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# Complete Detoxification of Olive Mill Wastewaters by Integrated Treatment Using the White Rot Fungus *Phanerochaete chrysosporium* Followed by Anaerobic Digestion and Ultrafiltration

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**Abstract:** In this study, we investigated an integrated technology for the treatment of the recalcitrant contaminants of OMW, allowing water recovery and reuse for agricultural purposes. We have developed a pilot plant based on fungal pretreatment using *Phanerochaete chrysosporium* followed by anaerobic digestion. *P. chrysosporium DSM 6909* was cultivated on pre-stored OMW as sole carbon and energy sources in a 120 L Air Lift Reactor (ALR) in a semi-continuous feed of OMW at a Hydraulic Retention Time (HRT) of 3 to 5 days. The *P. chrysosporium DSM 6909* pre-treated OMW was fed in a 300 l anaerobic filter after a decantation step. The anaerobic filter was loaded with undiluted pre-treated OMW at a starting loading rate of 2-3 g of COD per litre of reactor and per day. The COD loading was increased when no apparent toxicity was encountered. This anaerobic reactor worked continuously for 6 months at loading rates reaching 7 g $L^{-1}$ $d^{-1}$ of COD without any apparent toxicity. The percentages of inhibition of *Vibrio fisheri* luminescence after exposure to OMW showed that compared to the 100% inhibition of untreated OMW, *P. chrysosporium DSM 6909* decreased the relative toxicity to 74%. Consequently, the *P. chrysosporium DSM 6909* pre-treated OMW was well converted into biogas by anaerobic digestion which resulted in an effluent with 38% toxicity referred to as untreated OMW. Moreover, the use of ultrafiltration (UF) as post-treatment technology completely detoxified the anaerobic effluent and removed its black color. Results showed that compared to irrigation with water, treated effluent increased the Germination Index (GI) of *Lycopersicon esculentum* and enhanced plant growth while diluted untreated OMW decreased the GI and caused a pronounced growth inhibition.

**Key words:** Olive mill wastewaters, polyphenols, fungi, anaerobic digestion, ultrafiltration, toxicity

## INTRODUCTION

The efficiency in water use and the question of its sustainability are becoming major environmental issues especially in the Mediterranean countries where the fast population growth and industrial development together with climate changes have progressively lowered water resources and increased their pollution.

The worldwide olive oil production is about 2.5 million tons per year, over 95% of which is produced in the Mediterranean area. Because olive oil is a typical Mediterranean product, the treatment of olive Oil Milling Wastewater (OMW) is of crucial importance and a common problem in several European and Mediterranean countries. The waste resulting from olive oil production and its treatment constitutes a serious problem with a severe negative impact on soil and water quality and thus on agriculture, environment and health[1].

Several methods were investigated for OMW depollution such as wet flocculation/coagulation with $Al_2(SO_4)_3$,$18H_2O$ and $H_2O_2$[2], centrifugation and ultrafiltration[3,4] but most of the published research dealt with the anaerobic treatment[5]. Indeed, the high COD values of OMW favoured the choice anaerobic biological treatment as the best perspective for these reasons. First, it provides energy in the form of methane. Second, it protects the environment by reducing the polluting characteristics. Third, it yields a good quality of solids. In previous study on the anaerobic digestion of unmodified OMW, many problems such as the high toxicity, the lack of ammonia, the low alkalinity, the high lipid content, the low biodegradability and the acidification of reactors were studied[6,7]. Anaerobic bacteria are sensitive to the high phenolic content of OMW as are methanogens. These compounds are inhibitory factors in the anaerobic digestion. Several approaches were applied to overcome inhibition. Among these was the dilution[7]. Another approach was co-digestion with other effluents such as manure[8], other seasonal wastewater[9] or municipal wastewater[10,11].

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A pre-treatment step is required by using a physico-chemical or biological stage capable of decreasing the organic load and the toxicity of phenolic compounds, potential inhibitors of methanogenesis[3,12].

The use of *Phanerochaete chrysosporium* for a practical treatment of OMW was investigated. This fungus can significantly reduce the color of this effluent and degrade the high and low molecular-mass aromatics[13]. It secretes enzymes that break down lignin in wood to carbon dioxide and water. Previous work demonstrated the ability of this fungus and other fungi such as *Trametes versicolor, Pycnoporus cinnabarinus* to decolorise OMW[14]. The decolorisation of OMW corresponds to depolymerization of high molecular mass aromatics combined with mineralization of a wide range of monoaromatics. Lignin peroxidase was shown to be the key enzyme in this process[13,15].

Other fungi were then used for this same purpose[16-24]. Recently, new potent white rot fungi such as *Coriolopsis polyzona*, for decolorisation of OMW were reported[25]. All experiments were performed in bench scale (flasks) under batch mode and sterile conditions. A field continuous or semi-continuous experiment involving the treatment and detoxification of OMW with white rot fungi is needed for further feasibility studies.

This study attempted to investigate at a pilot scale, an integrated technology based on fungal pre-treatment using *Phanerochaete chrysosporium* followed by anaerobic digestion. Ultrafiltration was assayed as a post-treatment for complete detoxification and color removal allowing water recovery and reuse for agricultural purposes.

## MATERIALS AND METHODS

**OMW characterization:** OMW were obtained from a discontinuous olive oil processing plant located in Sfax (Southern Tunisia). The main characteristics of one OMW sample are indicated in Table 2. The phenolic monomers identified by gas chromatography coupled to mass spectroscopy technique were as reported earlier[26]: tyrosol 1, tyrosol 4, hydroxytyrosol, protocatechuic acid, syringic acid, p-coumaric acid, cafeic acid, ferulic acid, vanillyl alcohol, vanillic acid and vanillin.

