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Lipase production by *Aspergillus ibericus* using olive mill wastewater

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Abstract Olive mill wastewater (OMW) characteristics make it a suitable resource to be used as a microbial culture media to produce value-added compounds, such as enzymes. In this work, the ability of the novel species *Aspergillus ibericus* to discolor OMW and produce lipase was studied. An initial screening on plates containing an OMW-based agar medium and an emulsified olive oil/rhodamine-B agar medium was employed to select the strain *A. ibericus* MUM 03.49. Then, experiments in conical flasks with liquid OMW-based media showed that the fungus could growth on undiluted OMW, with a chemical oxygen demand (COD) of 97 ± 2 g/L, and to produce up to 2,927 ± 54 U/L of lipase. When pure OMW was used in the media, the maximum COD and color reduction achieved were 45 and 97 %, respectively. When OMW diluted to 10 % was used, *A. ibericus* was able to reduce phenolic and aromatic compounds by 37 and 39 %, respectively. Additionally, lipase production was found to be promoted by the addition of mineral nutrients. When the fermentations were scaled up to a 2-L bioreactor, *A. ibericus* produced up to 8,319 ± 33 U/L of lipase, and the maximum COD and color reduction were 57 and 24 %, respectively.

Keywords Olive mill wastewater · *Aspergillus ibericus* · Lipase · Chemical oxygen demand · Submerged fermentation

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Introduction

Olive oil is a traditional agricultural product that is originally from the Mediterranean basin; the majority (approx. 75 %) of the world's olive oil comes from southern European countries [1, 2]. According to the 2010 FAO data, the 10 most relevant producers of olive oil are Spain (with an annual production of 1.5×10^6 tons), Italy, Greece, Syria, Tunisia, Turkey, Morocco, Algeria, Portugal and Argentina [1]. In Portugal, approximately 71,800 tons of olive oil was produced in 2011/2012 campaign, which represents approximately 2 % of the world's production [2].

Olive oil is extracted from olives by physical methods. Olive crushing, malaxation of the resulting paste and oil phase separation are the most important stages of the extraction process. Currently, these steps are performed by three different methods: (i) the traditional discontinuous pressing process that is still used in many small olive mills, (ii) the continuous process with three-phase horizontal centrifuge systems that separate the olive pulp into oil, liquid waste and solid residue, and (iii) the continuous process with two-phase horizontal centrifuge systems that separate the olive pulp into oil and wet solid residue [3]. The first two processes require the addition of hot water, producing large amounts of liquid waste known as olive mill wastewater (OMW), which is a significant source of agro-industrial pollutants [4]. OMW characteristics are variable and depend on the properties of the olives processed and the kind of extraction method used. The discontinuous process produces a lower amount of more concentrated OMW, while the three-phase system generates approximately twice the volume of less-concentrated OMW. Typical volumes produced range from 0.4 to 1.2 m^3 per ton of olives processed [3]. In general, OMW has a dark brown color, is acidic and has a high content of organic matter. The main compounds found in OMW are

simple and complex sugars, residual oil (lipids), proteins, organic acids, mineral elements and recalcitrant compounds, such as tannins, lignins and phenols [5]. During the last decade, many olive mills have converted their production process to two-phase systems in order to improve presumably their environmental performances since the two-phase process is considered to be more eco-friendly because it consumes considerably less fresh water and produces only a wet solid residue (pomace). The shift in processes occurred mainly in Spain, where 98 % of the olive oil mills are equipped with the two-phase technology [6]. However, in other European countries, this conversion did not proceed as rapidly. Specifically, in 2003, 40 % of the olive mills were still using the traditional discontinuous process, 48 % were using the three-phase technology and only 11 % were using the two-phase process [7]. Currently in Portugal, 33 % of the olive mills use the two-phase process, 23 % use the three-phase process and 44 % still use the traditional discontinuous process [8]. OMW remains, therefore, an important effluent that needs to be properly treated before disposal.

Physical, chemical and biological technologies have been studied for the treatment of OMW [4]. Among these, the technologies that combine the treatment with the production of valuable byproducts have received particular attention. Several recent reviews on the subject have been written [5, 9, 10]. In particular, OMW is suitable for the production of enzymes, such as laccases [11], lignin peroxidases [12], Mn-dependent peroxidases [11], pectinases [13] and tannases [14]. Moreover, OMW is suitable for the production of lipases because it contains residual quantities of olive oil that induce lipase production [15, 16]. Many organisms, such as *Candida cylindracea* [15, 17], *Yarrowia lipolytica* [17, 18], *Geotrichum candidum* [19], *Penicillium citrinum* [20] and other fungi [15], have performed well in the production of lipase from OMW.

