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Strategies for dephenolization of raw olive mill wastewater by means of *Pleurotus ostreatus*

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Abstract The reduction of polyphenols content in olive mill wastewater (OMW) is a major issue in olive oil manufacturing. Although researchers have pointed out the potential of white-rot fungus in dephenolizing OMW, the results available in the literature mainly concern pretreated (sterilized) OMW. This paper deals with the reduction of polyphenols content in untreated OMW by means of a white-rot fungus, *Pleurotus ostreatus*. Dephenolization was performed both in an airlift bioreactor and in aerated flasks. The process was carried out under controlled non-sterile conditions, with different operating configurations (batch, continuous, biomass recycling) representative of potential industrial operations. Total organic carbon, polyphenols concentration, phenol oxidase activity, dissolved oxygen concentration, oxygen consumption rate, and pH were measured during every run. Tests were carried out with or without added nutrients (potato starch and potato dextrose) and laccases inducers (i.e., $CuSO_4$). OMW endogenous microorganisms were competing with *P. ostreatus* for oxygen during simultaneous fermentation. Dephenolization of raw OMW by *P. ostreatus* under single batch was as large as 70%. Dephenolization was still extensive even when biomass was recycled up to six times. OMW pre-aeration had to be provided under continuous operation to avoid oxygen consumption by endogenous microorganisms that might spoil the process. The role of laccases in the dephenolization process has been discussed. Dephenolization under batch conditions with biomass recycling and added nutrients proved to be the most effective configuration for OMW polyphenols reduction in industrial plants (42–68% for five cycles).

Keywords Olive mill wastewater · Airlift · Polyphenols · Laccases · Continuous cultures · *Pleurotus ostreatus*

Abbreviations

COD	Chemical oxygen demand, g/l
D_{ILAB}	Dilution rate, 1/h
DOT	Dissolved oxygen tension, mg/l
K_La_L	Mass transfer coefficient, 1/h
Lac	Laccase activity, U/ml
OCR	Oxygen consumption rate, g/(l h)
Ph	Polyphenols content, g/l
Q_L	Volumetric flow rate, ml/h
t	Time, h
TOC	Total organic carbon, g/l
X	Biomass concentration, g/l

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Introduction

About 2.5 million tonnes of olive oil is produced every year worldwide, mostly in the Mediterranean area. Typically, olive oil extraction is characterized by the production of a solid residue and a brown wastewater, the olive mill wastewater (OMW). The environmental impact of OMW is related to its large organic content (30–200 g/l COD) including sugars, nitrogenous compounds, fatty acids, and polyphenols. The phytotoxic and antibacterial action of polyphenols is a major drawback for conventional biological treatment [14, 20]. Hamdi [9] observed that OMW must be diluted at least 70 times before it can effectively undergo aerobic activated sludge treatment. Despite its inherent fertilizing potential [26], spreading on agricultural soils or discharging into water bodies is subject to severe restrictions due to OMW's dark color, polyphenols content, and low pH that ranges, generally, between 4 and 5. Remediation by means of physical and chemical methods (evaporation and incineration, ultrafiltration, reverse osmosis, anaerobic digestion, addition of chemicals, etc.) is generally technically or economically unfeasible [19], unless specific local factors come into play [21]. Recent advances regarding olive mill effluent treatments emphasize the need for sequential or integrated biological, physical, and chemical degradation processes to achieve complete mineralization at a reasonable cost [5, 12]. To reduce the OMW remediation cost, the simultaneous production of added-value compounds has been suggested [2, 6, 18].

The ability of white-rot fungi to degrade lignin and lignin-like compounds has long been recognized [10, 24]. Among them, *Pleurotus ostreatus* is active in the reduction of the phytotoxic and antibacterial activity of OMW by decreasing polyphenols concentration [11, 13, 22, 23, 25]. Remediation has been most typically investigated at bench scale, in batch-operated reactors under sterile conditions with addition of nutrients. The only exceptions are the studies by Tsioulpas et al. [25], Fountoulakis et al. [8], Aggelis et al. [1], and Olivieri et al. [16] who examined polyphenols abatement in sterilized OMW without supplementing nutrients. Bioremediation of raw non-sterile OMW by sequential aerobic (using *Phanerochaete chrysosporium*) and anaerobic digestion followed by ultrafiltration has been recently investigated by Dhouib et al. [7]. Olivieri et al. [16] reported results on bioremediation of non-sterile centrifuged OMW in both aerated flasks and internal loop airlift. The fungus effectively grew on the OMW at polyphenols concentrations as large as 1.4 g/l without any added nutrient with a half-life of about 2 days. The abatement of polyphenols was controlled by the availability of nutrients and was as large as 95%. On the basis of these promising findings the investigations on the OMW bioremediation process were continued. The results of an experimental campaign aimed at characterizing the dephenolization of raw OMW by *P. ostreatus* are reported in the present study. The investigation purpose was the definition of an optimal strategy for the dephenolization process, with a focus on the conditions that promote the activity of *P. ostreatus* in the presence of OMW endogenous microorganisms. Continuous and semi-continuous operating conditions with respect to the liquid phase were investigated in flasks and in an internal loop airlift bioreactor (ILAB). Dephenolization in water-diluted OMW was also studied to characterize the kinetics of the process at different polyphenols concentration.