**Fungal pre-treatment:** A stainless steel Air Lift Reactor (ALR) composed of Bubble Column Reactor (BCR) equipped with a draft tube was chosen for the pre-treatment of OMW by *Phanerochate chrysosporium* DSM 6909[15]. The working volume was 100 l. Air from a compressor was injected through an air Sparger located at the bottom of the reactor at a flow rate of 0.25 L of air per litre of fermenter and per minute. The temperature and the pH of the fermentation were not regulated. The COD of the OMW soluble fraction was determined before the feeding of the ALR. The COD varied between 70 and 130 g $L^{-1}$. For COD values higher than 100 g $L^{-1}$, OMW was diluted by a factor ranging between 1 and 1.3 with water to give a COD of 100 g $L^{-1}$. 1.75 g of N (5.2 g $NH_4NO_3$ 33.5%) and 0.35 g P (0.8 g $P_2O_5$ 43.66%) were added per litre of OMW (35 g $L^{-1}$ $BOD_5$) to fit the ratio $BOD_5$/N/P=100/5/1. The ALR was inoculated with *P. chrysosporium DSM 6909* grinded mycelium in non sterile conditions. OMW fermentation with *P. chrysosporium DSM 6909* was operated in a semi-continuous mode with a residence time of 3 to 5 days

**Anaerobic digestion and biogas analysis:** The anaerobic treatment of *P. chrysosporium DSM 6909* pre-treated OMW was performed in an anaerobic filter reactor (AF). This AF reactor already acclimated for degrading OMW, consisted of a 300 l (1.950 m long and 0.45 m internal diameter) double jacket stainless steel column packed with PVC rings. The working volume of the reactor was 275 l. The temperature of the reactor was regulated to 37°C. The OMW pre-treated with *P. chrysosporium DSM 6909* was fed into the reactor, without dilution and pH regulation, 6 times per day using a pump connected to a programmer. The mean loading rates varied from 2 to 8 g $L^{-1}$ $d^{-1}$ of COD. For monitoring the Volatile fatty acids (VFA) inside the reactor, three sampling points were made in the anaerobic filter. Level (a) was at the bottom of the reactor. Level (b) corresponded to the middle and level (c) was at the top of the reactor.

Gas flow rates were measured by a biogas counter. Gas samples were taken with a syringe from the tank of biogas. $CH_4$, $CO_2$ and $N_2$ were measured using a gas chromatograph GC11 (Delsi instruments) equipped with a Haye SepQ 60/80 (SUPELCO) column (maintained at 60°C), a thermal conductivity detector (current intensity of 160 mA) and a servotrace integrator (SEFRAM). Helium was used as a carrier gas at a pressure of 1.3 bars.

**Ultrafiltration treatment:** The experimental apparatus used for this study consisted of a stainless steel ultra-filtration system made by Gamma Filtration. A multi-tubular membranes (PCI France) having 2, 25 and 100 kDa and a surface of 0.85 $m^2$ were used.

**Description of the whole process and samples preparations:** The complete pilot plant used in this study is shown in Fig. 1. Raw OMW was pre-stored in a 500 l basin (1) before being transferred in a 300 l decanter (2) in

order to remove suspended solids. Decanted OMW was fed in the *P. chrysosporium DSM 6909* Air Lift Reactor (3) at a flow rate fixed by the pump P1 (20 to 35 L $d^{-1}$). After 3 to 5 days residence time, the effluent was recovered in the decanter (4) and successively in basin (5). The COD abatement during this first biological stage was determined by measuring the soluble COD in the sampling points S1 (Air lift inflow) and S2 (Air lift outflow). Decanted pretreated *P. chrsysosporium* OMW was fed into the anaerobic digester (6) in 6 times per day using the pump P2 connected to a programmer. The effluent of the digester flows in the basin (7) prior to be pumped by P3 for ultrafiltration (8). The COD abatement during anaerobic digestion was evaluated after determinations of soluble COD in the sampling points S3 (Digester inflow) and S4 (Digester outflow).

**Physicochemical analyses**

**Analysis of ortho-diphenols:** A 1/25 (v/v) aqueous extract, was shaken for 12 h in a mechanical shaker and concentrations in the extracts were quantified by means of Folin-Ciocalteau colorimetric method[27] using caffeic acid as standard. The absorbances were determined at $\lambda$ = 765 nm.

**Analysis of total polyphenols:** OMW was centrifuged at 7000 rpm for 20 min. The supernatant was extracted 3 times with ethyl acetate. The collected organic fraction was dried and evaporated under vacuum. The residue was extracted twice with dichloromethane in order to remove the non phenolic fraction (lipids, aliphatic, sugars). The liquid phase was discarded while the washed residue was weighed and analysed by gas chromatography coupled to mass spectroscopy technique to confirm the phenolic structure of the extracted compounds.

**COD determination:** COD was determined according to Knechtel[28] standard method and fading color was monitored by measuring the absorbance at 395 nm, the length of the maximum absorbance, using a spectrophotometer (ANTHLIE ADVANCED 5 SECOMAM). Samples (influent, effluent) were centrifuged 5 min at 10000 rpm and diluted appropriately before each COD determination.

**$BOD_5$ determination:** $BOD_5$ was determined by the manometric method with a respirometer (BSB-Controller Model 620 T (WTW)).

**Volatile Fatty Acids (VFA) determination:** Acetate, propionate, butyrate, isobutyrate and valerate were measured by HPLC (Waters) equipped with a PolyporeH column (250 mm by 7.8 mm [inside diameter]) connected to a differential refractometer (RI-401 Waters) and a CR-6A Shimadzu integrator. The mobile phase was 0.02 N $H_2SO_4$ at a flow rate of 0.6 mL $min^{-1}$ and the column temperature was maintained at 60°C. Samples were acidified with 1 M $H_2SO_4$, centrifuged 15 min at 13000 rpm, filtered through 0.22 μm filter (MILLIPORE) and 20 μL injected.

