Desalination and Water Treatment
www.deswater.com
doi: 10.5004/dwt.2019.23869

149 (2019) 129–137
May

Olive mill wastewater biological assessment: optimization and identification of compounds degrading yeasts

Aziz Boutafda[a,b], Yassine Zegzouti[a,c], Loubna El Fels[a,d,*], Yedir Ouhdouch[b,f], Ahmed Lebrihi[e], Faouzi Bekkaoui[f], Mohamed Hafidi[a,f]

[a]*Laboratory of Ecology and Environment (CNRST, URAC32), Faculty of Sciences Semlalia, Cadi Ayyad University, P.O. Box 2390, Marrakech, Morocco, Tel. +212666559755; email: loubna.elfels@gmail.com (L.E. Fels)*
[b]*Laboratory of Biology and Biotechnology of Microorganisms, Faculty of Sciences Semlalia, Cadi Ayyad University, P.O. Box 2390, Marrakech, Morocco*
[c]*Laboratory of Process Engineering, Faculty of Sciences, Ibn Zohr University, Agadir, Morocco*
[d]*Higher Institute of Nursing Professions and Health Technics, Marrakesh-Safi, Morocco*
[e]*ENSAT-INP Toulouse France*
[f]*Agrobiosciences Program, University Mohammed VI Polytechnic (UM6P), Benguerir, Morocco*

Received 25 July 2018; Accepted 26 January 2019

ABSTRACT

The present work aims to evaluate the capacity of a yeast strain to degrade the olive mill wastewater (OMWW) and to obtain a stabilized product that can be disposed without any environmental impact on the water–soil–plant system. The pollutant load, expressed by the chemical oxygen demand (COD) and the phenolic compounds (PC), are 187.63 and 4.30 g/L, respectively. The evaluation of the yeast strain capacity to develop on liquid and solid culture media based on the OMWW at different dilutions of 25%, 50%, 75%, raw matter, allowed us to optimize the abatement of the pollutant load, particularly, PC. The results show a significant reduction of 98% for the COD and 97% for the PC, after 1 month of treatment at a dilution of 25%. The PC extracts were characterized by high-performance liquid chromatography, the main identified PC are hydroxytyrosol, tyrosol, caffeic acid, *p*-coumaric acid and oleuropein. Most of which had disappeared after the treatment. The selected yeast strain was identified by 26S rDNA sequencing and shows that belongs to the species of *Rhodotorula mucilaginosa*.

Keywords: Olive mill wastewater; Biological treatment; *Rhodotorula mucilaginosa*; Phenolic compounds; Chemical oxygen demand

1. Introduction

Olive oil production is an important economic activity in the Mediterranean countries. In fact, these countries have 98% of the world's olive-growing areas and 97% of world olive production [1]. Given the growing global demand for olives and their derivatives, the olive sector is growing steadily. In Morocco, olive growing has received a great deal of attention. Indeed, a development plan for this sector has been implemented since the launch of the Green Morocco Plan in 2008. Indeed, at the national level, the planted areas with olive trees expanding from 773,000 ha in 2009 to more than 1 million ha in 2016, showing an increase of 31%. This agri-food business generates two by products: one solid called pomace and the other liquid called olive mill wastewater (OMWW), which is responsible for serious environmental problems such as inhibition of microbial activity, phytotoxic effect, release of foul odors, groundwater pollution and inhibition of auto-purification process [2–4]. Globally, the volume of OMWW generated is estimated at more than 30 million m^3/year during the seasonal extraction of olive oil [5]. This effluent is resulting

* Corresponding author.

from the olive leachate, olive washing operation, processing water, part of the olive pulp and residual oil. In addition, these effluents are characterized by a high acidity, a high organic matter load, being mainly composed by sugars, tannins, pectins, polyphenols, alcohols and lipids [6]. Moreover, the high level of the pollutant load which is estimated by the chemical oxygen demand (COD; 30–318 g/L) and the high load of phenolic compounds (PC), the average range between 0.79 and 24 g/L [7,8]. This creates a serious environmental problem in the Mediterranean countries.

After each trituration process, most of olive oil mill plants owners discharge these wastewaters without any treatment into nature or rarely stored in evaporation ponds. This could be attributed to the lack of suitable treatment methods. Hence, the need for a prior treatment that allows their detoxification and makes their use in fertigation for disposal without too much risk on the receiving environment. The treatment of OMWW is based on several biological, physicochemical and physical strategies. However, the biological processes of treatment with selected microorganisms such as *Aspergillus niger*, *Candida holstii* and *Azotobacter vinelandii* are among the most environmentally compatible and the least costly method [9]. The biological treatment of OMWW using fungi has shown a significant reduction of PC around 90% [3,10,11]. This reduction could be explained by the use of PC by microorganisms as an energy source [10]. Ascomycete and basidiomycete yeasts, white rot fungi are frequently used for PC degradation of OMWW [11]. Ben Sassi et al. [1] and Ntougias et al. [12] have shown that according to their ability to tolerate high concentrations of PC and acidic pH, yeasts appear to be the dominant microorganisms in these effluents compared with bacteria and other mould.

Several species of yeast were chosen to treat OMWW. The following species were reported as a potential candidate for the detoxification of these effluents: *Rhodotorula glutinis* [13], *Pichia membranifaciens* [14], *Candida* spp. [9]. In general, the results of the above studies have confirmed the promising prospects for the use of yeast to degrade organic pollutants from OMWW, in particular PC.

The aim of this work is to evaluate the performance of a selected yeast isolated from different biotopes for its ability to degrade raw OMWW in particular PC as the only carbon and energy source. This treatment has been carried out using a wide range of OMWW concentration (25%, 50%, 75% and raw matter).

2. Materials and methods

2.1. Olive mill wastewater sampling and composition

The OMWW used in this study was obtained from a three-phase processing plant (MOLINOVA TG, MOD 1000, Pieralisi, Italy) located in Chichaoua (Morocco). OMWW samples were collected at the beginning of the olive harvest season (December 2015). The OMWW was collected immediately after disposal to avoid any changes in the PC composition and maintained at –20°C until further utilization.