Materials and methods

Materials

OMW derived from the continuous three-phase centrifugation process and supplied by an olive oil factory in Southern Italy. OMW was delivered to the laboratory within 1 week after production, then stored frozen at −20°C. OMW was filtered with a 1-mm net to remove residual coarse solids before the experiments. The main properties of the OMW tested are total organic carbon 31 g/l, pH 5.2, COD 50 g/l, polyphenols concentration 5 g/l, and dry solid content (at 105°C) 10.5 g/l. The properties are typical of all OMW produced in Southern Italy.

Microorganism and inoculum preparation

Pleurotus ostreatus (Florida) (ATCC MYA-2306) was maintained, by means of periodic subculture every month, at 4°C on agar-potato dextrose (Difco Laboratories, Detroit, MI) plates containing 24 g/l potato dextrose (Difco Laboratories, Detroit, MI), 15 g/l agar (Sigma), and 5 g/l yeast extract (Difco). Plates were supplemented with OMW so as to reach a polyphenols concentration of 0.2 g/l. The mycelium was grown in a liquid medium for 5 days in agitated 500-ml Erlenmeyer flasks kept at 30°C in the dark. Each flask contained 500 ml of sterilized medium and five 10-mm plugs collected from the subculture plates. The 5-day-old culture was then filtered to remove the residual broth. Mycelial pellets from agar plugs were removed and only fresh mycelium was suspended in doubly distilled water, homogenized for a few seconds, and inoculated into reactors.

Apparatus

Tests were carried out in Erlenmeyer flasks and in a plant equipped with a lab-scale ILAB.

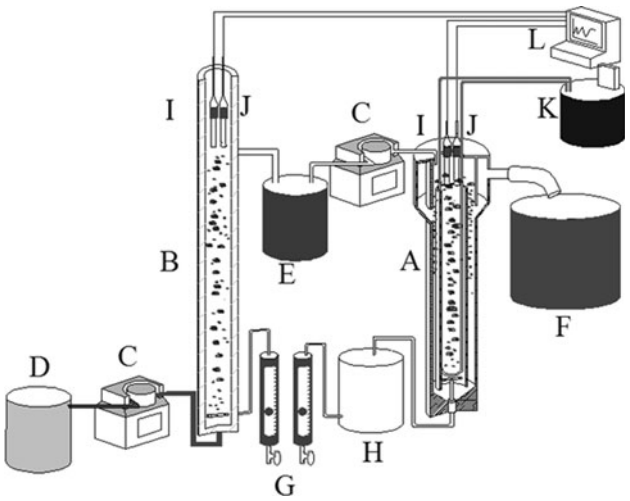

Fig. 1 Schematic of the plant equipped with the internal loop airlift. *A* ILAB, *B* pre-aeration bubble column, *C* liquid flow control unit, *D–E* OMW tank, *F* treated OMW tank, *G* gas flow control unit, *H* humidifier, *I* pH probe, *J* DOT probe, *K* thermostat bath, *L* data acquisition unit

The Erlenmeyer flasks (1 l) were immersed in a thermostatic bath and were continuously oxygenated by sparging with water-saturated air. The continuous pneumatic agitation of the suspension provided effective mixing.

The plant (Fig. 1) consists of a 7-l ILAB (A in Fig. 1), a 7-l bubble column (B in Fig. 1), OMW feeding vessels (D and E in Fig. 1), and a humidifier column (H). The plant was also equipped with a thermostatic bath, peristaltic pumps Miniplus III (Gilson), liquid and gas flow control units, and on-line diagnostics. The main characteristics of the ILAB are reported in Olivieri et al. [15]. The upper region of the ILAB was equipped with a liquid–solid decanter to confine the biomass to the reactor. The design/operational variables of the airlift were optimized with reference to bed hydrodynamics as discussed in Olivieri et al. [15].

The 7-l bubble column (BC)—120 mm ID, 1.5 m height—was adopted for pretreatment of OMW. Air was sparged into the BC by means of a perforated pipe distributor.

Air streams fed to the units were water-saturated to minimize water stripping from the vessels. The heating apparatus for both units consisted of an external heater/cooler connected to an internal heat exchanger.

Analytical methods

Oxygen consumption rate

Oxygen consumption rate (OCR) was measured by means of a respirometric assay according to the APHA Standard Methods [3]. An 800-ml BOD bottle equipped with a dissolved oxygen tension (DOT) probe was completely filled with flask culture, the bottle was aerated to saturate the liquid phase with oxygen and then hermetically closed. The DOT signal was continuously monitored and logged on a PC. Plots of oxygen concentration vs time were linear up to 1 mg/l. The initial slope of the plot was assumed to be the global oxygen consumption rate [4].

Biomass analysis

The biomass content of culture samples was separated from the liquid phase by centrifugation, dried at 40°C by means of a vacuum concentrator (Thermo RC Standard), and weighed.

