophila*

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Received 24 July 2007; received in revised form 1 November 2007; accepted 15 November 2007
Available online 11 February 2008

Abstract

In a previous work the ability of *Candida oleophila* to use phenolic compounds as sole carbon and energy source at high concentrations without an additional carbon source was reported. *C. oleophila* grown in bioreactor batch cultures in a diluted and sterilized olive oil mill wastewater (OMW) caused a significant decrease in the total tannins content but no significant alteration was observed in phenolic acid and fatty acid content. Both treated and untreated OMWs were tested to evaluate the capacity in interfering with mitochondrial bioenergetics. Mitochondrial respiration was not affected by treated OMW on the range of used concentrations, contrary to the untreated OMW. Furthermore, mitochondrial membrane potential and respiratory complexes were always significantly less affected by treated OMW in comparison with untreated OMW. However, supplementary treatment should be applied before OMW could be considered non-toxic.

Keywords: Olive mill wastewaters; Yeasts; Polyphenols; Tannins; Fatty acid; Biodegradation; Acute toxicity; Mitochondria; Bioenergetics

1. Introduction

The growing consumer demand for olive oil, as a consequence of its proved benefits for human health (Tuck and Hayball, 2002), has become a positive factor for higher productions of this natural fat. Olive oil extraction industries are mainly located in Mediterranean countries, producing seasonally large amounts of black olive wastewaters generally referred as olive mill wastewaters (OMWs). The amount of OMW produced is dependent on the extraction process used. In general, for each ton of olives processed, 1.3 m^3 of black waters are produced (Vitolo et al., 1999). This black liquid wastewater is highly pollutant, since it presents high biological oxygen demand (BOD) values (50–100 gO_2/L) as well as chemical oxygen demand (COD) values (80–200 gO_2/L) (Khoufi et al., 2006). These are 200–400 times higher than those of a typical municipal sewage (Cossu et al., 1993). Besides its high organic loading, the presence of polyphenols and tannins, high content of suspended solids, and acidity make these waters highly recalcitrant to conventional water treatment making the management and disposal of olive oil mill effluents a serious environmental problem (Paixão et al., 1999).

The research on OMW valorization has been focused on the degradation/elimination of phenolic compounds, since their breakdown is considered as the limiting step on the biotreatment of OMW (Fountoulakis et al., 2002). Therefore many different methods have already been proposed to decompose OMW (Aggelis et al., 2003; Benitez et al., 1999, 1997; Fadil et al., 2003; Gotsi et al., 2005; Pinto et al., 2002). In several of these works, microorganisms like filamentous fungi have been used to pre-treat OMW, since many are able to reduce polyphenol contents, making these

Abbreviations: OMW, olive mill wastewater; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; BSA, bovine serum albumin; EGTA, ethyleneglycol-bis(β-aminoethyl ether) *N*, *N*, *N*′, *N*′-tetraacetic acid; EDTA, ethylene-diaminetetraacetic; TPP^+, tetraphenylphosphonium.

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doi:10.1016/j.ecoenv.2007.11.003

waters suitable for secondary conventional treatment (Fadil et al., 2003).

Several investigations have already been carried out to evaluate OMW toxicity on microorganisms (Paixão et al., 1999). These clearly show that OMWs are highly toxicant not only to microorganisms but also to microcrustaceans as *Daphnia magna* or *Tamnocephalus platyurus* although the parameter responsible for this toxicity was not clear.

Furthermore, Sert et al. (1998) have shown that ferulic acid, a phenolic acid frequent in OMW, carries out an interference with both L-malate dehydrogenase and malic enzyme. Our previous studies have shown that mitochondrial bioenergetics is strongly disturbed by OMW (Martins et al., 2007). Interference with mitochondrial bioenergetics is known to be a part of the process of cell injury by assorted agents and by a variety of mechanisms (Wallace et al., 1997). Mitochondrial dysfunction promoted by any toxicant can lead to apoptotic cell death and to some neuronal degenerative diseases (Eckert et al., 2003). Furthermore, mitochondrion has proved itself to be a good model to study the action of many xenobiotics on cell toxicity, since data obtained from such studies are generally well correlated with cytotoxicity parameters reported in cell cultures and whole organisms (Knobeloch et al., 1990).

Filamentous fungi like *Geotrichum* sp., *Aspergillus* sp., *Pleurotus* sp., or *Phanerochaete* sp. have proved to be efficient in eliminating some organic loading and phenolic fractions of OMW (Borja et al., 1995a, b; Gharsallah et al., 1999). Yeasts are mainly unicellular fungi, which when used in full-scale wastewater plants do not present the bulking problems associated with filament formation. Besides, in previous works (Amaral et al., 2005), yeasts isolated by our working group, belonging to genus *Candida*, have shown the ability to use phenolic compounds as sole carbon and energy source at high concentrations (1000 mg/L). Based on these previous results, and considering the problem of technology application at large scale, the aim of this study was to evaluate the ability of *Candida oleophila* to remove phenolic and other toxic compounds from a real olive mill effluent, and analyse the effect of this biodegradation on the mitochondrial bioenergetics using isolated mitochondria from rat liver.

2. Materials and methods

2.1. Isolation of rat liver mitochondria

Wistar rats (200–300 g) were fasted overnight before being killed by cervical displacement. The isolation was performed by conventional methods (Gazzoti et al., 1979) with minor modifications. The homogenization medium contained 0.25 M sucrose, 5 mM Hepes (pH 7.4), 0.2 mM EGTA, and 0.1% fatty acid-free bovine serum albumin (BSA). EGTA and BSA were omitted from the final washing medium, which was adjusted to pH 7.2. The final concentration of the mitochondrial protein was determined by the biuret method (Gornall et al., 1949) using BSA as standard. The experiments were carried out in accordance with the National Requirements for Vertebrate Animal Research and European Convention for the Protection of Animals Used for Experimental and Other Scientific Purposes.