2.2. Physicochemical characterizations

OMWW samples were analyzed for the following parameters: pH was measured by pH-meter (HANNA instruments, HI 2210-02, Portugal). Electrical conductivity (EC) was measured by conductivity-meter (HANNA instruments, HI 9033, Portugal). Total suspended solids (TSS) were determined after filtering a sample through a filter (Whatman membrane filters nylon pore, size 0.45 μm, diam. 47 mm) and drying the retained residue at 105°C until constant weight was obtained. Total solids (TS) were determined by oven drying of 10 mL of sample at 105°C for 24 h, according to the APHA standard method. Total ash content was determined by incineration of TS at 600°C for 6 h. Total Kjeldahl nitrogen (TKN) was determined after mineralization and distillation, TKN analysis was carried out as follows: 5 mL of OMWW were mixed with a Kjeldahl catalyst tablet (0.5 g) and 10 mL of H_2SO_4 (96%). Digestion was carried out at 400°C during 2 h. After cooling, 100 mL of distilled water and 50 mL of NaOH solution (40 g/L) were added before distillation according to the AOAC analytical methods [15]. COD was determined by the Standard Methods for the Examination of Water and Wastewater [16]. The appropriate amount of wastewater samples was diluted up to 1,000 times and two milliliters of diluted OMWW was introduced into a lab-prepared digestion solution containing sulfuric acid, potassium dichromate and mercuric sulfate. The mixture was then incubated for 120 min at 150°C in a COD reactor and the COD concentration was measured calorimetrically at 600 nm. Total organic carbon (TOC) was determined after oxidation using dichromate of potassium according to the Anne's method as described by Aubert [17]. The total lipid concentration was determined using the method of Folch et al. [18]. This involved the OMWW being macerated with 60 mL of a 2/1 v/v chloroform/methanol mixture for 24 h at 4°C. The supernatant was then recovered and filtered through a Durieux filter. This was repeated twice more to ensure maximum recovery of the lipid. The pooled supernatants were then shaken with 60 mL of 1% NaCl to separate the methanol from the chloroform phase containing the lipid. The chloroform phase was then dried over anhydrous sodium sulfate (Na_2SO_4) and the total lipid content was determined after evaporation of the chloroform phase at 40°C. The PC were extracted and purified with ethyl acetate using the method of Macheix et al. [19], then assayed using Folin–Ciocalteu reagent [20]. The extraction yield, calculated on the basis of hydroxytyrosol, was 90.4%. Three replicates were used, and the mean values of the parameters were recorded. The amount of total saccharides was determined spectrophotometrically, according to the method described by Dubois et al. [21].

2.3. High-performance liquid chromatography analysis of phenolic compounds

PC were analyzed by high-performance liquid chromatography (HPLC). The system consists of a Knauer-type device with a Knauer diode array PDA detector for detection of compounds at wavelengths and software for data processing. The C18 reversed phase column (Eurospher II 100–5 C18, 250 × 4.6 mm) + precolumn was used. The column temperature was set at 25°C. The mobile phase consists of a gradient of acetonitrile and bidistilled water acidified to pH 2.6 with o-phosphoric acid and then filtered on Millipore (0.45 μm). The mobile phase composition was acetonitrile/water 5:95 (v/v). The flow rate was 1 mL/min and the

injection volume was 10 μL. The separation was performed on a gradient of 5% to 95% acetonitrile for 60 min. PC were simultaneously detected at a wavelength of 280 nm and were identified by comparison with controls for their retention time and UV spectrum. Co-injections with commercial standards (hydroxytyrosol, tyrosol, catechol, caffeic acid, *p*-coumaric acid, oleuropein, gallic acid and syringic acid) were carried out to confirm the identity of the compounds. Calibration curves of the analyzed compounds were constructed injecting 10 μL of standard solutions at five different concentrations.

2.4. Yeast strains isolation

Yeast strains were isolated from different biotopes such as sludge from OMWW evaporating ponds, soil irrigated with OMWW. The samples were collected from 8 to 10 cm depth using a sterile spatula and transferred to pre-autoclaved sterile glass bottles with rubber stoppers. The samples were brought to the laboratory and stored under refrigeration temperature. 1 g of each sample was suspended in 9 mL sterilized physiological water. The suspension was incubated at 28°C, 150 rpm for 2 h and a serial dilution (1:10) was prepared in sterilized physiological water. From each dilution, 0.1 mL was spread on potato dextrose agar (PDA) containing 50 μg/mL of chloramphenicol to inhibit bacterial growth. The Petri dishes were incubated for 48 h at 28°C. Colonies with distinct morphological differences such as color, shape and size were isolated and purified. The purified isolates were stored on yeast peptone glucose agar (YPGA) plates at 4°C.

2.5. Yeast strains screening

The isolated strains were tested for their ability to grow on the OMWW as culture medium. Different dilutions of OMWW (25%, 50%, 75%, and 100% (v/v)) were used. Each dilution was supplemented with Agar–Agar (15 g/L) before sterilization at 120°C for 30 min. The medium is poured into sterile Petri dishes. After solidification, the strains were seeded and incubated at 28°C for 7 d. The colonies cultivated in this medium based on OMWW have been purified on the PDA medium. This screening was followed by a culture of these strains on a liquid medium composed of sterilized OMWW, in order to make sure that the OMWW was used as a carbon and energy source for the growth of these strains.

2.6. Use of phenolic compounds as a sole carbon source

The ability of the strains to grow on PC as the only carbon source was tested on minimal synthetic medium (2 g/L sodium nitrate, 1 g/L potassium phosphate, 0.5 g/L magnesium sulfate) with different concentrations of the PC (1,000, 500, 250 and 125 mg/L) extracted from the OMWW as carbon and energy sources.