Characterization of liquid samples

Dissolved oxygen was measured with a DOT probe (Mettler Toledo, InPro 6050) connected to a DOT meter (Mettler Toledo, O_2 4100 Transmitter). pH was measured by means of a Broadley James probe connected to a pH meter (Consort R305). The liquid phase was separated from the biomass by centrifugation and characterized in terms of color, total organic carbon, polyphenols concentration, and laccase activity. OMW decolorization was monitored by measuring absorbance intensity at 395 nm. Total organic carbon (TOC) was measured with a Shimadzu TOC V-CSH analyzer.

The total polyphenols content (Ph) was determined using the Folin–Denis assay [3]. Tannic acid (Sigma-Aldrich) was used as the standard.

Enzyme assay

Lignin peroxidase (LiP) activity was determined using veratryl alcohol as substrate [16]. The assay mixture contained 2 mM veratryl alcohol and 0.4 mM H_2O_2 in 50 mM sodium tartrate buffer, pH 2.5. Absorbance at 310 nm was measured to monitor the oxidation of veratryl alcohol ($\varepsilon_{310} = 9{,}300\ M^{-1}\ cm^{-1}$).

Manganese peroxidase (MnP) activity was determined using $MnSO_4$ as substrate [16]. The assay mixture contained 0.5 mM $MnSO_4$ and 0.5 mM H_2O_2 in 50 mM sodium malonate buffer, pH 4.5. Oxidation of Mn^{2+} to Mn^{3+} was monitored by measuring the increase in absorbance at 270 nm due to the formation of Mn^{3+}–malonate ($\varepsilon_{270} = 115{,}900\ M^{-1}\ cm^{-1}$).

Laccase activity was determined using 2,2′-azino-bis (3-ethylbenzothialine-6-sulfonic acid) (ABTS) as substrate [16]. The assay mixture contained 2 mM ABTS in 0.1 M sodium citrate buffer, pH 3.0. Oxidation of ABTS was

monitored by measuring the increase of absorbance at 420 nm ($\varepsilon_{420} = 36{,}000\ M^{-1}\ cm^{-1}$).

Enzyme activities are expressed in IU.

Operating conditions and procedure

Table 1 reports the operating conditions of a representative set of runs carried out in the flasks and in the ILAB. No pH control was accomplished. Operating temperature was set at 30°C. Generally, tests were carried out in duplicate. The standard deviation of the reported data was always within 8%.

All experiments were carried out using raw OMW, coarse-filtered to remove suspended matter. Raw OMW was diluted with doubly distilled water to adjust the initial polyphenols concentration to preset values ranging between 0.2 and 1.5 g/l. The air stream fed to reactors was water-saturated, in order to limit water stripping from the reacting medium.

Procedure

Batch operations The tests were carried out both in the flasks and in the ILAB. The total volume of cultures was 0.8 l in experiments carried out in flasks and 7 l in the ILAB. The OMW charge was prepared by diluting raw OMW with distilled water to obtain the desired polyphenols concentrations. Initial *P. ostreatus* concentration (X_0) ranged between 0.1 and 0.2 g_{DM}/l. The reactor was run for the preset time and the treated OMW was then removed, paying particular attention to keep the biomass in the reactor. Fresh OMW was loaded into the reactor without any new inoculum and the was process monitored. Biomass recycling was typically iterated five times.

Continuous operations Three modalities of continuous process were adopted. Under all conditions the units were operated continuously with respect to the air streams at preset flow rate.

Modality A The ILAB was fed with pre-aerated OMW. The BC was operated under continuous conditions with respect to the liquid phase at a preset space and time. Once BC approached steady state conditions, the pre-aerated OMW was collected in the vessel E of Fig. 1 and fed to the ILAB. The ILAB was operated batchwise with respect to the liquid phase for 1 day. Then it was operated continuously with respect to the liquid phase at the preset space and time.

Modality B The ILAB was fed with pre-aerated OMW and operated batchwise with biomass recycling. The BC was operated under continuous conditions with respect to the liquid phase at a preset space and time. Once BC approached steady state conditions, the pre-aerated OMW was collected in the vessel E of Fig. 1 and fed to the ILAB. The ILAB was operated batchwise with respect to the liquid phase for the pre-fixed time. Then the liquid was withdrawn and the biomass was collected and inoculated into the successive batch of pre-aerated OMW. The cycle was repeated for a prefixed number of times.

Modality C The ILAB was fed with OMW without any pre-aeration step (C, B, and E of the plant in Fig. 1 were bypassed). The ILAB was operated batchwise with respect to the liquid phase for 1 day. Then it was operated continuously with respect to the liquid phase at the preset space and time.

Culture sampling was carried out typically every 3–4 h, both in batch and continuous operation. Samples were centrifuged and individual phases analyzed off-line. Data regarding biomass growth, polyphenols conversion, and TOC estimated during a run were calculated to determine the specific polyphenols uptake rate and the polyphenols to biomass yield defined in Olivieri et al. [16].

Results

Spontaneous OMW fermentation

Spontaneous OMW fermentation was carried out in aerated flasks at different initial polyphenols concentration without inoculum of *P. ostreatus* (Table 1, runs 1 and 2). Run 2 refers to the test carried out adding ampicillin (Amp) to the bioreactor to inhibit endogenous biomass growth. Ampicillin was fed as day-spots at a rate of about 50 mg/(l day). Spontaneous fermentation without ampicillin was characterized by significant OCR (Fig. 2) and no dephenolization (Table 1). OCR depletion was remarkably low during the tests carried out by adding ampicillin (Fig. 2).