**Size exclusion HPLC analysis:** A Progel TSK-G 2000-SW Supelco column (300x7.8 mm) was used with a Shimadzu 10AVP apparatus to analyse molecular-mass distribution of the OMW polyphenols. The elution was carried out using a phosphate buffer pH 6.8 and 0.6 mL $min^{-1}$ flow rate. The wave length of the detector was adjusted to 280 nm.

**Toxicity and reuse tests**

**Bioluminescence toxicity test:** The microtoxicity test consists of the inhibition of the bioluminescence of *Vibrio fischeri* LCK480 using the (Dr. Lange GmbH, Duesseldorf, Germany) LUMIStox system and according to ISO 11348-2[29]. Percentage inhibition of the bioluminescence was achieved by mixing 0.5 mL of OMW and 0.5 mL luminescent bacterial suspension. After a 15 min exposure at 15°C, the decrease in light emission was measured. The toxicity of the OMW is expressed as the percent of the inhibition of bioluminescence ($\%I_B$) relative to a non-contaminated reference. A positive control (7.5% NaCl) was included for each test.

**Phytotoxicity test:** Phytotoxicity was estimated by the determination of the germination index according to Wong *et al.*[30] using *Lycopersicon esculentum* (tomato) seeds.

## RESULTS

**Fungal pre-treatment:** After the first decantation (Fig. 1), the percentage of total suspended solids removal was 83%. Some colloids remained in the wastewaters. Decanted OMW fermentation with *P. chrysoporium* and the autochthonous micro flora was operated in a semi-continuous mode with a residence time of 3 to 5 days depending on the COD influent. The average values of the pH of the influent and the effluent were 4.9 and 6.4, respectively (Fig. 2a). Figure 2b shows the evolution of the COD of influent and the effluent of the aerobic bioreactor. The COD feeding was variable and ranged between 70 and 100 g $L^{-1}$ while the effluent COD ranged between 50 and 70 g $L^{-1}$. The aerobic treatment resulted in a COD abatement of 20 to 50%. The pre-storage of

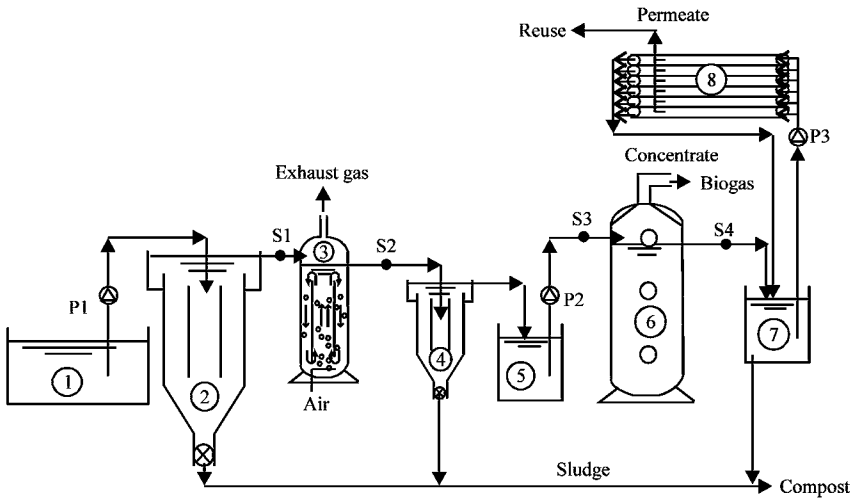


Fig. 1: Diagram of the integrated process used in this study. 2: decanter; 3: air lift reactor; 6: anaerobic filter reactor; 8: ultrafiltration membrane system

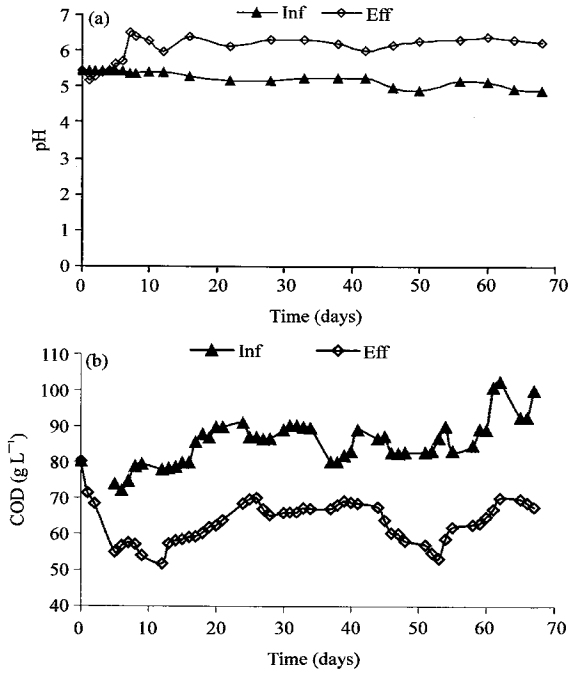


Fig. 2: Evolution of pH (a) and COD (b) in the influent and in the effluent as a function of time during *P. chrysosporium DSM 6909* pre-treatment in the 120 l air-lift reactor. Each point represents the mean of two determinations (SD <10% of the mean)

OMW (Fig. 3) in ambient temperature (20 to 35°C) resulted in the rapid consumption of easily biodegradable compounds and polyphenol polymerisation. This phenomenon was achieved by comparing the