2.2. OMW used

Samples of OMWs were collected from a continuous olive mill located in Northeastern Portugal during 2005–2006 extraction campaign. The samples were collected in order to obtain a representative effluent of the entire labouring period (December–February). After collection, samples were analysed for pH, DO, and temperature, *in situ*, using a multiparameter analyser Model 210i from WTW according to manufacturers' instructions. For biological treatment, samples were diluted with deionized water (75% v/v) and sterilized at 121 °C for 15 min. Sterilization was performed in order to ensure that biological treatment was made only by inoculated *Candida* strains. Hundred millilitre aliquots were aseptically collected at regular intervals and stored deep frozen (−70 °C). To perform the toxicological evaluation, the samples were defrosted, centrifuged at 8.000 rpm at 4 °C during 15 min. The pH was adjusted to 7.0 with NaOH (2 M). In each toxicological evaluation, concentrations of 0.5%, 1.0%, 1.5%, and 2.0% (v/v) of treated and untreated OMWs were prepared with each respective reaction medium.

2.3. Microorganisms

Pure young cultures of *C. oleophila* were prepared in Petri dishes with YM Agar from Difco®. Plates were incubated for 48 h at 28 °C and inoculated in Erlenmeyer flasks containing 500 mL of sterilized and previously diluted OMW. Inoculum was made at 1% v/v when in active growing phase ($A_{640\,nm} \approx 1.0$). Samples were made in duplicate with Erlenmeyer flasks with no inoculum which served as blanks.

2.4. Phenols assimilation assays

The reactors prepared as above were incubated at 28 °C, in an orbital shaker at 120 rpm, in order to ensure aerobic conditions in the reactors. After a 30 day incubation period, samples were aseptically collected for analysis. Samples for toxicological surveillance were collected in 100 mL polypropylene containers and stored deep frozen until analysis. Simultaneously 1 mL aliquots were aseptically collected in sterilized Eppendorf vessels for chromatographic analysis.

2.5. Phenolic acids analysis

Phenolic assimilation by yeasts was monitored by reverse-phase high-performance liquid chromatography (RP-HPLC). HPLC analysis was performed with a C18 column (150 × 4.6 mm) Hipersyl using gradient elution. The gradient was made as follows: 0–3 min 100% of A (water, methanol, and acetic acid, 88:10:2), 3–6 min 80% of A and 20% of B (water, methanol, 70:30), 6–9 min 60% of A and 40% of B, 9–12 min 100% of B, 12–30 min 100% of A. Detection was performed at 265 nm in a Merck-Hitachi L-4000 UV detector. The identification of phenol retention times was made by external standard's method.

2.6. Condensed tannin analysis

The tannins were determined by UV spectrophotometry method (Cary 50—Varian) based on acid hydrolysis and colour formation (Porter, 1986). Five hundred microlitres of the OMW was used for analysis with 1 mL of *n*-butanol ± HCl solution (95:5, v/v) and 40 μL of the iron reagent (2% ferric ammonium sulphate in 2 N HCl). For control samples, 500 μL of distilled water was used. The test tubes were covered with glass marbles and heated at 95 °C for 1 h using a heating block. The test tubes were cooled to room temperature, centrifuged, and absorbance measured at 550 nm.

2.7. Fatty acid analysis

OMW free fatty acids were analysed by gas chromatography according to Procida (Procida and Ceccon, 2006) with slight modifications. Samples (15 mL) were gushed by N_2 and the pH was adjusted to 2–3 with phosphoric acid. Acetone was added to the sample (water/acetone 1:2, v/v) and 0.1 g of polyvynilpirrolidone (PVP) to eliminate the interference by phenolic compounds. After 30-min incubation, the sample was clarified by centrifugation. Free fatty acids were analysed with a Perkin-Elmer gas chromatograph adapted for capillary columns. A FFAP-DB fused-silica capillary column, 30 m × 0.53 mm ID × 1 μm film thickness, was used. Oven temperature was programmed from 70 to 200 °C at 4 °C/min, then from 200 to 240 °C at 4 °C/min. Detector temperature was 260 °C; carrier gas (helium) flow rate, 2.0 mL/min. Peak heights were calculated using an appropriate integration software (JCL 6000 chromatography data system).

2.8. Mitochondrial respiratory activity

Oxygen consumption of isolated mitochondria was measured polarographically at 30 °C with a Clark oxygen electrode, in a closed chamber with magnetic stirring. The reaction medium consisted of 250 mM sucrose, 20 mM KCl, 2 mM $MgCl_2$, 5 mM KH_2PO_4, and 5 mM Hepes (pH 7.2). OMW was added in aliquots (0–20 μL) to 1 mL of the standard respiratory medium (25 °C) with mitochondria (1 mg protein), supplemented with 2 μM rotenone and allowed to incubate for 10 min before the addition of 10 mM succinate. State 4 respiration was achieved after phosphorylation of 50 nmol adenosine diphosphate (ADP). State 3 was elicited by adding adenosine 5′-diphosphate (ADP; 1 mM), and uncoupled respiration, by adding 1 μM FCCP. Controls were made as above with minor changes: first we exposed the mitochondria to a medium preimplemented with substrate and added OMW 5 min later. The purpose of this control was to verify whether the mitochondria pre-incubated with OMW prior to substrate addition resulted in an irreversible damage on the respiratory complexes, with a limitation in the maximal respiratory rates.