Aspergillus ibericus is a new species isolated from wine grapes [21] that it is not known to produce any relevant mycotoxins [22], which makes it safe for biotechnological applications. The aim of this study was to assess the ability of this new species to grow on OMW media and to produce lipase from it, contributing to the remediation and valorization of this effluent.

Materials and methods

OMW characteristics

The OMW samples used in the experiments were collected from a three-phase olive mill from the northern region of Portugal. On the same day of collection, the samples were mixed to obtain a standardized OMW that was stored at −20 °C and used throughout the study. The standardized OMW was characterized for pH, chemical oxygen demand (COD), total nitrogen (TN), total organic carbon (TOC), total solids (TS), total volatile solids (TVS), total suspended solids (TSS), reducing sugars, phenols and lipids. Analyses were performed in triplicate as described below in the analytical methods section.

Biological material

A. ibericus MUM 03.49 (MUM culture collection, Braga, Portugal) was revived on malt extract agar (MEA) plates (2 % malt extract, 2 % glucose, 0.1 % peptone and 2 % agar) from a preserved glycerol stock that had been stored at −80 °C. The culture was incubated in the dark at 25 °C for 7 days and subcultured on MEA slants that were incubated under the same conditions. The spore suspensions were prepared by vortex mixing 7-day-old culture slants with 4 mL of peptone solution (0.1 % peptone and 0.001 % Tween 80). The inoculum spore concentration was adjusted to 10^6 spores/mL using a Neubauer counting chamber. During the experimental period, the strain was preserved at 4 °C and was cultured monthly on fresh MEA slants.

Evaluation of OMW discoloration and lipase production

To determine the ability to decolorize OMW, *A. ibericus* was grown on agar plates that contained an OMW-based agar medium prepared with 100 % OMW supplemented with Triton X-100 (0.1 g/L), agar (20 g/L), and mineral nutrients: $NaNO_3$ (3 g/L), K_2HPO_4 (1 g/L), KCl (0.5 g/L), $MgSO_4{\cdot}7H_2O$ (0.5 g/L), $FeSO_4{\cdot}7H_2O$ (0.017 g/L), peptone (0.1 g/L), yeast extract (0.01 g/L), $ZnSO_4{\cdot}7H_2O$ (0.001 g/L) and $CuSO_4{\cdot}5H_2O$ (0.005 g/L). To evaluate the strain lipase activity, *A. ibericus* was grown on agar plates containing an emulsified oil medium composed of commercial olive oil (25 g/L), rhodamine-B (0.01 g/L), Triton X-100 (0.1 g/L), agar (20 g/L), and the mineral nutrients previously mentioned as adapted from Peterson et al. [23]. The culture plates were inoculated at their centers in triplicate with 10 μL of a spore suspension prepared as mentioned above. The plates were incubated at 25 °C for 5 days. The diameters of the colonies and the diameter of the clearing zone (halo) around the colonies were measured daily. The lipase activity was detected with UV irradiation (366 nm) to illuminate the orange-red fluorescent halos that are typical of rhodamine-B complexes containing free fatty acids.

Shake-flask experiments

The batch fermentations were performed in cotton-plugged 500-mL Erlenmeyer flasks that contained 250 mL of culture media. Liquid OMW-based culture media was prepared at

concentrations of 10, 50 and 100 % (v/v, in distilled water) for experiments A1, A2 and A3, respectively. They were also supplemented with the mineral nutrients previously mentioned. A control experiment with non-supplemented OMW at 100 % was also prepared (experiment A4). The final pH of the media was adjusted to 5.5 with 1 M KOH before it was autoclaved at 121 °C for 15 min. After the media had cooled to room temperature, the flasks were inoculated in triplicate with 1 mL of an inoculum suspension from *A. ibericus* MUM 03.49 and incubated at 27 °C in an orbital shaker incubator at 200 rpm for 7 days. Throughout the 7-day incubation period, 5-mL samples were collected daily under aseptic conditions and centrifuged (10,000×*g*, 15 min at 4 °C). The supernatants were preserved at −20 °C until analysis. At the end of the fermentation run, the culture broths were filtered through a 1.6-μm glass microfiber filter (904-AH, Whatman), and the fungal biomass was lyophilized and weighed.