Table 1: Effect of aeration rate on COD decrease, final pH and toxicity

| Aeration rate (vvm) | Initial COD (g $L^{-1}$) | COD decrease (%) | Final pH | Inhibition of *Vibrio fisheri* (%) |
|---|---|---|---|---|
| 0.25 | 115 | 32.3 | 5.0 | 73 |
| 0.5 | 118 | 30.6 | 5.0 | 71 |
| 0.75 | 114 | 32.9 | 4.9 | 68 |
| 1 | 121 | 35.4 | 7.5 | 62 |
| 1.5 | 118 | 46.0 | 7.8 | 49 |

polyphenols profile of fresh OMW with pre-stored OMW by Size Exclusion High Pressure Liquid Chromatography (Data not shown). This decantation step resulted in a slight decrease of the toxicity (5%). OMW became more resistant to biodegradation and an aeration rate of 0.25 vvm could be insufficient. The effect of the aeration rate on COD decrease, toxicity and final pH was realized in batch mode in 25 l bubble column reactor (Table 1). The aeration rate varied from 0.25 to 1.5 vvm. The initial COD was higher than 100 g $L^{-1}$. For aeration rates of 0.25, 0.5 and 0.75, the COD decrease was not improved and the pH was not changed. The percentages of inhibition of *Vibrio fisheri* luminescence showed that compared to the 100% inhibition of untreated OMW, *P. chrysosporium DSM 6909* decreased the relative toxicity in a range of 70%. However, for an aeration rate of 1 and 1.5 vvm, the COD decrease was more important and the final pH values were 7.5 and 7.8, respectively. The percentages of inhibition of *Vibrio fisheri* luminescence decreased to 62 and 49%, respectively. This is in good accordance with a high biological activity during this batch experiment. In these conditions *P. chrysosporium DSM 6909* grew during the first 3-4 days when bioconversion (depolymerization) took place. Subsequently, the other micro-organisms over-grew on the detoxified OMW. These results suggest that aeration should be around

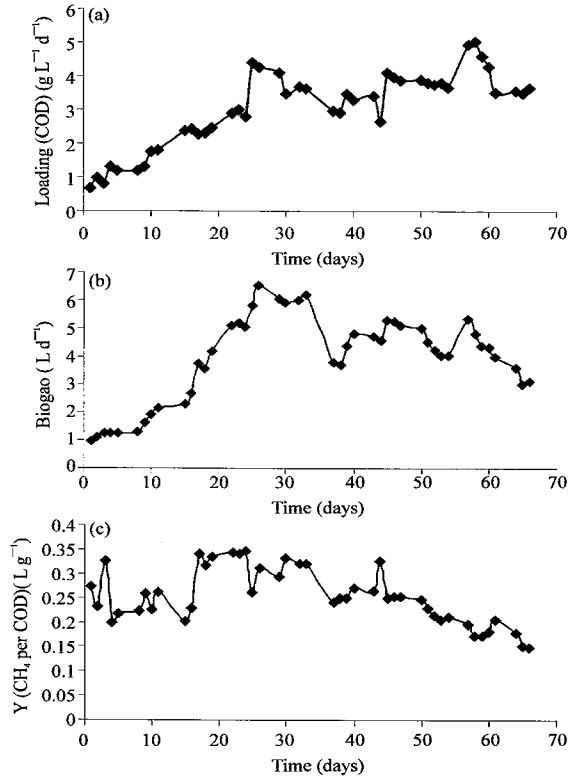


Fig. 3: Evolution of the loading rate (a), biogas productivity (b) and methane yield (c) as a function of time during methanisation of untreated OMW in a 3 L anaerobic filter. Each point represents the mean of two determinations and two independent experiments (SD <12% of the mean)

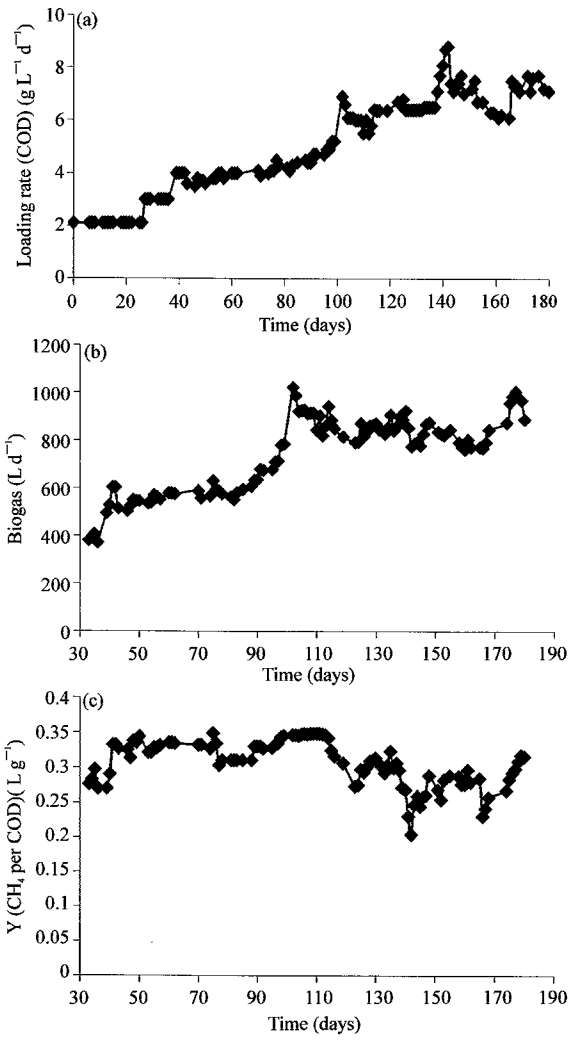


Fig. 4: Evolution of the loading rate (a), biogas productivity (b) and methane yield (c) as a function of time during methanisation of *P. chrysosporium DSM 6909* pre-treated OMW. Each point represents the mean of two determinations (SD <12% of the mean)

1 vvm when OMW are poorly biodegradable (in oxidized state). However, this aeration rate would have a negative impact on the cost of the treatment. Besides, decolorization of OMW was not observed even at 15 an aeration rate of 1.5 vvm.