2.9. Membrane potential

The mitochondrial transmembrane potential ($\Delta\psi$) was measured indirectly based on the activity of the lipophilic cation tetraphenylphosphonium (TPP^+) using a TPP^+-selective electrode in combination with and Ag/AgCl-saturated reference electrode, as previously described (Peixoto, 2005). Mitochondria (1 mg protein) were incubated in 1 mL of medium containing 250 mM sucrose, 20 mM KCl, 2 mM $MgCl_2$, 5 mM KH_2PO_4, and 5 mM Hepes (pH 7.2), supplemented with 2 μM rotenone and 3 μM TPP^+ and energized with 10 mM succinate. No correction was made for the "passive" binding of TPP^+ to the mitochondria membranes because the purpose of the experiments was to show relative changes in potential rather than absolute values. As a consequence, we can anticipate some overestimation for the values.

2.10. Enzymatic activities

Succinate dehydrogenase activity was measured spectrophotometrically by the reduction of 2,6-dichlorophenolindophenol (DCIP) at 600 nm in the presence of phenazine methosulfate (PMS) (Singer, 1974). The reaction was performed in 1 mL of the standard reaction medium supplemented with 5 mM succinate, 2 μM rotenone, 0.1 μg antimycin A, 1 mM KCN, 0.025% Triton X-100 at 25 °C, and 0.5 mg protein of disrupted mitochondria (two cycles of freezing and thawing).

Succinate cytochrome *c* reductase activity was measured spectrophotometrically (Tisdale, 1967) at 25 °C by following the reduction of oxidized cytochrome *c* by the increase in absorbance at 550 nm. The reaction was initiated by the addition of 5 mM succinate to 3 mL of the standard reaction medium supplemented with 2 μM rotenone, 1 mM KCN, 54 μM of cytochrome c, and 0.3 mg protein of broken mitochondria.

Cytochrome *c* oxidase activity was measured polarographically (Brautigan et al., 1978) at 25 °C in 1 mL of the standard reaction medium supplemented with 5 mM succinate, 2 μM rotenone, 10 μM cytochrome c, and 0.5 mg protein broken mitochondria. The reaction was initiated by the addition of 5 mM ascorbate plus 0.25 mM TMPD.

ATP-synthase activity was determined by monitoring the pH increase associated with ATP synthesis (Madeira et al., 1974). The reaction was carried out in 2 mL of the reaction medium containing 130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, and 2 mM KH_2PO_4 (pH 7.2), supplemented with 5 mM succinate and 1 mg of mitochondrial protein. The reaction was initiated by the addition of 200 μM ADP to the mitochondrial suspension. The pH change was evaluated with a Crison pH meter connected to a Hansatech acquisition data system. The addition of oligomycin (2 μg/mg protein) completely halted H^+ consumption. H^+ consumption was calculated after an elapsed time of 2 min from the start of the reaction.

ATPase activity was determined by monitoring the pH change associated with ATP hydrolysis (Madeira et al., 1974). The reaction was carried out in 2 mL of a medium containing 130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, and 0.5 mM Hepes (pH 7.2), supplemented with 2 μM rotenone and mitochondria (1 mg protein of disrupted mitochondria). The reaction was initiated by the addition of 2 mM Mg-ATP and was completely inhibited by the addition of oligomycin (2 μg/mg mitochondrial protein); this means that activity measured is due to a mitochondrial F_0–F_1 ATPase which is an Mg^{2+} ATPase. Proton production was again calculated 2 min after starting the reaction.

2.11. Mitochondrial swelling

Mitochondrial osmotic swelling was estimated from the decrease in the absorbance at 520 nm, measured in a UV/VIS spectrophotometer lambda 45, with magnetic stirring and thermostatic chamber (25 °C). Reaction medium contained 54 mM K^+-acetate, 5 mM HEPES-Na^+ buffer, pH 7.2, 0.1 mM EGTA, 0.2 mM EDTA, 15 μM atractyloside, 1 μM antimycin A, 100 μM Na^+-azide, 300 μM propranolol, 2 μM rotenone, and 0.1% BSA.

2.12. Chemicals

All reagents were of analytical grade for research.

2.13. Statistics

The results are presented as a percentage of the controls ± SEM from at least three independent experiments. When described, data obtained with different concentrations of OMW were compared with control (absence of OMW) by using one-way ANOVA with the Dunnett post-test. A value of $p < 0.05$ was considered statistically significant. Data were analysed by using GraphPad Prism 4.0 (GraphPad Software). Some figures are records of individual experiments representative of three or more replicates.

3. Results

3.1. Phenolic compounds analysis

The phenolic compounds identification was made using measured retention times in comparison with external standards prepared with deionized water. Lucas et al. (2006) have reported that *C. oleophila* was able to assimilate as sole carbon and energy source some phenolic acids and this yeast strain was also able to fully decolorize mediums containing the diazo dye Reactive Black 5. However, OMW treated with this yeast strain does not

show any detectable decolorization. Furthermore, on comparing the results for measured phenols in control and those obtained after treatment with *C. oleophila*, no significant differences were observed (data not shown).

3.2. Free fatty acid analysis

Fatty acids analysis of treated and untreated OMWs does not show any significant difference in the fatty acid profiles. As far as small chain free fatty acids (C2–C6) are concerned, no differences were established between treated and untreated OMWs. Long chain free fatty acids (C16–C18) generally present in olive oil were also measured and the percentages are presented in Table 1.