Bioreactor experiments

Lipase production by *A. ibericus* MUM 03.49 with OMW was scaled up to a 2-L fermenter (Biolab, B. Braun). To evaluate two different strategies of inoculation, two experiments were conducted with 1.5 L of OMW (supplemented with the mineral nutrients). In the first experiment (B1), the medium was inoculated with 5 mL of the fungus spore suspension, and in the second experiment (B2), the inoculation was performed with active mycelium that was obtained by growing the fungus in 250 mL of YPD medium (2 % glucose, 1 % yeast extract and 1 % peptone). Both fermentation runs were conducted for 7 days at 27 °C with a stirring speed of 200 rpm and an aeration rate of 1 L/min. The pH was maintained at 5.5 with 2 N KOH and 2 N HCl. When the 7-day fermentation cycle was complete, an additional 0.5 L of fresh OMW (supplemented with the mineral nutrients) was added to the bioreactor inoculated with active mycelium, and the fermentation time was extended for an additional 9 days. The results of this second fermentation cycle are presented below as experiment B3. The samples were collected daily under aseptic conditions and centrifuged (10,000×*g*, 15 min at 4 °C). The supernatants were preserved at −20 °C until analysis. The experiments were conducted in triplicate.

Analytical methods

COD, TN and TOC were determined using the Hach Lange kits LCK014, LCK338 and LCK387, respectively. TS, TVS and TSS were determined with standard methods [24]. The concentration of the reducing sugars was determined with a DNS-adapted method [25]. The amounts of the phenols were determined with the Folin–Ciocalteu method with tyrosol as the standard [26]. The lipids were extracted from the lyophilized samples with diethyl ether in a Tecator Soxtec system (model: HT2 with 1045 extraction unit and 1046 service unit) and weighed. Changes in the concentrations of the aromatic compounds were determined by absorbance readings at 254 nm [27], and changes in color were determined with absorbance readings at 390 nm [28]. Using a modified method [29], the activity of the extracellular lipase was assessed using *p*-nitrophenyl butyrate (*p*-NPB) as a substrate. One unit of lipase activity (U) was expressed as the amount of enzyme that produced 1 μmol of *p*-nitrophenol per minute under the assay conditions. All the analyses were performed in triplicate.

Results and discussion

OMW characteristics

The standardized OMW possessed the following characteristics (mean values ± SD; $n = 3$): pH, 4.9 ± 0.05; COD, 97 ± 2 g/L; TN, 3.4 ± 0.1 mg/L; TOC, 15.3 ± 3.4 g/L; TS, 58 ± 1 g/L; TVS, 46 ± 3 g/L; TSS, 31 ± 0.4 g/L; reducing sugars, 11.5 ± 5.1 g/L; phenols, 2.7 ± 0.2 g/L; and lipids, 3.5 ± 0.6 g/L. Those values are in accordance with OMW characteristics reported elsewhere [5, 9].

Growth on solid media

The colony and halo diameters obtained with *A. ibericus* MUM 03.49 on both types of media are shown in Fig. 1. The fungus grew well in the 100 % OMW agar medium with the colony reaching 8.5 cm in diameter after the 5-day incubation period. Additionally, the discolored halo reached the same diameter on day 4, indicating an active reduction in OMW-colored compounds. In contrast, the fungus growth on the emulsified olive oil/rhodamine-B medium was not as prolific; the colony size reached only 6.0 cm during the same time period. Nevertheless, an orange-red fluorescent halo was detected after 2 days of incubation, reaching a diameter of 6.6 cm around the colony at the end of the 5 days, which indicated the production of lipases.