After the aerobic treatment with *P. chrysosporium DSM 6909*, a low concentration of solids was separated by decantation steps (4) and (5) (Fig. 1). No detoxication effect was observed after this step. The *P. chrysosporium DSM 6909* pre-treated OMW was then fed in the anaerobic digester without dilution and pH regulation.

**Anaerobic bio-treatments**

**Anaerobic digestion of non pre-treated OMW:** The anaerobic treatment of non pre-treated OMW was performed in a 3 L Anaerobic Filter reactor (AF). The yield of methanisation of this untreated diluted OMW was higher than 0.3 L $g^{-1}$ ($CH_4$ per COD) at low loading rates. However, since the 26th day, when the loading rate reached a mean of 4 g $L^{-1}$ $d^{-1}$ of COD (Fig. 3a), a decrease in the biogas production and yield were observed (Fig. 3b). This toxicity was accompanied by a pH decrease in the three levels of the reactors and an accumulation of the Volatile Fatty Acids (VFA) (data not shown). This test of the anaerobic digestion of untreated OMW by an 8 years OMW-acclimated consortium will serve as a control for comparing the efficiency of the biological pre-treatment in the detoxification of this effluent.

**Anaerobic digestion of *P. chrysoporium* pre-treated OMW:** The anaerobic filter was loaded with undiluted

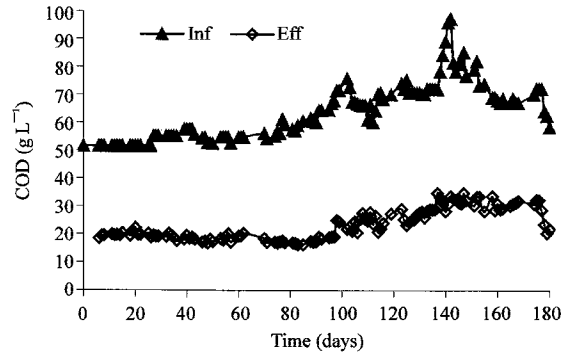


Fig. 5: volution of the COD in the influent and in the effluent as a function of time during methanisation of *P. chrysosporium DSM 6909* pre-treated OMW. Each point represents the mean of two determinations (SD <10% of the mean)

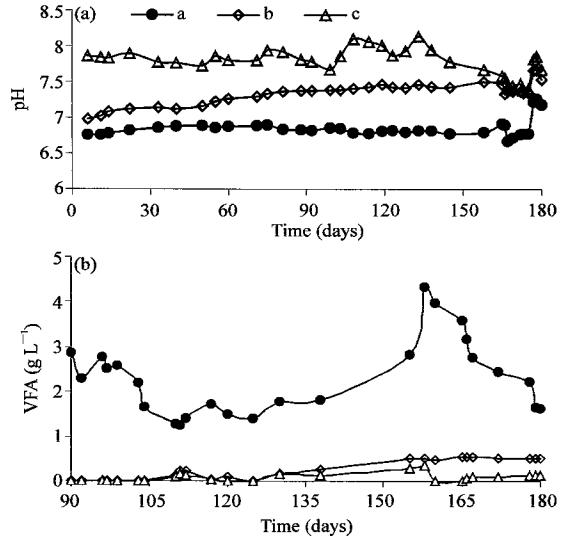


Fig. 6: Evolution of pH (A) and Volatile Fatty Acids (VFA) (B) in points a: bottom, b: middle and c: top of the 300 l digester as a function of time during methanisation of *P. chrysosporium DSM 6909* pre-treated OMW. Each point represents the mean of two determinations (SD <15% of the mean)

biological pre-treated OMW at a starting loading rate of 2-3 g COD per litre of reactor and per day. The COD loading was increased when no apparent toxicity was encountered.

The evolution of the loading rate, biogas production and methane yield are presented in Fig. 4a, b and c, respectively.

Table 2: Compositions of olive mill wastewaters before (point 1, Fig. 1) and after the treatment with the aerobic (*P. chrysosporium*)-anaerobic process (Point 7, Fig. 1)

| Parameter* | Untreated OMW | Biological treated OMW |
|---|---|---|
| pH (25°C) | 5.46 | 7.60 |
| Electric conductivity (25°C) (dS $m^{-1}$) | 8.70 | 11.30 |
| Salinity (g $L^{-1}$) | 5.90 | 9.70 |
| Colour (Absorbance 395 nm) | 82.00 | 44.00 |
| UV Absorbance 280 nm | 368.00 | 38.00 |
| $BOD_5$ (g $L^{-1}$) | 34.40 | 4.50 |
| COD (g $L^{-1}$) | 117.00 | 21.90 |
| $COD/BOD_5$ | 3.40 | 4.87 |
| Glucose (g $L^{-1}$) | 12.00 | ND |
| Reducing sugars (g $L^{-1}$) | 26.00 | ND |
| Total solids (%) | 11.40 | 2.50 |
| Total volatiles (%) | 9.30 | 1.42 |
| Total suspended solids (g $L^{-1}$) | 8.90 | 3.50 |
| Volatiles suspended solids (g $L^{-1}$) | 6.50 | 2.70 |
| Nitrogen (g $L^{-1}$) | 1.58 | 1.72 |
| Phosphorous (g $L^{-1}$) | 0.84 | 1.12 |
| Potassium (g $L^{-1}$) | 5.20 | 4.40 |
| O-Diphenols (g $L^{-1}$) | 8.39 | 1.26 |
| Total Polyphenols (g $L^{-1}$) | 9.20 | 1.57 |
| Residual oils (g $L^{-1}$) | 9.20 | ND |
| Toxicity by LUMIStox (% inhibition) | 100.00 | 38.00 |

* Total wastewaters

At the higher loading rates of COD (6-8 g $L^{-1}$ $d^{-1}$), the yields obtained were approximately 0.25 L $g^{-1}$ (methane per COD introduced). The volume of biogas reached 1000 L $d^{-1}$ (more than 3 fold of the volume of the digester). The higher values of yields (methane per COD introduced) 0.32 to 0.34 L $g^{-1}$ were obtained for loading rates lower than 6 g $L^{-1}$ $d^{-1}$ of COD. The mean COD reduction was 65% (Fig. 5). The biomethanisation yields of untreated and pre-treated OMW were compared at the same loading rate of COD 4 g $L^{-1}$ $d^{-1}$. Indeed, at the 65th day of operation, the methane yield ($CH_4$ per CODi) in (L $g^{-1}$) was 0.15 for untreated OMW (Fig. 3) and 0.34 for pre-treated OMW (Fig. 4).