3.3. Condensed tannin analysis

Tannins are assigned as benefic molecules due the popularity of some antioxidant activities observed on tannins isolated from fruits (e.g. grapes). However, like other phenols, not all tannins are benefic; some authors have reported disturbing effects of tannins on mitochondria (Spiridonov et al., 1997; Liu et al., 2004), like inhibition of succinate oxidation, loss of mitochondrial transmembrane potential, and mitochondria cytochrome *c* release. The analysis of OMW treated with *C. oleophila* shows a decrease of about 20.3±4.8 of the total condensed tannins when compared with untreated OMW.

3.4. Effects of OMW on mitochondrial respiration

The effect of OMW on rat liver respiratory rates of state 4 (succinate alone), state 3 respiration (ADP-stimulated), and FCCP-stimulated respiration (uncoupled) was studied in the presence of succinate as respiratory substrate (Fig. 1). A 5-min treatment of the mitochondrial suspension (1 mg protein/mL) with 0.5–1.5% (v/v) of untreated OMW results in a release of state 4 respiration, with an up to 200% increase in the O_2 consumption when compared with the control. With higher concentrations of OMW (2% v/v) the stimulation was not so marked. Probably at the highest OMW concentration (2% v/v) some inhibitory

Table 1
Free fatty acids found in a sample of olive mill wastewater

Fatty acid	Mol. (%)
C2	78.4±8.32
C3	1.37±0.04
C4	17.1±1.85
C6	0.221±0.02
C16	0.454±0.07
C16:1	0.086±0.01
C18	0.061±0.01
C18:1	1.93±0.37
C18:2	0.235±0.05

Data are the mean of three determinations±SD.

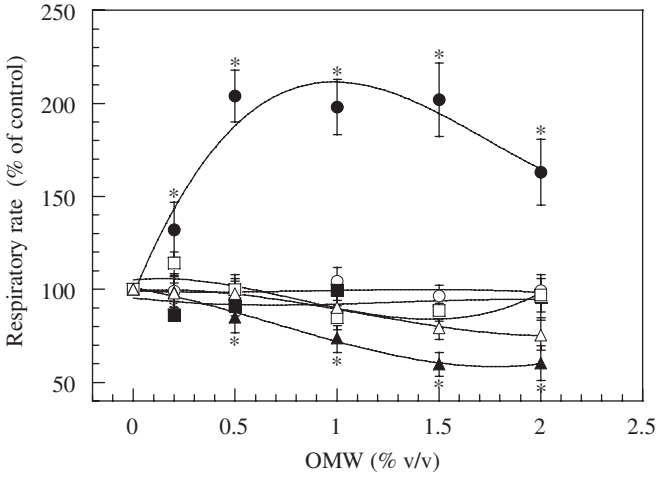

Fig. 1. Effect of untreated OMW (filled symbols) and treated OMW (open symbols) on respiratory rates of mitochondria. Mitochondria (1 mg) were incubated, for 10 min, in 1 mL of the respiratory standard medium in the presence of different OMW concentrations. (●, ○) State 4 respiration was initiated by the addition of 10 mM succinate, (■, □) state 3 respiration energized by 10 mM succinate was initiated by the addition of 1.5 mM ADP, (▲, □) FCCP-uncoupled respiration in the presence of 10 mM succinate was initiated by the addition of 1 μM FCCP. ADP or FCCP was added 2 min after the initiation of state 4 respiration. Values are the means±SD of four to five independent experiments performed in duplicates. *Values statistically different from control ($p<0.05$).

effect of the respiratory chain should acquire relevance. A 5–15 min treatment with treated OMW, at all tested concentrations, does not induce any significant alteration on the state 4 and 3 respirations. Furthermore, FCCP-uncoupled respiration was merely decreased 15% at the maximum used concentration (2% v/v treated OMW).

The initial stimulation on the respiratory state 4 rate is presumably caused by the membrane partition of some constituents of OMW, like free fatty acids and some other organic molecules, which can cause mitochondrial membrane permeabilization.

As a control we performed the assays described in Fig. 1 with a modification; first we exposed the mitochondria to a medium preimplemented with substrate and added OMW 5 min later. The purpose of this control was to verify whether the mitochondria pre-incubated with OMW prior to substrate addition resulted in an irreversible damage on the respiratory complexes, with a limitation in the maximal respiratory rates. From the results obtained, we can conclude that pre-incubation with OMW at 2% (v/v) before the substrate addition does not cause any irreversible damage on the respiratory complexes, since 5–15 min after OMW had been added the effects became noticed (data not shown); the results obtained are the same whether the substrate is added before or after pre-incubation with OMW.

The inhibition observed in the uncoupled respiration was about 40% of the control. This inhibitory effect of OMW on uncoupled respiration reflects its interaction with the mitochondrial redox chain. State 3 respiration was not so significantly inhibited as it was observed in the

uncoupled respiration. At the maximum concentration used in the assay (2% v/v), the inhibition was about 5% of the control.

3.5. Effects of OMW on mitochondrial membrane potential

The effects of OMW on the energization and phosphorylation capacities of mitochondria were investigated by following transmembrane potential ($\Delta\psi$) developed by mitochondria upon succinate oxidation. After succinate addition, mitochondria developed a transmembrane potential of about −212 mV (Fig. 2). Untreated OMW highly depolarized the membrane potential but treated OMW was much less efficient in decreasing mitochondrial membrane potential.