Shake-flask experiments

Aspergillus ibericus was able to grow in all of the liquid OMW-based media tested. The characteristics of the fermentation runs at the end of the 7-day incubation period are presented in Table 1. The final biomass concentration increased with the increase in the OMW content in the media, which was the only source of carbon-containing nutrients. The maximum concentration (28 g/L) was obtained in trial A3 (prepared with undiluted OMW and the

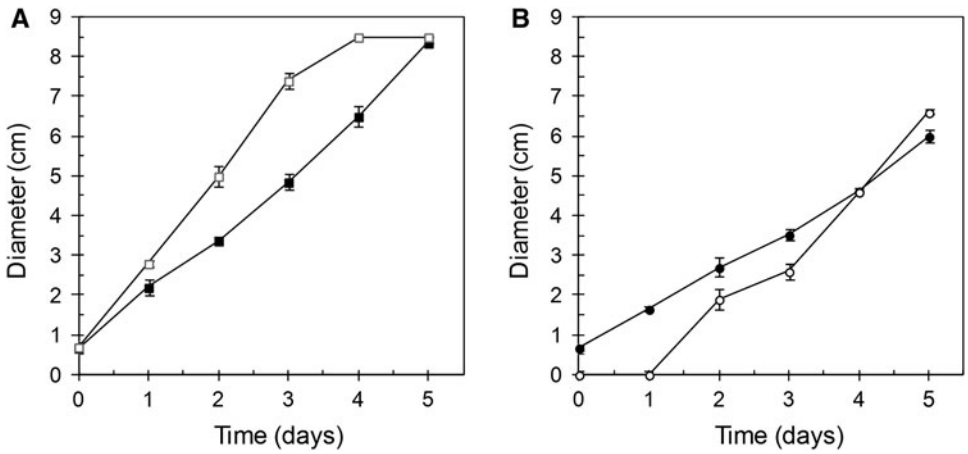

Fig. 1 **a** *Aspergillus ibericus* cultivated on plates with 100 % OMW agar medium: (*filled squares*) colony diameter over time, (*open squares*) diameter of discolored halo over time. **b** *A. ibericus* cultivated on plates with emulsified olive oil/rhodamine-B agar medium: (*filled circles*) colony diameter over time; (*open circles*) orange-red fluorescent halo diameter over time. Values are the averages of three independent experiments ± SD

mineral nutrients), and the minimum (4.6 g/L) was obtained in trial A1 (prepared with 10 % OMW and the mineral nutrients). Following the increase in biomass, there was an increase in reducing sugars consumption that reached a maximum of 98 % in trial A3. The mineral nutrients added to media did not contribute much to the fungal growth because the final biomass concentration obtained in trial A4 (prepared without mineral nutrients) was nearly identical to that of trial A3 (prepared with mineral nutrients). However, mineral addition substantially changed the fermentation pH. As shown in Table 1, the pH of trial A3 increased to 6.2, while the pH of trial A4 dropped to 2.9.

In these experiments, the highest lipase activity (2,927 ± 54 U/L) was obtained in trial A3, and the lowest activity was obtained in trial A1 (Table 2). An increase in the lipase activity was observed with the increase of the OMW content in the media, correlating biomass concentration with lipase activity. In addition, the lipase production was stimulated by the addition of mineral nutrients to media; the lipase activity increased approximately fivefold in trial A3 (2,927 ± 54 U/L) compared to trial A4 (600 ± 43 U/L).

The highest lipase activity obtained in these experiments was similar to lipase activities that have been obtained with other microorganisms in studies where fermentation with undiluted OMW was performed. For example, the results are in accordance with the lipase activities obtained with *C. cylindracea* (2,200 U/L) [17] and with *P. citrinum* (1,272 U/L) [20].

In the shake-flask experiments, *A. ibericus* could also effectively degrade the effluent; reductions in the OMW COD (39–58 %) and color (47–97 %) were obtained with all of the media tested after the 7-day incubation period. However, its capacity to reduce the levels of phenols and aromatic compounds was dependent on the organic load of the media; the reduction was only effective in the fermentations conducted with diluted OMW (Table 1). The reduction in phenols was 37 % in trial A1 and 28 % in trial A2. The reduction in aromatic compounds was 39 % in trial A1 and 25 % in trial A2.