2.7. Biological treatment of olive mill wastewater by the selected strain

The biological treatment was carried out in 500 mL mini-bioreactors containing 100 mL of OMWW medium. This is composed of 25%, 50%, 75% and 100% (v/v) of sterile OMWW in sterile distilled water. 1 mL of the selected yeast strain (54×10^3 CFU/mL), suspension (i.e., exponential phase), was used to inoculate the different dilutions of effluent. The sampling was performed typically every 3 d for 1 month and the samples were analyzed for their remaining COD and PC. Control sets consisting of uninoculated sterile OMWW were also prepared. The uninoculated controls were used to study the loss of PC by the abiotic processes. All the samples and controls were incubated under similar conditions and the experiments were performed in triplicate.

2.8. Taxonomic identification of the selected yeast strain

2.8.1. Identification of yeast strains

The Biolog system (Biolog Inc., Hayward, CA, USA) is a new semi-automated, computer-linked technology for yeast identification. It is based around a 96-well microtiter tray containing a range of dehydrated carbon sources for assimilation and oxidation tests [22,23]. The profile of growth responses provides a metabolic fingerprint for each isolate [22] and is compared with profiles of the 267 yeasts species in the Biolog database, to provide an identification. Purified isolates were transferred to Biolog universal growth agar and incubated at 26°C for 48 h. Pure colonies of yeast suspensions were prepared in 9 mL sterile distilled water and adjusted to 47 ± 2*T* (transmittance) using Biolog turbidimeter. 100 μL of inoculum was dispensed using digital pipettor to each of the 96 wells yeast microplates (YT) and incubated at 26°C for 24 to 72 h. The YT microplate was tagged with 96 carbon sources. The ability of an isolate to metabolize each carbon source was measured in the presence or absence of purple hue in the wells. The redox dye tetrazolium violet forms a purple color when oxidized by cellular respiration of microorganisms. The YT microplate measures both metabolic reactions as well as turbidity growth to produce identifications. YT microplate was read by the micro station reader at 24, 48 and 72 h at a single wavelength of 590 nm. The Biolog software (micro log 3 ver. 4.20.05) compared the results obtained with the test strain to the database and provided identification based on distance value of match and separation score produced similarity index value and probability for species identification [23].

2.8.2. Molecular identification of the selected yeast

The genotypic identification of the yeast strain was performed by DNA sequencing of the D1/D2 domain of the 26S rDNA gene. The DNA was extracted using the DNeasy tissue kit (QIAGEN, France), according to the protocol recommended by the manufacturer. Polymerase chain reaction (PCR) amplification with external primers NL1 5′-GCATATCAATAAGCGGAGGAAAAG-3′ and NL4 5′-GGTCCGTGTTTCAAGACGG-3′, was performed according to the method described by Kurtzman and Robnett [24]. PCR conditions were: initial denaturation at 95°C for 3 min, addition of 1 unit of Taq polymerase (Q-Biogene, France) followed by 34 cycles of denaturation at 95°C for 15 s, primer annealing at 59°C for 1 min and DNA extension at 72°C for 2 min. A final extension was completed at 72°C for

10 min. The amplified fragment was then purified by gel chromatography on Sephadex G-50 DNA Grade Super Fine (Sigma-Aldrich, Sweden). The purified PCR products were labeled using the CEQ Dye Terminator Cycle Sequencing (DTCS) with Quick Start Kit (Beckman Coulter, CA, USA) according to the manufacturer instructions. Sequencing was done in a CEQ 8000 XL Automatic Sequencer (Beckman Coulter, CA, USA). The consensus sequence was obtained by alignment of forward and reverse sequences with CEQ Investigator Software (Beckman Coulter, CA, USA). The identification of the closest relatives was obtained using the Basic Local Alignment Search Tool (BLAST) N (www.ncbi.nlm.nih.gov/BLAST/).

3. Results and discussions

3.1. Physicochemical characterization of olive mill wastewater

The results of the physicochemical characterization are presented in Table 1. The OMWW had a low pH (4.8), which is due to the presence of organic acids (phenolic acids, fatty acids, etc.). This acidic pH consistent with values (2.24–5.9) reported by literature [6]. The electrical conductivity (EC = 13.93 mS/cm) was due to the excessive use of salt (NaCl) for the conservation of olives before triturating. This value is similar to that found in the literature [1]. It contains large amounts of total solids up to about 120 g/L and a high concentration of TSS (10 g/L). The OMWW showed a high organic matter concentration (COD = 187.6 g/L). This COD content is very high compared with that recorded in other types of releases: different industrial wastewaters, slaughterhouse effluents, whey, municipal, manures, textile and food processing. In fact, COD does not exceed 4.02 g/L in slaughterhouse effluents, which are considered as the main dominant organic waste [27]. However, our value remains comparable with those obtained by several authors in OMWW from the three phase centrifugation systems [1,13].

Table 1
Physicochemical characterization of olive mill wastewater

Parameters	OMWW
pH	4.8 ± 0.04
EC, mS/cm	13.93 ± 0.1
TSS, g/L	10 ± 0.3
TS, g/L	122.9 ± 2.62
AshC, g/L	40.7 ± 1
COD, g/L	187.6 ± 19.11
TOC, g/L	73.26 ± 1.8
TKN, g/L	0.16 ± 0.001
Proteins, g/L	1 ± 0.02
Lipids, g/L	4.51 ± 0.4
Sugars, g/L	21.45 ± 0.4
PC, g/L	4.3 ± 0.1

Values are the average of three measurements ± standard error.
Note: EC, electrical conductivity; TSS, total suspended solids; TS, total solids; AshC, ash contents; COD, chemical oxygen demand; TOC, total organic compounds; TKN, total Kjeldahl nitrogen; PC, phenolic compounds; OMWW, olive mill wastewater.

The OMWW releases are also characterized by the predominance of toxic substances particularly PC with 4.3 g/L. This gives them antimicrobial potency [28], and could inhibit fauna and flora and thus limit any natural biodegradation and lead to a disruption of the entire ecosystem. This value is consistent with values (0.63–5.45 g/L) cited by Dermeche et al. [6]. These differences in phenol levels may be due to the variety and maturity level of processed olives.