When treated OMW was used, electrical membrane potential was only significantly decreased for an OMW concentration of 1.5% (v/v) (11% of the control value). At maximum used concentration (2% v/v), the decrease was 21% relatively to the control, while at this same concentration the untreated OMW almost completely collapses the electrical membrane potential (Fig. 2).

3.6. Enzymatic activities

Studies regarding enzymatic activities of respiratory complexes II–V allowed us to identify mitochondrial respiratory chain components affected by OMW.

In the presence of untreated OMW (1% v/v), succinate dehydrogenase was significantly inhibited (23%) while treated OMW did not induce any significant inhibition. For 2% (v/v) OMW dilutions, succinate dehydrogenase activity was inhibited 47% and 23% for untreated and treated OMWs, respectively (Fig. 3).

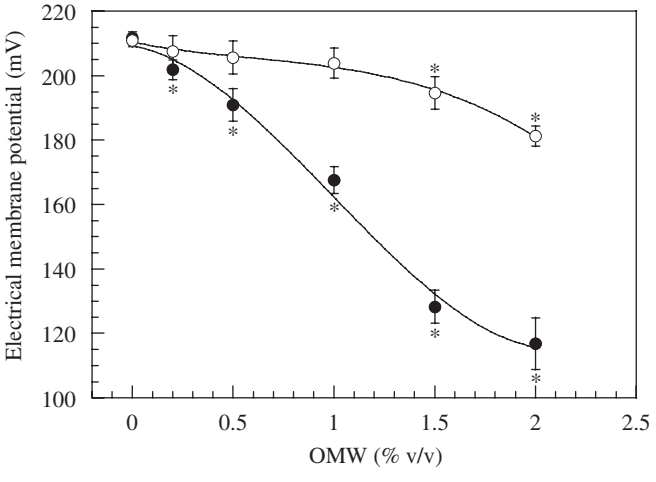

Fig. 2. Effect of untreated OMW (filled symbols) and treated OMW (open symbols) on mitochondrial membrane potential ($\Delta\psi$) supported by succinate. Mitochondria (1 mg) were added to the standard respiratory medium supplemented with 3 μM TPP^+. Maximum potential reached due to succinate (10 mM) oxidation after 10-min incubation with OMWs (●, ○). Values are the means ± SD of five independent experiments performed in duplicates. *Values statistically different from control ($p<0.05$).

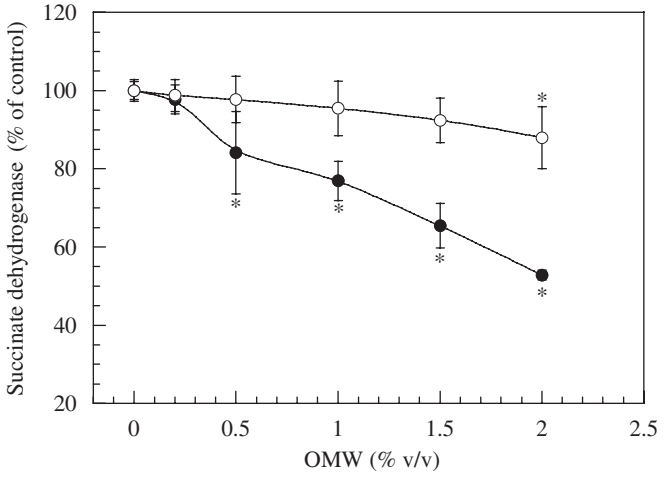

Fig. 3. Effect of untreated OMW (filled symbols) and treated OMW (open symbols) on the succinate dehydrogenase (●, ○). The activities were determined as described in the Materials and methods section. Values are the means ± SD of four independent experiments performed in duplicates. *Values statistically different from control ($p<0.05$).

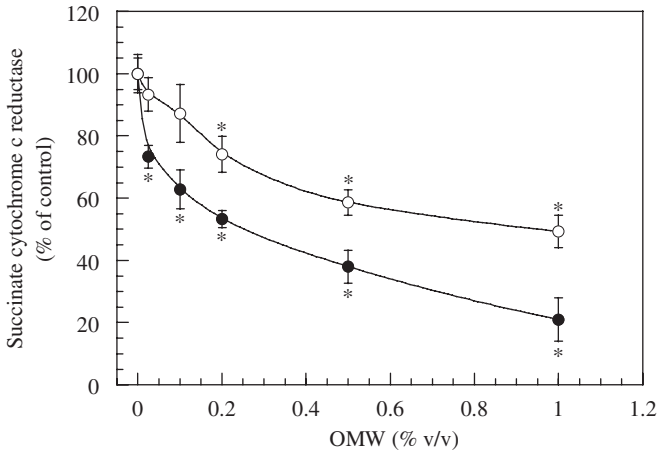

Fig. 4. Effect of untreated OMW (filled symbols) and treated OMW (open symbols) on the succinate cytochrome *c* reductase (■, □). The activities were determined as described in the Materials and methods section. Values are the means ± SD of four independent experiments performed in duplicates. *Values statistically different from control ($p<0.05$).

Succinate cytochrome *c* reductase respiratory complex (Fig. 4) was much more sensitive to OMW action when compared with the results obtained for succinate dehydrogenase. At maximum used concentration (1% v/v) untreated OMW decreased the enzymatic activity 80% compared with the control, whereas the treated OMW decreased the activity about 50% (Fig. 4).

Cytochrome *c* oxidase was not inhibited by any of the tested OMW (Fig. 5). Furthermore, a small, but significant, stimulation was observed for the lowest used concentration of untreated OMW.