Batch processes

Preliminary tests carried out with the raw filtered OMW inoculated with *P. ostreatus* were unsuccessful as regards the dephenolization process (data not reported).

Pre-aeration of OMW

Data reported in Table 1 (columns 3) refer to tests carried out in flasks with pre-aerated OMW. The OMW batches were aerated for 2 days and then inoculated with *P. ostreatus* (Fig. 3). It is interesting to note that effects of spontaneous fermentation apparently vanished within 2 days, even though pH increased up to about 8 as a consequence of the spontaneous fermentation. The main

Table 1 Operating conditions and selected results of a representative set of experimental runs

Run Bioreactor	1 Flask	2 Flask	3 Flask	4 Flask	5 Flask	6 ILA	7 BC-ILA	8 BC-ILA	9 ILA	10 ILA	11 Flask
Biomass											
Operating condition	Batch	Batch	Batch	Recycling	Recycling	Batch	Batch	Batch	Recycling	Recycling	Batch
Air											
Flow rate, l/h	250	250	250	250	250	300	500–300	500–300	300	300	250
OMW											
Operating condition	Batch	Batch	Batch	Batch	Batch	Continuous	Continuous	Continuous	Batch	Batch	Recycling
Pretreatment	–	–	Aeration	–	–	–	–	–	–	–	–
Cycle time, h	–	–	–	55	40	–	–			–	–
Mean residence time, h	–	–	–	–	–	160	90	90	110	80	–
Ph^0	0.2–1.4	0.2–1.4	1	1.5	1.1	1.2	1.3	1.2	1	0.6	1.25
Potato dextrose, g/l	–	–	–	–	2.4	–	–	2.4	–	–	–
Yeast extract, g/l	–	–	–	–	0.5	–	–	0.5	–	–	–
Potato starch, g/l	–	–	–	–	–	–	–	–	–	1	–
Amp, mg/(l day)	–	50	–	–	–	–	–	–	–	–	–
$CuSO_4$, μM	–	–	–	–	–	–	–	20	–	–	–
Results											
Polyphenols conversion, %[a]	0	0	69	57–0–0	45–54–64–63–61	No steady state	No steady state	No steady state	50–36–0–0	44–42–60–68–58–48	50–54
Polyphenols uptake rate, g/(g_{DM} day)	–	–	8.2	12	0.96	–	–	–	–	–	–
Polyphenols to biomass yield, g/g	–	–	0.64	0.9	2.0–1.4–0.2–0.1–0	–	–	–	–	–	–

[a] Value at the end of the run or of each cycle, if applicable

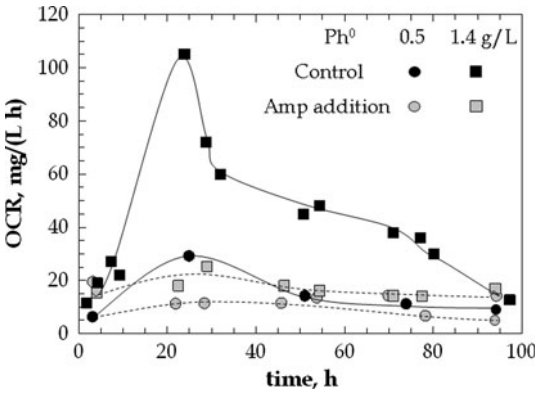

Fig. 2 OCR during spontaneous OMW fermentation without (control) and with periodic addition of ampicillin (Amp). Reactor: flasks (Table 1, runs 1 and 2)

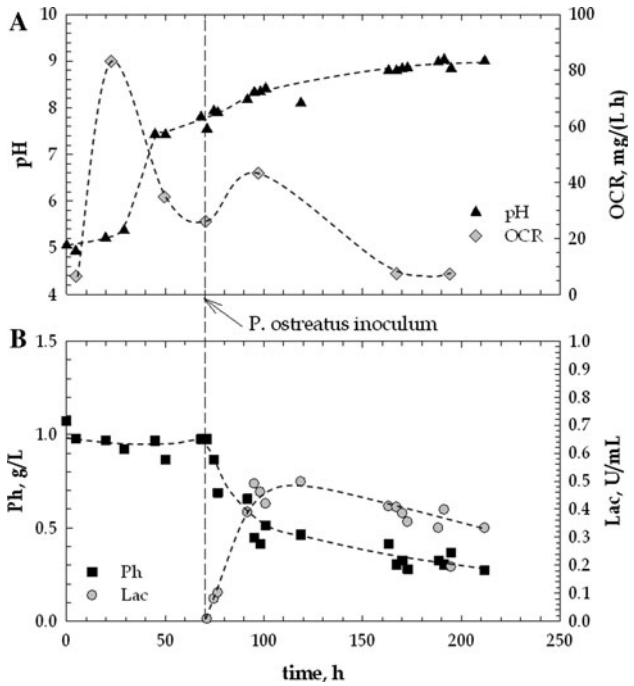

Fig. 3 Dephenolization in the OMW pre-aerated. Reactor: flasks (Table 1, run 3). *Filled triangles* pH, *shaded diamonds* oxygen consumption rate (OCR), *filled squares* polyphenols concentration (Ph), *shaded circles* laccase activity (Lac). The *vertical dashed line* marks the time at which *P. ostreatus* was inoculated