3.2. Isolation, screening and phenolic tolerance tests

In this study, five yeast isolates were selected. Table 2 presents the results of the yeast tolerance test. It is found that the isolate Y1 can develop better on liquid and solid media based on OMWW diluted up to 50%. Given their tolerance to high loads of PC and acidic pH, yeasts are designated as the most dominant microorganism in OMWW [9]. However, the Y1 isolate can grow better on the minimum synthetic medium based on solid and liquid phenolic extract up to 1,000 mg/L (Table 3). The results suggest that yeast Y1 can use PC as the sole source of carbon, which justified its use for the bioremediation of OMWW. Studies of the ability of yeasts to degrade PC have shown that yeasts are able to tolerate large concentrations of PC and have the power to metabolize them and use them as a carbon and energy source for their growth. Katayama-Hirayama et al. [29] studied the effect of phenol concentration on the rate of oxygen uptake by cells

Table 2
Tolerance results of yeast strains on solid and liquid medium based on olive mill wastewater with different dilutions

	Olive mill wastewater							
	Solid				Liquid			
	100%	75%	50%	25%	100%	75%	50%	25%
Y1	–	–	+	+	–	–	+	+
Y2	–	–	–	+	–	–	–	+
Y3	–	–	–	–	–	–	–	–
Y4	–	–	–	+	–	–	–	+
Y5	–	–	–	+	–	–	–	+

Legend: Y = Yeast.

Table 3
Results of the growth of yeast strains on solid and liquid synthetic minimal medium (SMM) based on phenolic extract

	MMS (mg/L)									
	Solid					Liquid				
	1,000	500	250	125	C	1,000	500	250	125	C
Y1	+	+	+	+	–	+	+	+	+	–
Y2	–	–	+	+	–	–	–	–	+	–
Y3	–	–	–	–	–	–	–	–	–	–
Y4	–	–	–	–	–	–	–	–	–	–
Y5	–	–	–	+	–	–	–	–	+	–

Legend: Y = Yeast, C = Control.

from 12 yeast strains belonging to the genus *Rhodotorula*. In this experiment, the oxygen uptake rate was not inhibited in strains at the phenol concentration of 500 mg/L, where as 50% inhibition of oxygen uptake was observed at a concentration of 2,000 mg/L. The *Rhodotorula rubra* IFO 0870, 0889, 0892, 1,100 and 1,101 cells grew well in a medium containing 250 mg/L of phenol. *Rhodotorula rubra* IFO 0870 shows slight growth in media containing 1,000 mg/L of phenols. In recent studies, Fakharedine et al. [14] showed the ability of *Pichia membranifaciens* to grow on a minimal synthetic medium based on phenolic extract from OMWW.

3.3. Chemical oxygen demand abatement

The kinetics of degradation of the COD by the selected yeast is illustrated in Fig. 1. The results show that COD in the raw sample (100%) inoculated with the strain is 18.98% after 30 d of treatment. This percentage exceeds that recorded in the control with 1.06% (Table 4). Nevertheless, the growth of the selected yeast on the OMWW is accompanied by a decrease of the COD with 37.83% during the first 3 d and of more than 98.16% after 1 month of treatment for the dilution 25% (Fig. 1). Indeed, the concentration of COD has decreased following the biodegradation of organic matter. This could explain that selected yeast uses the compounds which can easily metabolized such as sugar, proteins and simple PC.

Fakharedine et al. [30] have treated OMWW in the laboratory scale by aerobic digestion for 20 d. This treatment reduced the COD by 58.33% and 86.45%, respectively, for the inoculated raw effluent and the inoculated raw effluent with neutral pH. Karakaya et al. [13] obtained a COD reduction of about 80% on OMWW diluted to 25% and added ammonium sulfate and yeast extract after treatment with *Rhodotorula glutinis* for 14 d. During 6 d of treatment, Jarboui et al. [25] recorded a COD reduction of 38%, 47.69% and 56.91% at initial concentrations of 26.70, 14.40 and 6.50 g/L by *R. Mucilaginosa*, respectively.

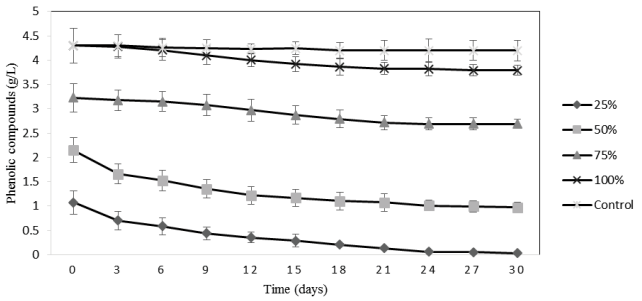


Fig. 1. Abatement rate of phenolic compounds as a function of time.

Table 4
Percentage control reduction of phenolic compounds (PC) and chemical oxygen demand (COD) for 1 month of treatment

	25%	50%	75%	100%
PC %	3.9	3.2	3.25	2.32
COD%	1.87	1.7	1.42	1.06

3.4. Abatement of phenolic compounds

The reduction of PC by the selected yeast in the culture media with different dilutions in OMWW is illustrated in Fig. 2. The abatement of PC differs according to each ratio. 96.98%, 58.56%, 16.59% and 11.86% abatement were recorded for the 25%, 50%, 75% and 100% (v/v), respectively. This level of abatement is much higher than that recorded in the controls of each dilution without inoculum (Table 4). This suggests that the strain selected has a remarkable effect on the degradation of the PC. Direct inoculation of the raw OMWW (100%) with the strain without prior dilution gives unsatisfactory results. The percentage of reduction of PC (11.86%) in this case exceeds that recorded in the control with 2.32% after 30 d of treatment. This is probably due to the nature of OMWW that are too loaded with organic pollutants, particularly with the presence of large concentrations of toxic compounds such as tannins and PC and some of these compounds may have an antimicrobial effect with alteration of cell membranes [28].