Like it was observed for succinate dehydrogenase and succinate cytochrome *c* reductase, ATPase activity was significantly inhibited by untreated OMW compared with

treated OMW (Fig. 6). At maximum OMW used concentrations (2% v/v), the differences between the treated and untreated OMWs was nearly 24%. ATP synthase was also much more inhibited by untreated OMW than by treated OMW. Mitochondrial incubation with 1% (v/v) of untreated OMW decreased the ATP-synthase activity about 78% compared with control, while treated OMW at the same concentration just inhibited by 37% in relation with the control. IC_{50} values obtained with treated and untreated OMWs are presented in Table 2 (Fig. 7).

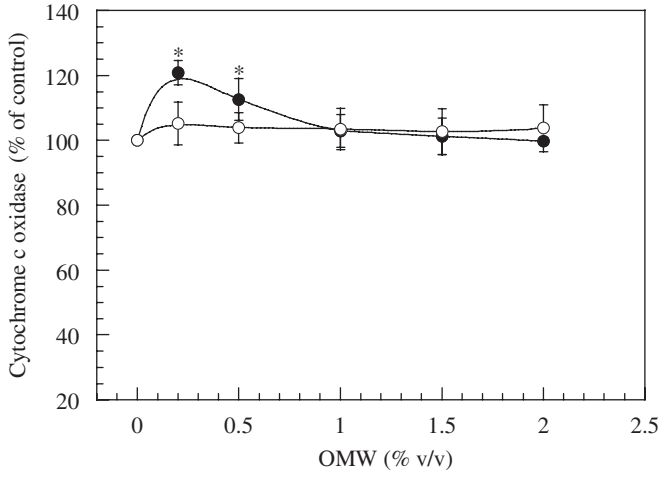

Fig. 5. Effect of untreated OMW (filled symbols) and treated OMW (open symbols) on the cytochrome *c* oxidase (▲, □). The activities were determined as described in the Materials and methods section. Values are the means ± SD of four independent experiments performed in duplicates. *Values statistically different from control ($p<0.05$).

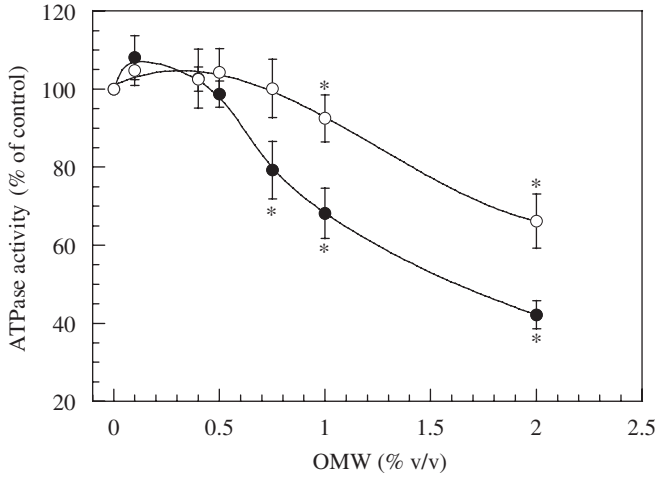

Fig. 6. Effect of untreated OMW (filled symbols) and treated OMW (open symbols) on rat liver mitochondrial ATPase (◆, ◇) activities. Experimental conditions are described in the Materials and methods section. Values are the means ± SD of four independent experiments performed in duplicates. *Values statistically different from control ($p<0.05$).

3.7. Proton-dependent mitochondrial swelling

In order to demonstrate the protonophoric properties of OMW, we performed mitochondrial swelling in isosmotic K^+-acetate medium in the presence of different concentrations of OMW (Fig. 8). The maximal valinomycin-dependent swelling stimulation was observed upon the addition of FCCP (1 μM). Mitochondrial swelling occurs in the presence of a protonophore which enables the passage of protons from the matrix to the extramitochondrial reaction medium, allowing further acetate and K^+ influx (Nicholls, 1982). Therefore, valinomycin-induced swelling resulting from OMW is a consequence of a protonophoric action. OMW increases inner mitochondria membrane's permeability to protons (Fig. 8). Nevertheless, treated OMW was not so efficient.

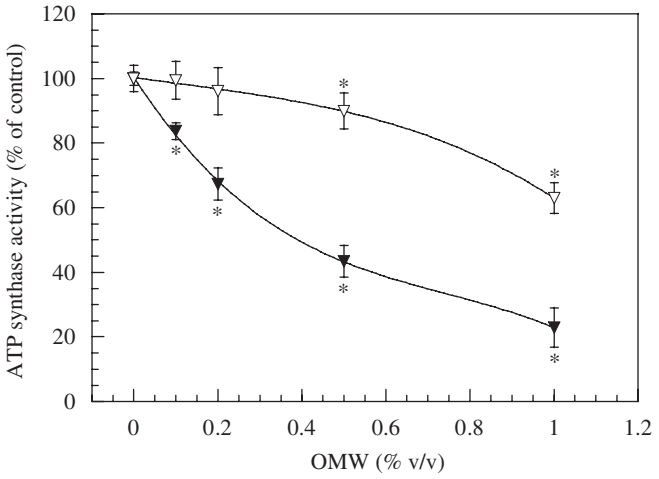

Fig. 7. Effect of untreated OMW (filled symbols) and treated OMW (open symbols) on rat liver mitochondrial ATP-synthase (▼, ▽) activities. Experimental conditions are described in the Materials and methods section. Values are the means ± SD of four independent experiments performed in duplicates. *Values statistically different from control ($p<0.05$).