features of dephenolization were (1) Ph decreased rapidly to about 50% of the initial value the first day after the inoculation, then kept on decreasing at a lower rate up to about 30% of the initial value; (2) a non-monotonic trend of both the laccase activity and OCR, which peaked the first day after inoculation; (3) pH lightly increased up to 9. The fermentation features observed from inoculation—polyphenols conversion, laccase activity, and OCR—reproduce those observed during the remediation process of pre-centrifuged OMW carried out with *P. ostreatus* [16], even though the pre-aeration step changed the value of TOC and pH at the beginning of the remediation step. The progressive reduction of laccase activity and OCR after about 1 day from inoculation may be due to a change in the fungal metabolism.

No appreciable lignin and manganese peroxidase activities were detected throughout the tests. In particular, the assays for these enzymes were negative for all the tests carried out.

Biomass recycling

Preliminary tests were carried out with the coarse-filtered OMW and *P. ostreatus* in aerated flasks adopting the procedure reported in Olivieri et al. [16]. The features of fermentation during these tests—in terms of time course of the polyphenols conversion, laccase activity, and pH—confirm those observed with the pre-centrifuged OMW [16]. Single incubation under batch conditions of both the biomass and the liquid phase was characterized by (1) levels of polyphenols abatement as large as 70% over 4–7 days of incubation, (2) 50% conversion of polyphenols over about 2 days, (3) almost no decolorization during bioremediation.

The data reported in Table 1, run 4 refer to the OMW phenol reduction tests—at pre-fixed dilution ratios—carried out in flasks with biomass recycling: the biomass harvested by filtration at the end of each culture was inoculated in fresh OMW. The incubation time of each cycle was set at about 4 days in agreement with the typical timescale of polyphenols conversion estimated under single-stage tests (half-life = 2 days). At $Ph^0 = 1.5$ g/l polyphenols conversion was negligible from the second cycle on. Tests carried out at lower Ph^0 highlighted that polyphenols conversion was extensive in the following cycles, even though the laccase activity was below the detectable value after the first cycle.

The overall polyphenols conversion rate was estimated at the beginning of the first cycle in agreement with the procedure proposed by Olivieri et al. [16]. The rate was 12 g/(g_{DM} day). Comparison of our results with the data reported by Olivieri et al. [16] shows that OMW pretreatment does not affect the remediation rate.

Addition of nutrients

Data reported in Table 1, run 5 concern the effects of nutrient addition on polyphenols abatement with biomass recycling. In particular, extra nutrients were PDY (potato dextrose 2.4 g/l and yeast extract 0.5 g/l) and potato starch (1 g/l).

The results of the tests carried out in flasks with OMW supplemented at each cycle with PDY are reported in

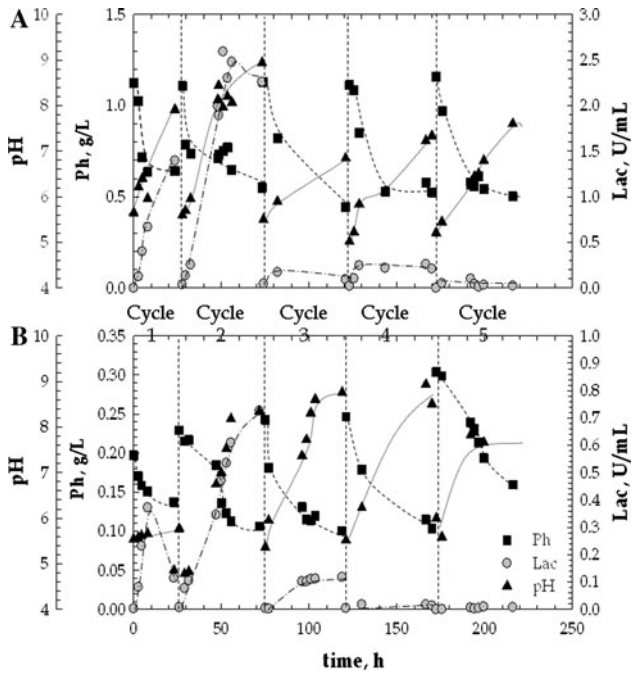

Fig. 4 OMW treatment with biomass recycling: PDY supplement. Reactor: flasks. Data refer to run 5 of Table 1. *Filled triangles* pH, *filled squares* polyphenols concentration (Ph), *shaded circles* laccase activity (Lac)