In a similar study, D'Annibale et al. [15] reported that *C. cylindracea* produced a reduction in the COD by

Table 1 Characteristics of the shake-flask experiments at the end of the fermentation run

Trial	OMW-based media	Final values		Reduction amount (%)				
		Biomass (g/L)	pH	Reducing sugars	COD	Color	Total phenols	Aromatic compounds
A1	10 % OMW[a]	4.6 ± 0.5	4.0 ± 0.01	60 ± 0.7	39 ± 2.1	55 ± 0.4	37 ± 2.9	39 ± 1.7
A2	50 % OMW[a]	12.0 ± 1.0	5.1 ± 0.7	50 ± 5.8	58 ± 1.6	47 ± 1.1	28 ± 0.4	25 ± 4.2
A3	100 % OMW[a]	28.1 ± 1.2	6.2 ± 0.03	98 ± 1.1	45 ± 0.9	97 ± 0.4	0	0
A4	100 % OMW[b]	27.3 ± 0.7	2.9 ± 0.1	85 ± 3.8	41 ± 2.5	58 ± 0.6	0	0

Values are the averages of three independent experiments ± SD

COD chemical oxygen demand

[a] Media supplemented with the mineral nutrients

[b] Media not supplemented with the mineral nutrients

Table 2 Extracellular lipase produced by *A. ibericus* in the different experiments conducted

Shake-flask experiments				2-L bioreactor experiments			
Trial	OMW-based media	Lipase activity (U/L)	Lipase productivity (U/L/h)	Trial	OMW-based media	Lipase activity (U/L)	Lipase productivity (U/L/h)
A1	10 % OMW[a]	291 ± 31	1.7 ± 0.2	B1	100 % OMW[a]	2,108 ± 46	12.5 ± 0.3
A2	50 % OMW[a]	1,517 ± 73	9.0 ± 0.4	B2	100 % OMW[a]	5,391 ± 18	32.1 ± 0.1
A3	100 % OMW[b]	2,927 ± 54	17.4 ± 0.3	B3	100 % OMW[a]	8,319 ± 33	21.7 ± 0.1
A4	100 % OMW[b]	600 ± 43	3.6 ± 0.3	–	–	–	–

Values are the averages of three independent experiments ± SD

B1 fermentation inoculated with spore suspension, *B2* fermentation inoculated with active mycelia, *B3* extension of the fermentation B2 after the addition of fresh OMW

[a] Media supplemented with the mineral nutrients

[b] Media not supplemented with the mineral nutrients

48 % and a reduction in phenolic compounds of 36 %. Gonçalves et al. [17] corroborated these data and reported similar results for the same yeast with a COD reduction between 46 and 70 % and a reduction in phenolic compounds by 27 %. Similar reductions in COD were also achieved with other microorganisms. For example, *G. candidum* produced a reduction in COD of 62 % [19], *P. citrinum* a reduction of 68 % and *Y. lipolytica* a reduction of 41 % [30].

These preliminary data confirmed that *A. ibericus* was able to grow on undiluted OMW, produce lipase and reduce the levels of some of the pollutants in the effluent. Based on these encouraging results, the fermentations were then scaled up to bioreactor volumes.

Bioreactor experiments

Two fermentation trials were performed using a 2-L fermenter. In one trial, the OMW medium was inoculated with a spore suspension of the fungus (B1), and in the other trial, active mycelia was used for the inoculation (B2). The consumption of reducing sugars and the production of lipase during the fermentation runs are shown in Fig. 2. After the first 7 days of fermentation, a lipase activity of 2,108 ± 46 U/L was detected in fermentation B1, and an activity of 5,391 ± 18 U/L was obtained in fermentation B2, representing a 2.6-fold increase in lipase production (Table 2). This increase in production may be due to a shorter lag phase in B2 because the spore inoculum usually requires a germination period that extends the lag phase, and the active mycelium can better adapt to a new environment and initiate more quickly the synthesis of the enzymes required for substrate utilization and growth [31]. Moreover, the production of lipase in B2 was further increased, reaching an activity of 8,319 ± 33 U/L, with the extension of the fermentation time and addition of fresh

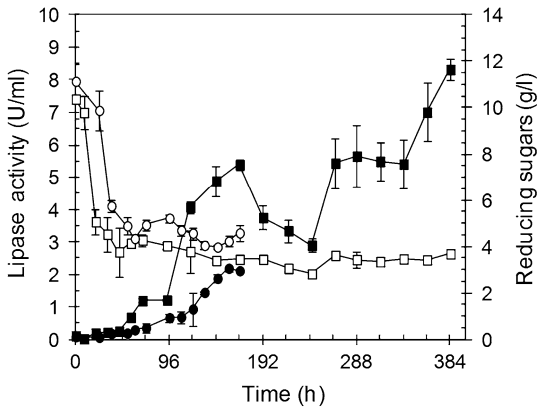

Fig. 2 Time course of reducing sugar consumption (*open symbols*) and extracellular lipase activity (*closed symbols*) obtained in the experiments conducted in the 2-L fermenter. Fermentation B1 started with the addition of the spore suspension (*circles*); and fermentation B2 started with active mycelium (*squares*). Values are the averages of three independent experiments ± SD