Table 2
Inhibitory results for different enzymes from mitochondrial respiratory chain after incubation with treated and untreated olive oil wastewaters

OMW	SD IC_{50} 5 min (%)	SCR IC_{50} 5 min (%)	COX IC_{50} 5 min (%)	ATPase IC_{50} 5 min (%)	ATP synthase IC_{50} 5 min (%)
Untreated	1.90	0.28	#	1.65	0.39
Treated	#	0.84	#	#	#

SD, succinate dehydrogenase; SCR, succinate cytochrome *c* reductase; COX, cytochrome *c* oxidase. # values are out of range of the used concentrations.

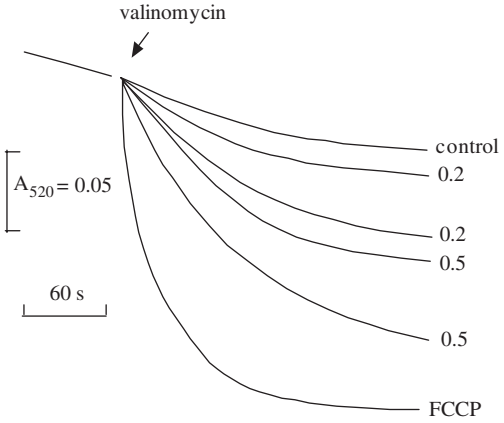

Fig. 8. Effect of untreated OMW and treated OMW on mitochondrial swelling. OMW was added at concentrations of 0.2% and 0.5% (v/v)/mg mitochondrial protein. The control experiment was made in the absence of FCCP and OMW. Maximum swelling was obtained with FCCP (1 μM). Valinomycin (1 μM) was added where indicated. The traces are representative of a group of four independent experiments.

4. Discussion

Different olive wastes are known to contain high concentration of phenolic compounds (Fountoulakis et al., 2002; Lesage-Meessen et al., 2001) to whom several toxic effects are pointed out (Paixão et al., 1999; Martins et al., 2007). The degradation of phenolic compounds is considered as the limiting step in the biotreatment of OMW, since their natural breakdown is not easy (Fountoulakis et al., 2002). Long chain free fatty acids (C16 and C18) are naturally occurring fats from olive fruits, which not only exhibit toxic effects towards microorganisms (Guilloux-Benatier et al., 1998) but also mitochondrial depolarization (Ray et al., 2002; Schönfeld et al., 2004).

Biological treatment of OMWs with *C. oleophila* was conducted in batch bioreactor cultures. *C. oleophila* grew well in sterilized and diluted OMW (75%), without any addition of nutrients and any specific pre-treatment. During treatment no significant phenolic removal was observed. However, it is considered in this work that sterilized OMW should function as a model of phenolic rich wastewater, since sterilization may cause important physicochemical alterations on several compounds such as oxidation followed by precipitation (Fountoulakis et al., 2002).

From the HPLC chromatogram analysis, phenolic compounds present in OMW were not significantly metabolized by *C. oleophila*, although we may conclude that these compounds present no toxicity towards this species. Hamdi (1992) reported that phenols, responsible for OMW's black colour, present little toxicity and are not biodegradable. Other studies regarding the dephenolization of OMW, or production of valuable products, using as source an OMW effluent, used other *C. oleophila* yeast related species (Lanciotti et al., 2005; Fadil et al., 2003; Ettayebi et al., 2003; D'Annibale et al., 2006; Papanikolaou et al., 2007). These works have proven that phenol removal from OMW by yeasts seems to be a strain-dependent process (Papanikolaou et al., 2007), since in similar conditions some yeast strains are able and others cannot grow in media with OMW, even when the amount of phenolic compounds is low. Previous reports (Lucas et al., 2006) showed that *C. oleophila* can use phenolics as carbon source when there is no other source, but is much more efficient when other easier source of carbon (as glucose) is available. In this work, and by the obtained results, the low metabolism of phenolic compounds may result from the fact that the isolate possesses many other sources of carbon and energy in the sterilized OMW. Tsioulpas et al. (2002) developed a new toxicity index, based on phenolic concentrations, and suggested (based on the same index) that although (*Pleurotus* sp.) treated OMW revealed to be less toxic, this decrease was not proportional to phenolics removal, as we found in our work.

As already reported by other authors, phenolic acids (e.g. ferulic acid) inhibit L-malate dehydrogenase and malic enzyme from soya bean mitochondria (Sert et al., 1998), *p*-coumaric acid inhibits plasmatic and mitochondrial monocarboxylate carrier and also mitochondrial respiration dependent on pyruvate oxidation (Lima et al., 2006). Mitochondrial proton F_0–F_1 ATPase/ATP synthase was also affected by polyphenolic acids (Zheng and Ramirez, 2000). However, considering the results obtained in this work, the toxicological effect of OMW previously reported (Martins et al., 2007) cannot be assigned to the effect of phenolic compounds. Instead, our results lead to the presence of condensed tannins as a possible cause for the deleterious effect observed on rat liver bioenergetics (Spiridonov et al., 1997; Liu et al., 2004), since treatment of OMW with *C. oleophila* has a reducing effect on tannins content. Again, in the above-mentioned report (Hamdi, 1992), it is suggested that, on the contrary to polyphenolic compounds, tannins are highly toxic, but biodegradable by some species. In our working conditions, the *C. oleophila* inoculums were not affected by tannins toxicity, and furthermore, they were able to somehow metabolize these compounds reducing their content in the samples. This seems to be the most important factor in reducing the toxic effect on mitochondrial bioenergetics.