The reduction of PC is optimal in OMWW diluted to 25%, a net decrease of polyphenols of the order of 34.65% was noted during the first 3 d. However, the reduction rate obtained after 1 month of treatment is 96.98%. This is due to the strain's ability to degrade the polyphenols. Yeast strains belonging to the genera *Rhodotorula* have already demonstrated that they can reduce the concentration of phenols [12,19,22]. Jarboui et al. [25] reported that the abatement of PC by *Rhodotorula mucilaginosa* decreased with increasing initial phenol concentration. A reduction of only 34.81%, 27.89% and 5.84% was achieved for OMWW diluted to 830, 1,660 and 3,300 mg/L of PC, respectively, during 6 d of treatment. *Rhodotorula glutinis* resulted in 83% abatement of PC in OMWW diluted to 900 mg/L of PC with addition of nitrogen sources and yeast extract [13].

In the presence of PC, the enzymatic system of alternative metabolism is activated. Some species form large peroxisomes that play an important role in the degradation and metabolism of PC [31]. However, in the presence of high concentrations of polyphenols, most PC could bind to enzymes, the enzymatic activity would be altered, and the cells would be deprived of intermediate metabolites, energy and reducing power [27]. PC in high concentration also act on membrane structure and can alter macromolecules such as proteins.

The reduction of total phenols could also be attributed to the decomposition of PC and their adsorption on yeast

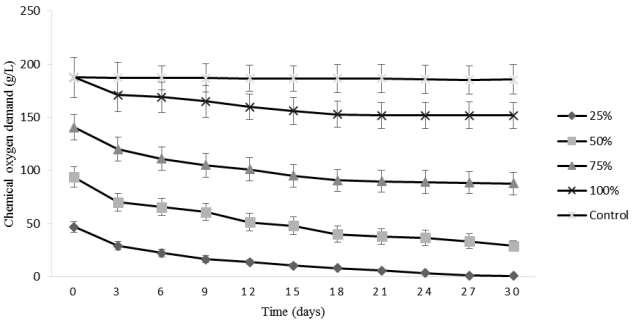


Fig. 2. Degradation rate of organic matter as a function of time.

walls with formation of polyphenol–protein complexes [14]. Indeed, Hafidi et al. [10] attributed such changes during aerobic digestion of the neutralized effluents in the presence of microorganisms to a strong decomposition of biodegradable compounds and less condensed structures. In addition, these changes could also be the result of the neoformation of more resistant compounds and poly-condensed structures [26,32]. These polymerization and poly-condensation mechanisms are similar to the usual humification processes. Under our experimental conditions, the PC can be polymerized, probably after conversion of phenols into quinones by specific enzymes, which are then self-polymerized or recombined with nitrogen compounds to form humic macromolecules [8]. This neoformation of more condensed molecules will enrich OMWW in more stable compounds and promote their reuse as an organic amendment.

The biological properties of the yeast strain capable of degrading PC have been studied to evaluate their potential use in detoxification. In fact, it has been found that their growth accentuated the decrease of PC responsible for the toxicity of OMWW. Fakharedine et al. [30] reported a 60% reduction in PC after 20 d of treatment with aerobic microorganisms isolated from OMWW neutralized by the addition of phosphate rock.

3.5. Identification of phenolic compounds by HPLC

The identification of the polyphenols was carried out by comparing their retention time with those of the standards (Fig. 3). Three classes of polyphenols have been identified in our effluents: simple phenols and secoiridoidal derivatives in significant quantities, followed by phenolic acids.

Analysis of phenolic extract of the crude OMWW shows the presence of five compounds, most of which absorb at 280 nm; hydroxytyrosol, tyrosol, caffeic acid, *p*-coumaric acid, oleuropein (Table 5). The identified PC have already been reported in Marrakech effluents by Hafidi et al. [10], Zenjari et al. [3] and Fakharedine et al. [14].

In general, more than 20 different phenolic compounds have been identified in OMWW. The phenolic compounds identified in the effluent extracts have never been exhaustive, the composition varies from one region to another. The phenolic composition of OMWW has been studied in several

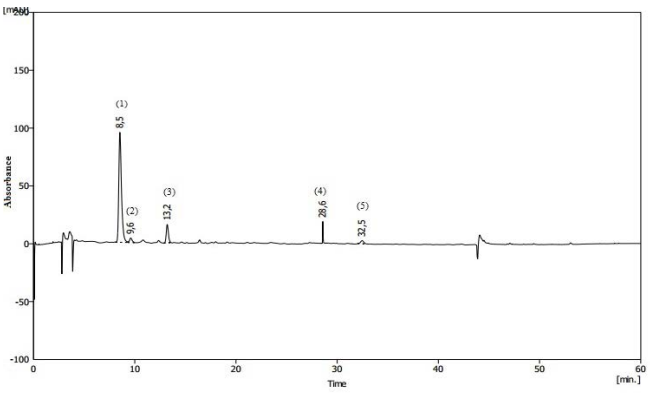
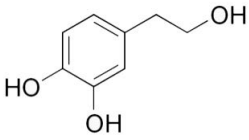
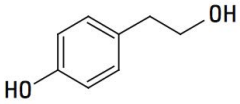
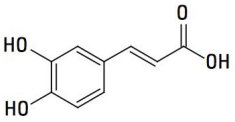
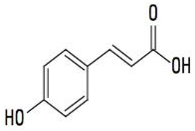
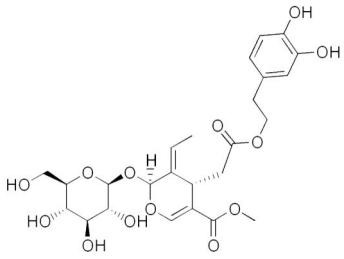


Fig. 3. HPLC chromatogram of the phenolic extract: (1) hydroxytyrosol, (2) tyrosol, (3) caffeic acid, (4) *p*-coumaric acid, and (5) oleuropein.