OMW for a second batch cycle (fermentation B3). However, it can be observed that the increase in the fermentation time resulted in the reduction in the productivity per volume from 32.1 ± 0.1 U/L/h at 168 h to 21.7 ± 0.1 U/L/h at the end of the experiment (Table 2).

When comparing the present results with the literature, we observed that the maximum lipase activity achieved in this study is greater than the lipase activity reported by Gonçalves et al. [17] when using the non-conventional yeast *C. cylindracea*, which produced up to 3,511 U/L in a bioreactor; or greater than the activity reported by D'Annibale et al. [20] with *P. citrinum*, which produced a maximum of 735 U/L. Additionally, it is important to note that *A. ibericus* lipase production was not negatively affected by high COD contents like what was observed with *P. citrinum*, which showed to produce more lipase when cultivated on OMW with high organic loads [20]. On the other hand, the results obtained also show that the production of lipase by

Table 3 Characteristics of 2-L bioreactor experiments at the end of the fermentations run

Trial	Final values		Reduction amount (%)							
	Biomass (g/L)	pH	Reducing sugars	Lipids	COD	TOC	Color	TN	TS	TVS
B1	73.9 ± 1.3	5.2 ± 0.1	59 ± 3.0	34 ± 5.7	57 ± 4.3	49 ± 2.3	24 ± 0.6	31 ± 1.7	14 ± 0.5	26 ± 3.0
B2	86.1 ± 2.5	5.3 ± 0.05	67 ± 0.4	82 ± 3.1	30 ± 1.9	49 ± 1.2	0	17 ± 1.3	18 ± 2.7	44 ± 1.2
B3	102 ± 2.7	5.4 ± 0.03	65 ± 0.5	97 ± 4.5	33 ± 2.3	48 ± 0.6	0	18 ± 1.5	18 ± 1.4	23 ± 2.0

Values are the averages of three independent experiments ± SD

B1 fermentation inoculated with spore suspension, *B2* fermentation inoculated with active mycelia, *B3* extension of the fermentation B2 after the addition of fresh OMW, *COD* chemical oxygen demand, *TOC* total organic carbon, *TN* total nitrogen, *TS* total solids, *TVS* total volatile solids

A. ibericus was not repressed by the sugar content of the fermentation media because only 59–67 % of the initial sugar content had been consumed. So, lipase expression in *A. ibericus* may not be repressed by glucose as in other microorganisms such as *C. rugosa* [16].

The reduction in the levels of OMW pollutants obtained with these fermentation trials is shown in Table 3. The extent of COD reduction varied from 30 to 57 %, while the amount of TOC reduction reached nearly 50 % in all fermentations. In contrast, a reduction in the levels of the phenolic and aromatic compounds was not detected in any of the bioreactor fermentations, similarly to what was observed in the flask fermentations conducted with undiluted OMW. Regarding this aspect, the involved *A. ibericus* enzymatic system appears to be repressed when the organic load is high and easily degradable compounds are more available. This behavior was similarly reported for other filamentous fungi by Aissam et al. [32]. According to the authors, the degradation of phenols can be adversely affected by media containing more than 20–25 % OMW. A color reduction of 24 % was only obtained in the fermentation trial (B1) that was inoculated with the spores. Therefore, in contrast with lipase production, the bioremediation of OMW was not positively affected by the inoculation with active mycelium.

In the available literature, white rot fungi appear to be more effective in achieving a reduction in the OMW pollution characteristics [33]. Nevertheless, *Aspergillus niger*, a closely related species to *A. ibericus*, has also already showed its good suitability to treat OMW [14, 34–36], to degrade phenolic compounds [33], and even, to produce several extracellular laccases from the multicopper oxidases group [37]. Therefore, it can be expected that the observed reductions in OMW pollution characteristics may be due to an enzymatic complex similar to *A. niger*.