Many works concerning OMW biodegradation can be found in the literature (Ahmadi et al., 2006; Di Gioia et al., 2001; Jaouani et al., 2005; Lanciotti et al., 2005), and although some perform toxicological evaluations of OMW after treatment (Dhouib et al., 2006; El Asli et al., 2005; Fiorentino et al., 2004; Isidori et al., 2005), these toxicological tests evaluate mainly the effects on seed germination.

Mitochondrion supports the energy-dependent regulation of many cell functions, namely intermediary metabolism, protein folding, ion regulation, cell motility, and cell proliferation (Wallace, 1999). Therefore, disruption of the coupling efficiency between oxidation and phosphorylation

promotes large bioenergetic deficits leading to the loss of several vital functions both to the cell and the organism. OMW toxicity could then be monitored by the disturbances induced on mitochondrial functions.

In the present work, rat liver mitochondria were used as *in vitro* monitors to validate the effect of OMW treatment with *C. oleophila* on the OMW toxicity. Previous work (Martins et al., 2007) by our group clearly demonstrate OMW's ability to interact with mitochondrial oxidative phosphorylation. Alterations of basic mitochondrial functions were detected by changes induced in mitochondrial respiration and membrane energization ($\Delta\psi$).

The results obtained in this study clearly show that the treatment of OMW with *C. oleophila* considerably decreased the injurious effect of OMW on the mitochondrial electrical membrane potential. In fact, at tested concentrations, treated OMW has no significant effect on mitochondrial respiration, and the effects on mitochondrial electrical membrane potential appear only for dilutions up to 1.5%. Furthermore, untreated OMW at 0.2% induced a small, but significant, decrease on the electrical membrane potential, and at 1.5% dilutions almost completely collapsed the electrical membrane potential. OMW have many free fatty acids (Filidei et al., 2003; Procida and Ceccon, 2006). These fats are originally from the olive oil itself and others from microbial metabolism, which can significantly contribute to the observed effect on the transmembrane potential since long chain free fatty acid can easily disrupt the inner mitochondrial membrane. However, the differences observed in the effect of the two OMWs could not be attributed to free fatty acids, since no differences were observed on the composition and content between treated and untreated OMW. Papanikolaou and Aggelis (2003), as well as other reports, have proven the existence in *Yarrowia lipolytica* and other related strains of carrier systems for fatty acids (one for C_{12} and C_{14} fatty acids, and other for C_{16} and C_{18} fatty acids). According to these reports, these strains, specially *Y. lipolytica*, preferably incorporates from the medium unsaturated fatty acids. Furthermore, Papanikolaou and Aggelis (2003) suggested the use of fatty wastewaters as growth medium to produce "new" pre-determined lipids by selected *Y. lipolytica* strains. To the best of our knowledge, there are no reports concerning *C. oleophila* ability to selectively remove free fatty acids from an oleaginous mixture. Nevertheless, *C. oleophila* proved to be a well-adapted species in OMW. The aim of our study could not determine our isolate ability to use fatty acids, but is an issue to be studied in further works.

Comparing the effects of untreated and treated OMW on the enzymes of mitochondrial respiratory chain, it is clear that the previous treatment with *C. oleophila* was very significant in reducing the inhibitory effect in some enzymatic activities. However, the effect was not similar for all the tested enzymes. Untreated OMW shows a very low IC_{50} for succinate cytochrome *c* reductase (0.28) and ATP synthase (0.39) (Table 2). These values are lower than that of those reported for other toxicity tests with *D. magna* and *V. fischeri* (Gotsi et al., 2005; Paixão et al., 1999). The inhibitory effect on ATP-synthase activity may result from a direct inhibition of some OMW compound on the complex, which was not tested (ATPase activity); by inhibitory effects on other respiratory chain complexes, as seen by the results; or by changes in electrical membrane potential, also observed in the results. On the other hand, the observed effect in succinate cytochrome *c* reductase is a consequence of direct inhibition of the enzyme (complexes II and III). We so believe that this enzyme could be considered as a target of OMW inhibition effects, especially caused by tannins present in these wastewaters, since only the variation of these compounds presented significance between *C. oleophila* treated and untreated OMWs.

5. Conclusions

Ultimately, condensed tannins could, probably, interact with mitochondrial membranes, causing an alteration on the surface charge density and a disturbance in the physicochemical and structural properties of the inner membrane. These would then lead to a disturbance in the electron delivery between redox complexes and, additionally, to an increase of the permeability to protons.

In conclusion, OMW treatment with *C. oleophila* shows a significant decrease in the interference with the mitochondrial bioenergetics; the observed decrease is not connected to the presence of free fatty acids or phenolic acid compounds on the OMW, since no differences were observed with the treatment. However, the differences could be related with diminishing of some mitochondrial active tannins. Considering the potential effect of some of these compounds on the rat liver bioenergetics, we can consider that the decrease observed on the OMW after *C. oleophila* treatment could probably be explained by the degradation of this kind of compounds. From these results we can also conclude that *C. oleophila* could be efficient in decolorizing some commercial dyes but in complex matrixes like OMW the results were not totally satisfactory.

Acknowledgments

This study was fully supported by the Foundation for Science and Technology (FCT) through the financial support attributed to the Center of Animal and Veterinary Science (CECAV) from the University of Trás-os-Montes and Alto Douro, Portugal.

Disclaimer. The experiments were carried out in accordance with the National (DL 129/92; DL 197/96; P 1131/97) and European Convention for the Protection of Animals Used for Experimental and Other Scientific Purposes and related European Legislation (OJ L 222, 24.8.1999).

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