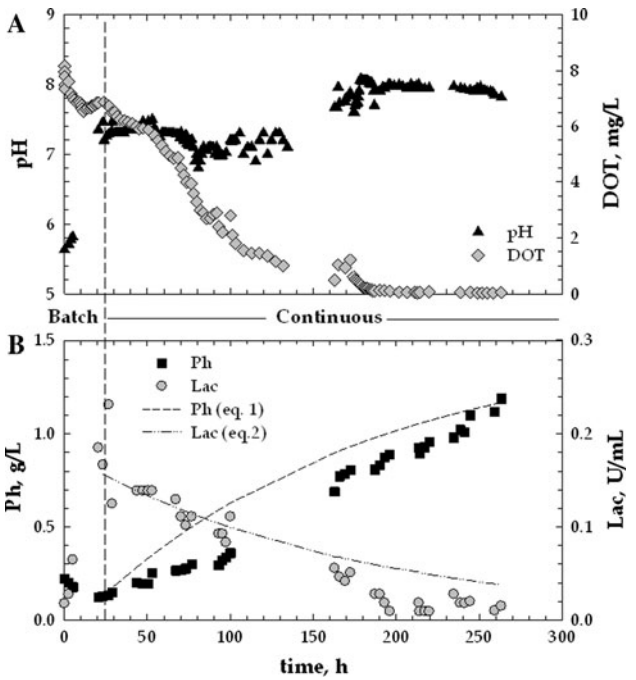

Fig. 5 Continuous treatment of OMW. Reactor: ILAB (Table 1, run 6). *Filled triangles* pH, *shaded diamonds* dissolved oxygen tension (DOT), *filled squares* polyphenols concentration (Ph), *shaded circles* laccase activity (Lac)

Fig. 4. The fermentation process was a reproduction of the fermentation observed during the tests carried out without extra nutrients. The main difference was the enhanced intensity of all the fermentation features. In particular, laccase activity was larger with than without extra nutrients, and laccase activity was significant in the first two cycles.

Continuous processes

Bioremediation without pre-aeration

Figure 5 shows selected results of the experimental continuous treatment of OMW in the ILAB operated according to modality C, i.e., without OMW pre-aeration. The main operating conditions are reported in Table 1, run 6. The initial loading of mycelium was produced during the 1-day batch culture in diluted OMW ($Ph^0 = 0.2$ g/l). At $t^0 = 22$ h the reactor was fed with the OMW stream (polyphenols concentration $Ph^{IN} = 1.2$ g/l) at volumetric flow rate $Q_L = 42$ ml/h, corresponding to a dilution rate $D_{ILAB} = 0.006\ h^{-1}$. In particular the D_{ILAB} was fixed considering the polyphenols conversion rate [16] and the amount of mycelium that was likely to be present in the ILAB at the end of the batch phase. Figure 5 reports also the plots of polyphenols concentration and laccase activity (Lac) versus time (t) estimated assuming that the ILAB conformed to an evenly mixed tank operated under continuous conditions:

$$Ph(t) = Ph^{IN} - \left(Ph^{IN} - Ph^0\right) \cdot e^{-D\left(t-t^0\right)} \quad (1)$$

$$Lac(t) = Lac^0 \cdot e^{-D\left(t-t^0\right)} \quad (2)$$

where Ph^0 and Lac^0 are the polyphenols concentration and laccase activity measured at the beginning of the continuous operation (t^0). The difference between the Ph measured at the time $t > t^0$ and the estimated value Ph(t) from Eq. 1 is a measure of polyphenols conversion. The difference between the Lac measured at the time $t > t^0$ and the estimated value Lac(t) from Eq. 2 is a measure of the rate of laccases expression by *P. ostreatus*.

Figure 5 shows that (1) OCR was nearly constant during the batch stage and increased remarkably during the continuous process, so that anoxic conditions were established after 6 days of continuous culture; (2) a finite polyphenols conversion degree could be observed over 9 days of continuous culture; (3) the laccases were washed out from the beginning of the continuous process, in agreement with Eq. 2.

Bioremediation with pre-aeration

Data reported in Table 1, runs 7 and 8 refer to the continuous treatment of pre-aerated OMW according to modality A) (see Sect. "Operating conditions and

procedure"). The liquid phase was continuously fed to the BC ($D_{BC} = 0.011\ h^{-1}$) operated at an air flow rate of 500 l/h (mass transfer coefficient $K_L a_L = 80\ h^{-1}$) and then pumped to the ILAB to reduce polyphenols by means of *P. ostreatus*. During run 8 the stream pumped to the ILAB was supplemented with a solution of PDY and $CuSO_4$, a laccases expression inducer [17]. The dilution rate of the bubble column (D_{BC}) was set at values smaller than the timescale of OCR controlled by OMW endogenous microorganisms (maximum at $t = 25$ h in Fig. 2). The dilution rate of the ILAB (D_{ILAB}) was $0.011\ h^{-1}$.

Figure 6 reports a selected data from run 7 (Table 1). The time at which aeration of the BC started was assumed to be $t = 0$ of the run. According to modality A (see Sect. "Operating conditions and procedure"), OMW issuing from the BC was pumped into the ILAB when steady state conditions in the BC were established. The initial concentration of polyphenols in the ILAB was fixed at 0.6 g/l—corresponding to a dilution ratio 1:2 of the pre-aerated OMW—at the beginning of the batch phase. After a 1-day incubation, the ILAB was continuously fed with pre-aerated OMW without any dilution. As expected, OCR in the BC peaked at 130 mg/(l h) during the first week of operation and then reached a steady value of nearly 40 mg/(l h). At the same time, DOT in BC first decreased and then approached the solubility-limiting value, 6–7 mg/l. Polyphenols concentration in the BC did not change with respect to the raw OMW. The analysis of the time-resolved profiles of polyphenols concentration and laccase activity in the ILAB indicates that (1) the time course of the main fermentation features observed during the tests carried out in flasks under batch conditions are fairly well reproduced in the ILAB, (2) wash-out of both polyphenols and laccase activities may take place during continuous operation. It is remarkable that DOT does not depart significantly from the limiting value of oxygen solubility.