Conclusions

The present study shows that *A. ibericus* is a suitable microorganism to produce lipase using undiluted OMW as substrate, since it simultaneously contributes to reduce the COD and TOC in OMW. It was also shown that lipase production was promoted by the addition of mineral nutrients to OMW, indicating that some essential nutrients for lipase synthesis are missing in the effluent. Moreover, the lipase production by *A. ibericus* was successfully conducted in a 2-L bioreactor and a maximum lipase activity of 8,319 ± 33 U/L was obtained.

It is important to note that in many olive oil producing countries, OMW is still one of the most important types of waste generated by this industry. Therefore, this work leads to new perspectives in the valorization of OMW through fermentation with *A. ibericus*.

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References

1. FAO (2010) Food and Agriculture Organization of the United Nations Statistics. http://faostat.fao.org/site/636/DesktopDefault.aspx?PageID=636#ancor. Accessed 15 Apr 2012
2. IOC (2012) International Olive Oil Council series of world statistics on production, imports, exports and consumption. http://www.internationaloliveoil.org/estaticos/view/131-world-olive-oil-figures. Accessed 15 Apr 2012
3. Caputo AC, Scacchia F, Pelagagge PM (2003) Disposal of by-products in olive oil industry: waste-to-energy solutions. Appl Therm Eng 23:197–214
4. Paraskeva P, Diamadopoulos E (2006) Technologies for olive mill wastewater (OMW) treatment: a review. J Chem Technol Biotechnol 81:1475–1485
5. Gonçalves C, Pereira C, Alves M, Belo I (2010) Olive mill wastewater as a renewable resource. Environ Eng Manag J 9:319–325
6. Niaounakis M, Halvadakis CP (2006) Olive processing waste management: literature review and patent survey. Pergamon Press, Oxford, UK
7. IMPEL (2003) IMPEL Olive oil project report. http://impel.eu/wp-content/uploads/2010/02/2003-03-olive-oil-FINAL-REPORT.pdf. Accessed 15 Apr 2012
8. INE (2010) Portuguese type of oil press unit and type of extraction system. http://www.ine.pt/xportal/xmain?xpid=INE&xpgid=ine_indicadores&indOcorrCod=0000702&contexto=bd&selTab=tab2. Accessed 15 Apr 2012