The biomethanisation process was found to be stable during 6 months of operation although the COD of the influent was very high (exceeding 80 g $L^{-1}$ in some cases). We did not observe any toxicity phenomenon. This experiment was interrupted due to cross-mixing phenomenon at very high gas activity (more than 31 $L^{-1}$ $d^{-1}$). VFA and pH were analysed in the three levels of the anaerobic filter. Figure 6A shows that pH values at points a, b and c were optimal since the anaerobic filter was a compartmentalized reactor involving hydrolytic and acidogenic bacteria in the lower part of the reactor and methanogenic ones in the upper part. The VFA concentrations were low even at the higher loading rates (Fig. 6B).

**Characterisation of the final effluent:** As shown in Table 2, untreated OMW exercised 100% inhibition on *Vibrio fischeri*. This toxicity was due essentially to its

Table 3: Effect of membrane cut-off on COD, color and suspended solids SS abatements

| | Reduction (%) | | |
|---|---|---|---|
| Membrane cut-off | COD | Coloration | SS |
| 2 kDa | 57.5 | 94.8 | 100 |
| 25 kDa | 45.0 | 95.9 | 100 |
| 100 kDa | 36.0 | 87.0 | 100 |

Table 4: Inhibition of *Vibrio fisheri* luminescence after exposure with different OMW samples during 15 min

| OMW sample | Inhibition of *Vibrio fischeri* (%) |
|---|---|
| Untreated OMW | 100 |
| *P. chrysosporium DSM 6909* treated OMW | 74 |
| *P. chrysosprium*-anaerobic treated OMW | 38 |
| Ultra filtered anaerobic effluent | 0 |

high phenolic content (9.2 g $L^{-1}$). It was reduced to 38% in treated OMW which contained 1.26 g $L^{-1}$ of ortho-diphenolics. C18-HPLC analysis showed that almost all monoaromatic compounds were removed in the treated OMW except of a small concentration of hydroxytyrosol (data not shown). The COD of treated OMW remained high (21.9 g $L^{-1}$). This value far exceeded the standard for direct discharge to a natural water body. Several costly steps are necessary if we want to reach the Tunisian standard requirements (0.09 g $L^{-1}$). Table 2 showed also that the residual COD was poorly biodegradable ($COD/BOD_5$ = 4.87). The organic compounds which remained in the effluent could be attributed to inert COD formed by highly polymerised polyphenolic compounds such as humic-like substances. These compounds could undergo biochemical modifications during the humification process in soil. Treated OMW contained important concentrations of nitrogen, phosphorous and potassium. This effluent was free of pathogens, less toxic and contained low concentrations of heavy metals. Apart from the COD, $DBO_5$ and black color, the quality of treated OMW was high and could be used for irrigation after field tests. We investigated the removal of the residual black color by ultrafiltration processes. For this purpose, membranes having 2, 25 and 100 kDa cut-offs were tested in order to remove the residual compounds.

**Improvement of the quality of the effluent using membrane technology and reuse test:** The best membrane flux 59 l $h^{-1}$ $m^{-2}$ was found using the PVDF membrane having a cut-off of 100 kDa at 1.75 bars. At this same pressure, the use of 25 and 2 kDa cut-off resulted in lower flux: 50 and 41 l $h^{-1}m^{-2}$, respectively. This could be explained by the colloidal characteristics of the biological treated effluent (high molecular-mass polyphenolic compounds). These compounds strongly affected the flux rate and stability during the first 15 min of operation.

The determination of the physico-chemical parameters of the ultrafiltered anaerobic effluent showed that the 25 kDa cut-off membrane was able to remove 100% of the suspended solids, 95% of the color and 45% of the residual COD (Table 3). Moreover, the results showed that high polyphenolic compounds (monitored by SE-HPLC analysis) as well as residual monoaromatic compounds (monitored by C18-HPLC analysis) were retained by the UF membrane (data not shown). Hence, the UF treated anaerobic effluent was free of toxic compounds as can be seen in Table 4. Indeed, the percentages of inhibition of *Vibrio fisheri* luminescence after exposure with the OMW samples showed that compared to the 100% inhibition of untreated OMW and 38% inhibition of the biologically treated effluent, the UF post-treatment completely detoxified the wastewater and removed the black color. Ultrafiltration of the *P. chrysosporium DSM 6909*-anaerobic digestion effluent improved the quality of the effluent by reducing its COD, darkness and toxicity. This effluent could be tested for irrigation at large scale experiments.

Reuse test experiments of the UF *P. chrysosporium DSM 6909* anaerobic digestion effluent were carried out using the Germination Index (GI) and growth of the *Lycopersicon esculentum* (tomato) seeds in arable soil. Results showed that application of ultrafiltered *P. chrysosporium DSM 6909*-anaerobic digestion effluent increased the GI percentage of *L. esculentum* to 128% compared to 100% for control (irrigated with water) while irrigation with diluted untreated OMW led to a decrease of the GI to 12%. Moreover, treated OMW enhanced the plant growth compared to the control while untreated OMW caused an important growth inhibition.