Table 5
Identification of phenolic compounds from olive mill wastewater extracts and their chemical structures

RT(mn)	Name	Molecular structure
8.5	Hydroxytyrosol	
9.6	Tyrosol	
13.2	Caffeic acid	
28.6	*p*-Coumaric acid	
32.5	Oleuropein	

relatively recent works [6,20,27] and the entire phenolic fraction was identified as rich in hydroxytyrosol and highly complexed secoiridoid derivatives [33]. These data are consistent with our results (Fig. 3; Table 5). Indeed, our results show that hydroxytyrosol is the most predominant compound identified in the phenolic extracts of Moroccan effluents. Tyrosol has also been detected, quantified and characterized in OMWW. The secoiridoid derivatives, especially oleuropein, are esters of elenolic acid and hydroxytyrosol, also identified in the extract of the OMWW. This compound has been identified in various studies [6]. Phenolic acids, namely caffeic and *p*-coumaric acids in the phenolic extract are the phenols most frequently reported in OMWW [33].

The PC present at different dilutions of OMWW (25%, 50%, 75% and 100%) after 1 month of treatment with the selected yeast strain were identified and summarized in Figs. 4(a)–(d). The quantitative and qualitative transformations were determined by HPLC chromatogram analysis. All the compounds identified in the effluent without treatment disappeared after 30 d of treatment for the dilutions of 25%. The hydroxytyrosol compound persists at levels of 37% and 73.37% for dilutions of 50% and 75%, respectively,

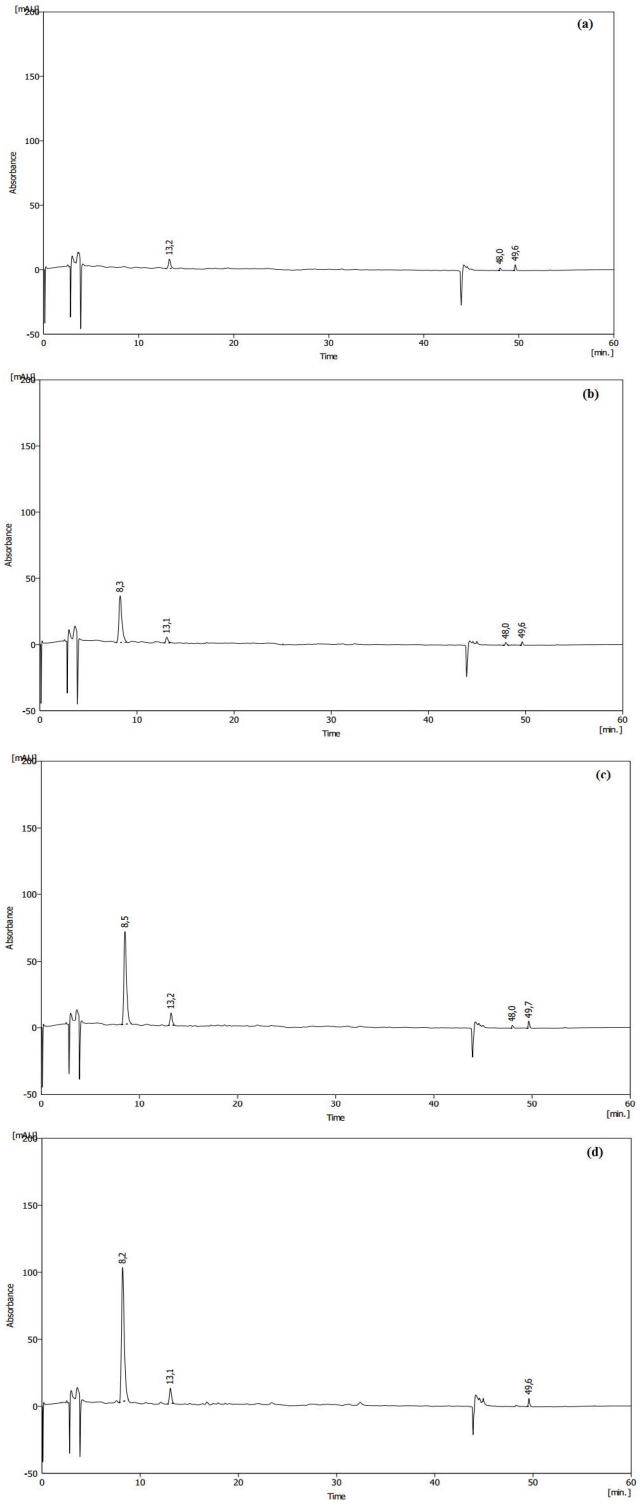


Fig. 4. HPLC chromatogram of phenolic extract from wastewater after treatment, (a) 25% dilution, (b) 50% dilution, (c) 75% dilution and (d) crude (100%).

whereas for the raw effluent the level of this PC increases due to the degradation of oleuropein. This is probably due to the increased activity of some hydrolytic enzymes [14]. Zenjari et al. [3] and Hafidi et al. [10] observed complete degradation of hydroxytyrosol for 20 d of treatment of OMWW by soil microorganisms.

However, the percentage of abatement is 100% for tyrosol, *p*-coumaric acid and oleuropein, while the reduction of caffeic acid is of 73.37%, 40.24% and 27.91% for dilutions of 50%, 75% and 100%, respectively. This is due to the degradation of PC by the selected yeast.

3.6. Identification of yeast species using Biolog microstation

The primary identification of the yeast isolates was done on the basis of morphological characteristics of colonies on solid media. The biochemical characteristics were generated using Biolog. Biolog microstation read at 24, 48 and 72 YT microplate (Biolog Inc.) and incubated at 26°C for 24, 48 or 72 h until a sufficient metabolic pattern is formed. During incubation, yeast respiration in wells containing compounds that can be utilized will either reduce the tetrazolium dye forming a formazan purple color or initiate growth leading to an increase in turbidity. Each metabolic pattern was read by a Micro Station (Biolog Inc.) at a single wavelength of 590 nm and interpreted by micro log software ver. 4.20.05 (Biolog, Hayward, CA). For Micro Plates read at 24 h of incubation, the similarity index must be at least 0.75 to be considered as acceptable species identification. At 48 or 72 h of incubation, the similarity index must be at least 0.50 to be considered as acceptable ranges. Microplate incubation result revealed that yeast ≥0.5 similarity index (25%) *Rhodotorula mucilaginosa* (old name *Rhodotorula rubra*; Table 6). Unicellular pigmented yeast, part of the division Basidiomycota. Tsegaye et al. [34] they used Biolog microstation for yeast species identification which is involved in kocho and bulla fermentation process. Among these yeast species Omni log 100% probability and >0.615 similarity results read was observed in yeast species such as *Cryptococcus albidus* var. *aerus*, *Guilliermondella selenospora*, *Rhodotorula achenionum* and *Trichosporon beigelii*. In another study. which evaluated the Biolog system for the identification of 21 species (72 strains) of yeasts of food and wine origin. Species correctly identified included *Saccharomyces cerevisiae*, *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Kluyveromyces marxianus*, *Kloeckera apiculata*, *Dekkera* and *Schizosaccharomyces pombe* [35].