9. Roig A, Cayuela ML, Sanchez-Monedero MA (2006) An overview on olive mill wastes and their valorisation methods. Waste Manag 26:960–969
10. Morillo J, Antizar-Ladislao B, Monteoliva-Sánchez M, Ramos-Cormenzana A, Russell N (2009) Bioremediation and biovalorisation of olive-mill wastes. Appl Microbiol Biotechnol 82:25–39
11. Fenice M, Sermanni GG, Federici F, D'Annibale A (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
12. Sayadi S, Ellouz R (1995) Roles of lignin peroxidase and manganese peroxidase from *Phanerochaete chrysosporium* in the decolorization of olive mill wastewaters. Appl Environ Microbiol 61:1098–1103
13. Federici F, Montedoro G, Servili M, Petruccioli M (1988) Pectic enzyme production by *Cryptococcus albidus* var. *albidus* on olive vegetation waters enriched with sunflower calathide meal. Biol Wastes 25:291–301
14. Aissam H, Errachidi F, Penninckx MJ, Merzouki M, Benlemlih M (2005) Production of tannase by *Aspergillus niger* HA37 growing on tannic acid and olive mill waste waters. World J Microbiol Biotechnol 21:609–614
15. D'Annibale A, Sermanni GG, Federici F, Petruccioli M (2006) Olive-mill wastewaters: a promising substrate for microbial lipase production. Bioresour Technol 97:1828–1833
16. Lotti M, Monticelli S, Luis Montesinos J, Brocca S, Valero F, Lafuente J (1998) Physiological control on the expression and secretion of *Candida rugosa* lipase. Chem Phys Lipids 93: 143–148
17. Gonçalves C, Lopes M, Ferreira JP, Belo I (2009) Biological treatment of olive mill wastewater by non-conventional yeasts. Bioresour Technol 100:3759–3763
18. Lopes M, Araujo C, Aguedo M, Gomes N, Gonçalves C, Teixeira JA, Belo I (2009) The use of olive mill wastewater by wild type *Yarrowia lipolytica* strains: medium supplementation and surfactant presence effect. J Chem Technol Biotechnol 84:533–537
19. Asses N, Ayed L, Bouallagui H, Ben Rejeb I, Gargouri M, Hamdi M (2009) Use of *Geotrichum candidum* for olive mill wastewater treatment in submerged and static culture. Bioresour Technol 100:2182–2188
20. D'Annibale A, Brozzoli V, Crognale S, Gallo AM, Federici F, Petruccioli M (2006) Optimisation by response surface methodology of fungal lipase production on olive mill wastewater. J Chem Technol Biotechnol 81:1586–1593
21. Serra R, Cabañes FJ, Perrone G, Castellá G, Venâncio A, Mulè G, Kozakiewicz Z (2006) *Aspergillus ibericus*: a new species of section Nigri isolated from grapes. Mycologia 98:295–306
22. Nielsen KF, Mogensen JM, Johansen M, Larsen TO, Frisvad JC (2009) Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. Anal Bioanal Chem 395:1225–1242
23. Peterson RA, Bradner JR, Roberts TH, Nevalainen KMH (2009) Fungi from koala (*Phascolarctos cinereus*) faeces exhibit a broad range of enzyme activities against recalcitrant substrates. Lett Appl Microbiol 48:218–225
24. APHA, AWWA, WPCF (1989) Standard methods for the examination of water and wastewater. American Public Health Association, Washington
25. Gonçalves C, Rodriguez-Jasso RM, Gomes N, Teixeira JA, Belo I (2010) Adaptation of dinitrosalicylic acid method to microtiter plates. Anal Methods 2:2046–2048
26. Singleton VL, Orthofer R, Lamuela-Raventós RM (1999) Analysis of total phenols and other oxidation substrates and antioxidants by means of folin–ciocalteu reagent. In: Lester P (ed) Methods in enzymology—oxidants and antioxidants Part A. Academic Press, San Diego
27. Weishaar JL, Aiken GR, Bergamaschi BA, Fram MS, Fujii R, Mopper K (2003) Evaluation of specific ultraviolet absorbance as an indicator of the chemical composition and reactivity of dissolved organic carbon. Environ Sci Technol 37:4702–4708
28. Ben Othman N, Ayed L, Assas N, Kachouri F, Hammami M, Hamdi M (2008) Ecological removal of recalcitrant phenolic compounds of treated olive mill wastewater by *Pediococcus pentosaceus*. Bioresour Technol 99:2996–3001
29. Gomes N, Gonçalves C, Garcia-Roman M, Teixeira JA, Belo I (2011) Optimization of a colorimetric assay for yeast lipase activity in complex systems. Anal Methods 3:1008–1013
30. Lanciotti R, Gianotti A, Baldi D, Angrisani R, Suzzi G, Mastrocola D, Guerzoni ME (2005) Use of *Yarrowia lipolytica* strains for the treatment of olive mill wastewater. Bioresour Technol 96:317–322
31. Papagianni M (2004) Fungal morphology and metabolite production in submerged mycelial processes. Biotechnol Adv 22: 189–259
32. Aissam H, Penninckx M, Benlemlih M (2007) Reduction of phenolics content and COD in olive oil mill wastewaters by indigenous yeasts and fungi. World J Microbiol Biotechnol 23: 1203–1208
33. García GI, Peña JPR, Venceslada BJL, Martín MA, Santos MMA, Gómez RE (2000) Removal of phenol compounds from olive mill wastewater using *Phanerochaete chrysosporium*, *Aspergillus niger*, *Aspergillus terreus* and *Geotrichum candidum*. Process Biochem 35:751–758
34. Crognale S, D'Annibale A, Federici F, Fenice M, Quaratino D, Petruccioli M (2006) Olive oil mill wastewater valorisation by fungi. J Chem Technol Biotechnol 81:1547–1555
35. Hamdi M, Bouhamed H, Ellouz R (1991) Optimization of the fermentation of olive mill waste-waters by *Aspergillus niger*. Appl Microbiol Biotechnol 36:285–288
36. Hamdi M, Khadir A, Garcia JL (1991) The use of *Aspergillus niger* for the bioconversion of olive mill waste-waters. Appl Microbiol Biotechnol 34:828–831
37. Ramos JT, Barends S, Verhaert R, de Graaff L (2011) The *Aspergillus niger* multicopper oxidase family: analysis and overexpression of laccase-like encoding genes. Microb Cell Factories 10:78