## DISCUSSION

A technical application of OMW treatment does not exist. Therefore, the development of a suitable technology involving the white rot fungi *P. chrysosporium DSM 6909* is attractive due to its possible application in the degradation of other recalcitrant wastewaters such as textiles, tanneries and landfill leachates wastewaters. In combination with an anaerobic post-treatment, an economic treatment of the wastewaters discharged mostly by small and medium sized olive mills would be possible and will guarantee the long term survival of these enterprises.

Through this pilot experiment, the use of *P. chrysosporium DSM 6909* was shown to be possible even in the worst conditions e.g. not sterile condition, not regulated pH and temperature, without inducers and in semi-continuous mode of cultivation. *P. chrysosporium DSM 6909* was inoculated in a medium where other fungi and bacteria co-existed in competition. During the

experiments, *P. chrysoporium* hyphae and spores were abundant. However, bacteria were omnipresent and overgrew after the detoxification with *P. chrysoporium* although OMW feeding imposed a selective pressure in the medium due to the physico-chemical characteristics of the influent such as high COD and polyphenol concentrations, pH 5, lipids. Nevertheless, the reactor was bio-augmented with grinded mycelium every 2 weeks when the proportion of mycelium compared to bacteria decreased.

The problem with the use of *P. chrysosporium DSM 6909* fungus would be the high oxygen need for biological reactions especially when the OMW is not frozen before treatment. Results in this study showed that 0.25 vvm of aeration was not sufficient for the biological treatment of the high strength OMW effluent. The percentage of toxicity removal by *P. chrysosporium DSM 6909* is low (26%). Indeed, the depolymerization activity of such a strain which resulted in the generation of intermediate molecular-mass aromatic compounds, did not allow high reduction of toxicity as was reported by Fukui[31]. Aggelis *et al.*[17] used a white rot fungi *Pleurotus ostreatus* in bioreactor batch cultures to reduce phenolic content and toxicity of sterilized OMW. However, they reported that high OMW dilutions should be used and/or additional treatment should be applied for complete detoxification and use of the OMW for irrigation.

An aeration of 1 vvm gave better results with respect to residence time, COD removal and detoxification. On the other hand, the increase of aeration rate would increase the cost of treatment. For the lower aeration rate of 0.25 vvm and a residence time of 5 days, the specific energy consumption was 3.5 kWh $kg^{-1}$ of COD degraded. This value is higher than that of municipal wastewater treatment which is approximately 1kWh $kg^{-1}$ of COD degraded. This could be explained by the nature of organic compounds present in OMW (polyphenols) which are resistant to biodegradation.

On the other hand, the anaerobic digestion of undiluted pre-treated OMW was realized in 300 l reactor. This anaerobic reactor worked for 6 months at loading rates reaching 7-8 g $L^{-1}$ $d^{-1}$ of COD. During a 2 successive olive harvest years without any apparent toxicity. Knowing that in this study untreated OMW causes inhibition of methanization at a loading rate of COD 2 to 4 g $L^{-1}d^{-1}$[32,33], it can be concluded that aerobic pre-treatment of OMW with *P. chrysosporium DSM 6909* resulted in decreasing the toxic effect of this wastewater on anaerobic digestion. Moreover, this experiment was stopped at a loading rate of COD 7-8 g $L^{-1}$ $d^{-1}$ while the biological process did not show any apparent toxicity. Indeed, at the last days of this experiment, when the biogas production became more than 900 L $d^{-1}$, cross-mixing phenomena were observed and the pressure inside the reactor increased which was resulted in a biogas escape through the gas outlet tube as well as the liquid outlet. This problem was solved in further experiments by modifying the conception of the anaerobic filter. In this way, parallel feed entrances and lateral gas exits were designed. The liquid to be treated entered the reactors by parallel entrances in order to prevent clogging. A portion of the gas formed during the treatment would rise to another compartment while the rest of the gas leaved the reactor by a gas exit situated under the same plate.

In this regard, Beccari *et al.*[12] reported that phenolic fraction below 500 D is removed by the methanogenic process whereas the phenolic fraction above 1000 D were significantly adsorbed on bentonite suggesting the necessity of an activated sludge post treatment[12].

This anaerobic step would produce an excess of energy generated from the methane (approximately 1.2 kWh $kg^{-1}$ of COD transformed). Part of the calorific energy would be used for maintaining the digester at 37°C while the co-generated electricity could be used for the aerobic step.

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## REFERENCES

1. Capasso, R., A. Evidente, I. Schivo, G. Orru, M.A. Marciallis and G. Cristinzo, 1995. Antibacterial polyphenols from olive oil mill waste waters. J. Applied Bacteriol., 79: 393-398.
2. Flouri, F., D. Sotirchos, S. Ioannidou and C. Balis, 1996. Decolorization of olive oil mill liquid wastes by chemical and biological means. Intl. Biodeterioration and Biodegradation, 9: 189-192.
3. Andreozzi, R., G. Longo, M. Majone and G. Modesti, 1998. Integrated treatment of olive oil mill wastewater: Study of ozonation coupled with anaerobic digestion. Water Res., 32: 2357-2364.
4. Beccari, M., M. Majone, C. Riccardi, F. Savarese and L. Torrisi, 1999. Integrated treatment of olive oil mill effluents: Effect of chemical and physical pretreatment on anaerobic treatability. Water Sci. Technol., 40: 347-355.