Polyphenols abatement carried out by feeding the stream of PDY and $CuSO_4$ to the ILAB (run 8) closely reproduced the behavior observed during run 7.

The main results of the test carried out in the ILAB with biomass recycling operated according to modality B are reported in Table 1, runs 9 and 10. In particular, run 10 is characterized by potato starch supplementation.

Figure 7 reports data from run 10. During each batch stage it is possible to note that (1) TOC conversion is even larger than that measured for polyphenols, (2) DOT is characterized by a minimum immediately after the beginning of each cycle as a consequence of biomass growth. Interestingly, polyphenols conversion was extensive (up to 68%) in each cycle, laccases expression occurring even under the anoxic conditions established during the last two cycles. All together, the results observed in the flasks (Table 1, runs 4 and 5) are confirmed.

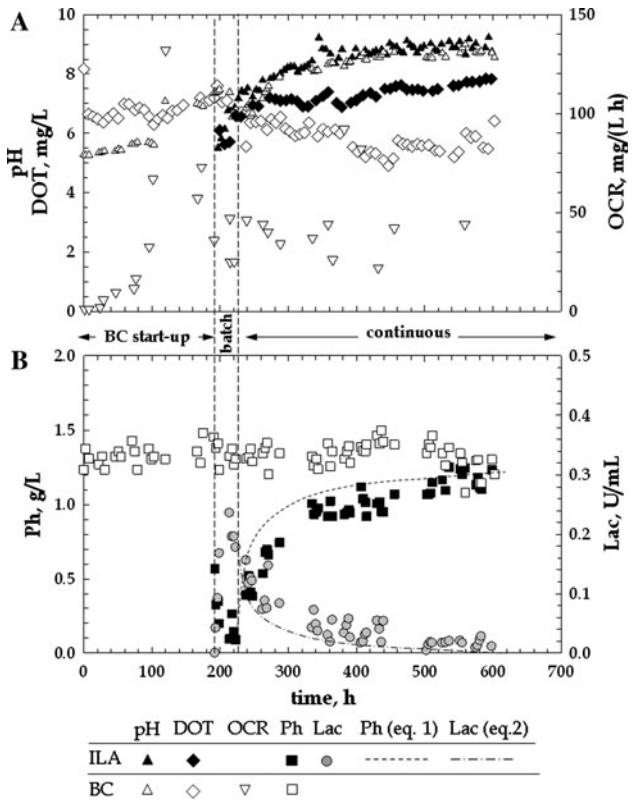

Fig. 6 Continuous treatment of OMW. Reactor: ILAB and BC (Table 1, run 7)

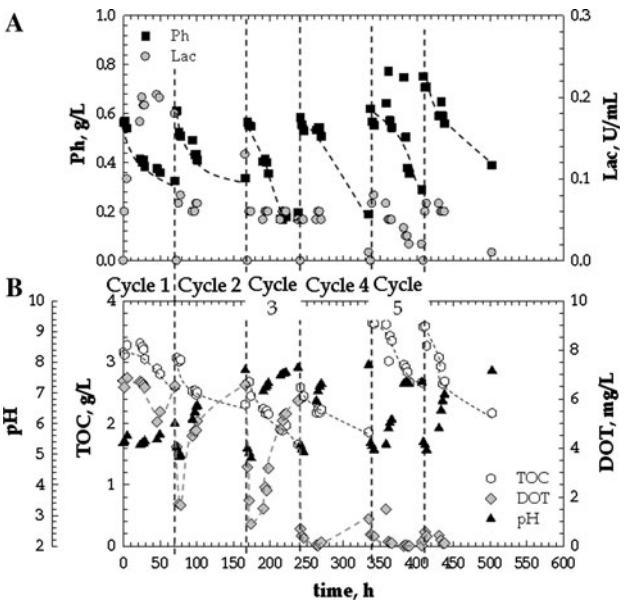

Fig. 7 OMW treatment with biomass recycling: potato starch supplement. Reactor: ILAB (Table 1, run 10). *Filled triangles* pH, *shaded diamonds* dissolved oxygen tension (DOT), *filled squares* polyphenols concentration (Ph), *shaded circles* laccase activity (Lac), *open diamonds* total organic carbon (TOC)

The results of run 9, carried out without any potato starch addition, confirmed those of run 10 except for the number of successful cycles. The dephenolization was active for two cycles instead of six cycles.

Role of the laccases

Data reported in Table 1, run 11 regard the role of the extracellular enzymes in OMW bioremediation. The raw OMW was inoculated with *P. ostreatus* and the liquid phase of 1-day culture was recovered by filtration. A 200-ml sample of the recovered liquid was inoculated in fresh OMW (600 ml)—at the preset dilution ratio—and the remediation process characterized. Polyphenols conversion was still observed in fresh OMW as a consequence of the presence of recycled laccases, whose activity was practically constant over the 5-day run.