3.7. Identification of yeast strain by molecular characteristics (26S rDNA sequencing)

The capacity to sequence nucleic acids has supposed a great advance in biology and medicine. It has brought the possibility to know the primary structure of genes and to infer then the encoded function/s by comparison with well-known sequences. Several studies have used the direct sequencing of the ITS regions and the D1/D2 domains for yeast identification. The yeast isolates in this case were identified by sequencing of the domains D1 and D2 of the 26S rDNA gene, which is performed for the genotypic identification of the selected yeast strain. The strain showed similarity score higher than 99% with *Rhodotorula mucilaginosa* (old name *Rhodotorula rubra*). The divergence must not exceed 1% according to Kurtzman and Robnett [36]. The similarity (%) means identical percentage in the sequence obtained from D1/D2 region of 26S rDNA gene and the sequence found in Genbank. The

Table 6
Biolog microstation yeast fungi identification result read

Index value	Yeast ≥ similarity index 25%
Fungus species	*Rhodotorula mucilaginosa*
Probability	100
Similarity	0.604
Distance	6.1

fragment length is 572 of base pairs. Białkowska et al. [37] studied the genetic and biochemical characterization of yeasts isolated from Antarctic soil samples. The isolates were characterized physiologically and biochemically and identified at the molecular level using the D1/D2 and ITS1-5.8S-ITS2 regions of rDNA. The results show that the most abundant microorganisms in soil samples belonged to the genera *Cryptococcus*, *Rhodotorula* and *Debaryomyces* and six isolates belonging to the phylum Basidiomycota were conclusively classified as *Rhodotorula mucilaginosa*.

4. Conclusion

Biological treatments of OMWW by the selected yeast in a mini-bioreactor were conducted for 30 d. The microbiological activity during treatments resulted in a degradation of polyphenols and COD abatement, with a reduction of 96.98% and 98.15%, respectively, when OMWW is diluted to 25%. Using the polyphasic, the taxonomic position of the selected yeast isolate showed that it belonged to *Rhodotorula* genus and partial 16S similarity with *Rhodotorula mucilaginosa*. The synthesis of all these results shows that the treatment of OMWW, with no nutrient supply or pH correction, by a selected yeast strain, gave very promising results in terms of COD and PC abatement, which paves the way for the extrapolation of large-scale experiment.

Acknowledgment

The authors would like to acknowledge the managers of the Abayno Chichaoua Cooperative "3-phase olive pulping unit" who supplied them with samples of the OMWW.