5. Marques, I.P., 2001. Anaerobic digestion treatment of olive mill wastewater for effluent reuse in irrigation. Desalination, 137: 233-239.
6. Andreoni, V., A. Ferrari, G. Ranali and C. Sortini, 1984. The influence of phenolic acids present in oil mill waters on microbial groups for the methanogenesis. In: Process of International Symposium on Olive By-products Valorisation. FAO 5-7 March Espana.
7. Boari, G., A. Brunetti, R. Passino and A. Rozzi, 1984. Anaerobic digestion of olive oil mill wastewaters. Agric. Wastes, 10: 161-175.
8. Angelidaki, I., L. Ellegaard and B.K. Ahring, 1997. Modelling anaerobic co-digestion of manure with olive oil mill effluent. Water Sci. Technol., 36: 263-270.
9. Gavala, H.N., I.V. Skiadas and G. Lyberatos, 1999. On the performance of a centralised digestion facility receiving seasonal agro-industrial wastewaters. Water Sci. Technol., 40: 339-346.
10. Boari, G. and I.M. Mancini, 1990. Combined Treatments of urban and olive mill effluents in Apulia Italy. Water Sci. Technol., 22: 235-240.
11. Croce, F., S. Poulsom and D.W. Hendricks, 1994. Combined treatment of olive mill effluent and municipal wastewater in a small tourist community. Water Sci. Technol., 29: 105-110.
12. Beccari, M., G. Carucci, A.M. Lanz, M. Majone and M. Petrangeli Papini, 2002. Removal of molecular weight fractions of COD and phenolic compounds in an integrated treatment of olive oil mill effluents. Biodegradation, 13: 401-410.
13. Sayadi, S. and R. Ellouz, 1992. Decolourization of olive mill waste-waters by *Phanerochaete chrysosporium*, involvement of the lignin degrading system. Applied Microbiol. Biotechnol., 37: 813-817.
14. Sayadi, S. and R. Ellouz, 1993. Screening of white rot fungi for the treatment of olive mill waste-waters. J. Chem. Technol. Biotechnol., 57: 141-146.
15. Sayadi, S. and R. Ellouz, 1995. Role of lignin peroxidase and manganese peroxidase from *Phanerochaete chrysosporium* in the decolourization of olive mill waste-waters. Applied Environ. Microbiol., 61: 1098-1103.
16. Martirani, L., P. Giardina, L. Marzullo and G. Sannia, 1996. Reduction of phenol content and toxicity in olive oil mill waste waters with the ligninolytic fungus *Pleurotus ostreatus*. Water Res., 30: 1914-1918.
17. Aggelis, G., D. Iconomou, M. Christou, D. Bokas, S. Kotzailias, G. Christou, V. Tsagou and S. Papanikolaou, 2003. Phenolic removal in a model olive oil mill wastewater using *Pleurotus ostreatus* in bioreactor cultures and biological evaluation of the process. Water Res., 37: 3897-3904.
18. Fenice, M., G.G. Sermanni, F. Federici and A. D'Annibale, 2003. Submerged and solid-state production of laccase and Mn-peroxidase by *Panus tigrinus* on olive mill wastewater-based media. J. Biotechnol., 100: 77-85.
19. Yesilada, O., S. Sik and M. Sam, 1998. Biodegradation of olive mill wastewaters (OOMW) by *Coriolus versicolor* and *Funalia trogii*: Effects of agitation, initial COD concentration, inoculum size and immbilization. World J. Microbiol. Biotechnol., 14: 37-42.
20. D'Annibale, A., C. Crestini, V. Vinciguerra and G.G. Sermanni, 1998. The biodegradation of recalcitrant effluents from an olive mill by a white-rot fungus. J. Biotechnol., 61: 209-218.
21. D'Annibale, A., M. Ricci, D. Quaratino, F. Federici, and M. Fenice, 2004. *Panus tigrinus* efficiently removes phenols, color and organic load from olive-mill wastewater. Research in Microbiology, (In Press).
22. Perez, J., T. De la Rubia, O.B. Hamman and J. Martinez, 1998. *Phanerochaete flavido-alba* laccase induction and modification of manganese peroxidase isoenzyme pattern in decolorized olive oil mill wastewaters. Applied Environ. Microbiol., 64: 2726-2729.
23. Kissi, M., M. Mountadar, O. Assobhei, E. Gargiulo, G. Palmieri, P. Giardina and G. Sannia, 2001. Roles of two white-rot basidiomycete fungi in decolorisation and detoxification of olive mill waste water. Applied Microbiol. Biotechnol., 57: 221-226.
24. Fountoulakis, M.S., S.N. Dokianakis, M.E. Kornaros, G.G. Aggelis and G. Lyberatos, 2002. Removal of phenolics in olive mill wastewaters using the white-rot fungus *Pleurotus ostreatus*. Water Res., 36: 4735-4744.
25. Jaouani, A., S. Sayadi, M. Vanthournhout and M. Penninckx, 2003. Potent fungi for decolourization of olive oil mill wastewater. Enzyme and Microbial Technol., 33: 802-809.
26. Sayadi, S., N. Allouch, M. Jaoua and F. Aloui, 2000. Detrimental effects of high molecular-mass polyphenols on olive mill wastewaters biotreatments. Process Biochemi., 35: 725-735.
27. Box, J.D., 1983. Investigation of the Folin-Ciocalteau phenol reagent for the determination of polyphenolic substances in natural waters. Water Res., 17: 511-522.
28. Knechtel, R.J., 1978. A more economical method for the determination of chemical oxygen demand. J. Water Pollution Control Federation, pp: 25-29.
29. ISO 11348-2, 1998. Water quality-Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test) - Part 2: Method using liquid-dried bacteria.

30. Wong, J.W.C., K. Li, M. Fang and D.C. Su, 2001. Toxicity evaluation of sewage sludges in Hong Kong. Environ. Intl., 27: 373-380.
31. Fukui, H., 1992. Dechlorination and detoxification of bleach plant effluent by *Phanerochaete chrysosporium*. J. Biotechnol., 24: 267-275.
32. Hamdi, M., 1991. Effects of agitation and pretreatment on the batch anaerobic digestion of olive mill wastewater. Biores. Technol., 36: 173-178.
33. Rozzi, A., R. Passino and M. limoni, 1989. Anaerobic treatment of olive mill effluents in polyurethane foam bed reactor. Process Biochem., 4: 68-74.