Some tests were carried out with a solution of crude laccases and raw OMW with different initial volumetric ratios. Particular attention was paid to the ratio between initial laccase activity and the initial polyphenols concentration. The solution of crude laccases was the culture broth of the *P. ostreatus* inoculum culture. The analysis of the time-resolved data of polyphenols concentration and laccase activity showed that (1) the laccase activity in the solution decreased as soon as the broth and the OMW were mixed and that the higher the initial polyphenols concentration the larger the activity decrease, (2) after about 10 h of incubation the laccase activity approached the initial value and the polyphenols concentration was smaller.

Discussion

The results presented in this study are directed to support the design of industrial dephenolization of raw OMW. The OMW used in the experiments was raw matter, filtered only to remove coarse suspended solids. Conversely to previous studies on OMW bioremediation with heat pretreatment, no decolorization was observed during the tests [8, 16].

Raw filtered OMW is characterized by the presence of endogenous microorganisms that are typically removed by heat pretreatment or centrifugation. Both the batch tests and the continuous tests carried out with the raw filtered OMW inoculated with *P. ostreatus* were unsuccessful as regards the dephenolization process. The presence of endogenous microorganisms in OMW may cause two problems for the activity of the microorganism in charge of the bioremediation: (1) the competition for the common substrates, oxygen, and TOC; and (2) the change of environment features, pH, etc. The high oxygen demand may be fulfilled either by higher oxygen transfer rate in the process unit or in a pre-aeration unit. However, both solutions have drawbacks. On one hand, the high shear rate typically associated with higher oxygen transfer rates may inhibit the microorganism activity. On the other hand, the pre-aeration unit increases the complexity and the costs of the process.

The batch tests carried out with pre-aerated OMW inoculated with *P. ostreatus* were successful as regards the dephenolization process. Both the pH increase and the TOC uptake observed during the endogenous fermentation did not affect the *P. ostreatus* performance. On the basis of the success of the pre-aeration strategy, two process modalities were investigated: modality A, i.e., continuous process with respect to both liquid and biomass phases, and modality B, i.e., continuous process with respect to the liquid phase and biomass recycling.

Modality A

The dephenolization tests carried out in the ILAB operated under continuous conditions with OMW pre-aeration were characterized by a decrease in the laccase activity that could be attributed to the reactor approaching wash-out conditions (Eq. 2). The observed behavior can be interpreted according to two different explanations:

- The rate at which laccases are expressed by *P. ostreatus* is not large enough to balance wash-out: accordingly the laccase activity in the ILAB decreases monotonically.
- On the basis that polyphenols are complexed by free laccases, the feeding of polyphenols under continuous conditions results in a continuous subtraction of free laccases from the liquid phase. In this case, the overall conversion process is a combination of the rate at which complexed enzymes are formed, the rate at which they undergo further conversion, and the wash-out rate.

Modality B

The dephenolization tests carried out in the ILAB operated under successive batchwise conditions and fed with pre-aerated OMW were successful. The dephenolization was extensive in up to six cycles. TOC starvation was never observed. Laccases were expressed up to the sixth cycle. Accumulation of microorganisms in the recycled biomass increases the OCR of the resulting suspension, thus favoring the establishment of an anoxic environment in the bioreactor.

The performances of the ILAB operated under modality B may further be enhanced if the strategy proposed by Dhouib et al. [7] is adopted. The periodic re-inoculation of the ILAB would improve the dephenolization performances.

The above results suggest that the productivity in the continuous bioremediation of OMW depends on the rate at

which laccases are expressed. The conditions to enhance laccases expression may be offered by iterated batch operation with biomass recycling, as successfully demonstrated in the present study. Notwithstanding the rate at which laccases are expressed during the cycles, the conversion of polyphenols by *P. ostreatus* remains fairly constant when the broth is supplemented with auxiliary carbon sources such as potato dextrose or potato starch.

Conclusions

Dephenolization of raw OMW—subjected to no pretreatment other than filtration to remove coarse suspended solids—was successfully carried out by *P. ostreatus*. Oxygen is the limiting substrate as regards competition between the endogenous microorganisms in raw OMW and *P. ostreatus*. In no case did the endogenous microorganisms contribute to polyphenols conversion.

Continuous treatment of OMW by *P. ostreatus* was effectively performed in an ILAB provided that pre-aeration of the water is accomplished in a separate bubble column to meet the oxygen demand of the endogenous microorganisms. Bioremediation rate was limited by the rate at which laccases were expressed by *P. ostreatus*.

As an alternative to the continuous process, treatment of raw OMW in a battery of batch reactors with biomass recycling was successfully demonstrated in the present study. Polyphenols abatement was quantitative when contacted with each of the successive batches of *P. ostreatus*, particularly at low initial concentration (0.2 g/l) of polyphenols. For large initial concentrations of polyphenols ($\approx$1 g/l) the conversion was still quantitative in each successive batch but addition of extra nutrients was necessary. In particular, the same biomass could be recycled up to six times with freshly charged OMW at high polyphenols concentration when the OMW was supplemented with cheap nutrients—PDY or potato starch.

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