References

[1] A. Ben Sassi, A. Boularbah, A. Jaouad, G. Walker, A. Boussaid, A comparison of olive oil mill wastewaters (OMW) from three different processes in Morocco, Process Biochem., 41 (2006) 74–78.
[2] G. Ait Baddi, M. Hafidi, V. Gilard, J.C. Revel, Characterization of humic acids produced during composting of olive mill wastes, elemental and spectroscopic analyses (FTIR and 13C NMR), Agronomy, 23 (2003) 661–666.
[3] B. Zenjari, H. El Hajjouji, G. Ait Baddi, J.R. Bailly, J.C. Revel, A. Nejmeddine, M. Hafidi, Eliminating toxic compounds by composting olive mill wastewater-straw mixtures, J. Hazard. Mater., 138 (2006) 433–437.
[4] M. Dourou, A. Kancelista, P. Juszczyk, D. Sarris, S. Bellou, I.E. Triantaphyllidou, A. Rywinska, S. Papanikolaou, G. Aggelis, Bioconversion of olive mill wastewater into high-added value products, J. Clean. Prod., 139 (2016) 957–969.
[5] A. El-Abbassi, H. Kiai, A. Hafidi, Phenolic profile and antioxidant activities of olive mill wastewater, Food Chem., 132 (2012) 406–412.
[6] S. Dermeche, M. Nadour, C. Larroche, F. Moulti-Mati, P. Michaud, Olive mill wastes: biochemical characterizations and valorization strategies, Process Biochem., 48 (2013) 1532–1552.
[7] L. Ioannou-Ttofa, I. Michael-Kordatou, S.C. Fattas, A. Eusebio, B. Ribeiro, M. Rusan, A.R.B. Amer, S. Zuraiqi, M. Waismand, C. Linder, Z. Wiesman, J. Gilron, D. Fatta-Kassinos, Treatment efficiency and economic feasibility of biological oxidation, membrane filtration and separation processes, and advanced oxidation for the purification and valorization of olive mill wastewater, Water Res., 144 (2017) 1–13.
[8] H. El Hajjouji, N. Fakharedine, G. Ait Baddi, P. Winterton, J.R. Bailly, J.C. Revel, M. Hafidi, Treatment of olive mill wastewater by aerobic biodegradation: an analytical study using gel permeation chromatography, ultraviolet-visible and Fourier transform infrared spectroscopy, Bioresour. Technol., 98 (2007) 3513–3520.
[9] A. Ben Sassi, N. Ouazzani, G.M. Walker, S. Ibnsouda, M. El Mzibri, A. Boussaid, Detoxification of olive mill wastewaters by Moroccan yeast isolates, Biodegradation, 19 (2008) 337–346.
[10] M. Hafidi, G. Ait Baddi, A. Chetoui, I. El Hadrami, Traitements des effluents liquides d'huileries d'olive par des micro-organismes aerobies, Agrochimica, 48 (2004) 1–12.
[11] S. Ntougias, K. Bourtzis, G. Tsiamis, The microbiology of olive mill wastes, Biomed. Res. Int., 2013 (2013) 1–16.
[12] S. Ntougias, P. Baldrian, C. Ehaliotis, F. Nerud, T. Antoniou, V. Merhautová, G.I. Zervakis, Biodegradation and detoxification of olive mill wastewater by selected strains of the mushroom genera *Ganoderma* and *Pleurotus*, Chemosphere, 88 (2012) 620–626.
[13] A. Karakaya, Y. Laleli, S. Takaç, Development of process conditions for biodegradation of raw olive mill wastewater by *Rhodotorula glutinis*, Int. Biodeterior. Biodegrad., 75 (2012) 75–82.
[14] N. Fakharedine, M. Ouadghiri, M. Amar, P. Winterton, M. Hafidi, Y. Ouhdouch, Isolation and identification of a yeast strain involved in the degradation of Marrakech olive mill wastewater, EurAsian J. Biosci., 137 (2011) 127–137.
[15] AOAC, Official Methods of Analysis of Chemists, 15th Ed., Association of Official Analytical Chemists. Arlington, VA, USA, 1990.
[16] APHA, WEF, AWWA, Standard Methods for the Examination of Water and Wastewater, L.S. Clesceri, A.E. Greenberg, A.D. Eaton, Eds., American Public Health Association, Water Pollution Control Federation and American Water Works Association, 20th Ed., Washington, DC, USA, 1998.
[17] G. Aubert, Méthodes d'analyses des sols, Centre Nat., 1978.
[18] J. Folch, M. Lees, G. Sloane-Stanley, A simple method for the isolation and purification of total lipids from animal tissues, J. Biol. Chem., 226 (1957) 497–509.
[19] J.J. Macheix, A. Fleuriet, J. Billot, Fruit Phenolics, CRC Press, Inc., Boca Raton, FL, 1990.
[20] R.A. Vasquez, D.R. Maestro, C.E. Graciani, Componentes fenolicos de la aceituna II. Polifenoles del alpecin, Grasas Y Aceites, 25 (1974) 341–345.
[21] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem., 28 (1956) 350–356.
[22] B.R. Bochner, Sleuthing out bacterial identities, Nature, 339 (1989) 157–158.
[23] Biolog, Y.T. Microplate: Instructions for use. Biolog Inc., Hayward, California, 1993.
[24] C.P. Kurtzman, C.J. Robnett, Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5′ end of the large-subunit (26S) ribosomal DNA gene, J. Clin. Microbiol., 35 (1997) 1216–1223.
[25] R. Jarboui, H. Baati, F. Fetoui, A. Gargouri, N. Gharsallah, E. Ammar, Yeast performance in wastewater treatment: case study of *Rhodotorula mucilaginosa*, Environ. Technol., 33 (2012) 951–960.
[26] H. El Hajjouji, G. Ait Baddi, A. Yaacoubi, H. Hamdi, P. Winterton, J.C. Revel, M. Hafidi, Optimisation of biodegradation conditions for the treatment of olive mill wastewater, Bioresour. Technol., 99 (2008) 5505–5510.

[27] H. Aissam, K. Sendide, M. Benlemlih, Etude et traitement biologique préliminaire de la pollution azotée et phosphorée contenue dans les effluents d'industries agro-alimentaires, Ann. Chim. Sci. Mat., 26 (2001) 391–396.
[28] R. Capasso, A. Evidente, L. Schivo, G. Orru, M.A. Marcialis, G. Cristinzio, Antibacterial polyphenols from olive oil mill waste waters, J. Appl. Bacteriol., 79 (1995) 393–398.
[29] K. Katayama-Hirayama, S. Tobita, K. Hirayama, Degradation of Phenol by Yeast *Rhodotorula*, J. Gen. Appl. Microbiol., 37 (1991) 147–156.
[30] N. Fakharedine, H. El Hajjouji, G. Ait Baddi, J.C. Revel, M. Hafidi, Chemical and spectroscopic analysis of organic matter transformation during aerobic digestion of olive-mill waste-waters, Process Biochem., 41 (2006) 398–404.
[31] H. Horiguchi, H. Yurimoto, N. Kato, Y. Sakai, Antioxidant system within yeast peroxisome. Biochemical and physiological characterization of CbPmp20 in the methylotrophic yeast *Candida boidinii*, J. Biol. Chem., 276 (2001) 14279–14288.
[32] G.A. Ait Baddi, J.A. Alburquerque, J. Gonzálvez, J. Cegarra, M. Hafidi, Chemical and spectroscopic analyses of organic matter transformations during composting of olive mill wastes, Int. Biodeterior. Biodegrad., 54 (2004) 39–44.
[33] P. Ramos, S.A.O. Santos, Â.R. Guerra, O. Guerreiro, L. Felício, E. Jerónimo, A.J.D. Silvestre, C.P. Neto, M. Duarte, Valorization of olive mill residues: antioxidant and breast cancer antiproliferative activities of hydroxytyrosol-rich extracts derived from olive oil by-products, Ind. Crops Prod., 46 (2013) 359–368.
[34] Z. Tsegaye, B. Gizaw, G. Tefera, Isolation, Identification and characterization of yeast species from Kocho and Bulla collected from Gedeo Zone, South Nation, Int. J. Curr. Trends Pharmacobiol. Med. Sci., 1 (2016) 37–44.
[35] W. Praphailong, M. van Gestel, G.H. Fleet, G.M. Heard, Evaluation of the Biolog system for the identification of food and beverage yeasts, Lett. Appl. Microbiol., 24 (1997) 455–459.
[36] C.P. Kurtzman, C.J. Robnett, Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences, Antonie van Leeuwenhoek, 98 (1998) 331–371.
[37] A.M. Białkowska, K.M. Szulczewska, J. Krysiak, T. Florczak, E. Gromek, H. Kassassir, J. Kur, M. Turkiewicz, Genetic and biochemical characterization of yeasts isolated from Antarctic soil samples, Polar Biol., 40 (2017) 1787–1803.