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Screening of strains of *Lentinula edodes* grown on model olive mill wastewater in solid and liquid state culture for polyphenol biodegradation

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ABSTRACT

In Morocco, the olive industry produces great amounts of olive mill wastewater (OMW) yearly in a short period (250 000 m^3 of liquid wastes in four months, November–February). Phenolic compounds are largely responsible for the phytotoxicity and antimicrobial effects of OMW. Several studies have been carried out on biological and enzymatic treatments of OMW. However, the use of OMW to produce value-added products, *e.g.* mushroom cultivation, are less explored. This research aimed to select shiitake mushroom strains capable of growing on OMW, involving decolorization, removal of total phenol, and high production of mycelial biomass. Sixteen strains of *Lentinula edodes* were evaluated for their tolerance to OMW, apical growth rate, and biomass production on agar media. The highest biomass yields were recorded in four strains (Le118, Le119, Le121, Le122) grown in the presence of 20% (v/v) OMW. The ability of these pre-selected strains to decolorize and to remove total phenol from OMW was then assessed in liquid culture, without nutritional supplements. The strain Le119 of *L. edodes* showed 65% decolorization, and 75% elimination of total phenol according to the Folin–Ciocalteu assay. Laccase production was the main lignolytic activity observed, and one of its isoforms stained on native PAGE with *p*-phenylenediamine as substrate at pH 5.0.

1. Introduction

The production of olive oil is expanding in the Mediterranean region, which accounts for 95% of total worldwide production (Ismaili-Alaoui and Heddoun, 2006). Main producers of olive oil are Spain, Italy, Greece, Tunisia and Morocco (International Olive Oil Council, 2008). The extraction of oil from olives generates large quantities of liquid by-products, which have been estimated in excess of 30 × 10^6 m^3 per year (Crognale et al., 2006). Chemical characteristics of these liquid wastes depend on olive varieties, as well as the olive oil extraction system.

In Morocco, about 50% of olive oil production comes from traditional mills called "Maâsra", which use the conventional "three-phase system" (Ismaili-Alaoui and Heddoun, 2006). A major drawback of this system is that huge amounts of olive mill wastewater (OMW) are discarded, containing a high concentration of polyphenols (Ben Sassi et al., 2006). OMW are now generating pollution problems in the Mediterranean region, particularly in Morocco, where suitable treatments for these effluents are poorly developed (Ben Sassi et al., 2006).

Detrimental effects of OMW (Sayadi et al., 2000) on plant growth (Isidori et al., 2005), microbial activity, and soil properties have already been described (Saadi et al., 2007). The presence of phenolic compounds in high concentrations is considered the main factor from OMW causing harmful effects (Sayadi et al., 2000; El Hajjouji et al., 2007). Extensive research on white-rot basidiomycetous fungi has been conducted in order to select strains showing high detoxification capability (*i.e.*, simultaneous removal of phenolic compounds and color) on OMW (Sayadi and Ellouz, 1993; D'Annibale et al., 2004). Other research efforts are focused on the use of fungal enzymes involved in the degradation of polyphenols (D'Annibale et al., 2000; Tsioulpas et al., 2002). The cultivation of edible mushrooms (*Pleurotus* or *Lentinula*) using OMW as a wetting agent has also been carried out (Zervakis et al., 1996; Kalmis and Sargin, 2004; Kalmis et al., 2008).

Lentinula edodes (shiitake) is a white-rot mushroom cultivated commercially, which has medicinal properties, as well as

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biodegradation and biotransformation abilities (Philippoussis et al., 2007; Israilides et al., 2008; Philippoussis, 2009). This species produces extracellular oxidizing enzymes capable of biodegrading lignin-related recalcitrant compounds, such as polyphenols (D'Annibale et al., 1998). The efficiency of polyphenol degradation by *L. edodes* is associated with several enzymes, including lignin peroxidase, manganese peroxidase and laccase (D'Annibale et al., 2004; Ayed et al., 2005; Matos et al., 2007). However, the production of these enzymes depends on substrate composition, cultivation system and environmental factors affecting fungal growth (Alaoui et al., 2008; Elisashvili et al., 2008).

In previous research work, it has been shown that mycelial growth rate, biomass yield and enzymatic activities are important factors for successful mushroom cultivation (Philippoussis et al., 2003; Silva et al., 2005). In this study, we selected strains of *L. edodes* capable not only of tolerating the phenolics present in OMW, but also of using them as a carbon source to yield high mycelial biomass. Decolorization and removal of phenolic compounds were also criteria of selection.

2. Materials and methods

2.1. Substrates

OMW was obtained from an olive oil plant at Beni-Mellal (central Morocco), after the three-phase extraction. OMW was stored at −20 °C until used. The composition of untreated OMW is shown in Table 1.

2.2. Microorganisms

Sixteen strains of *L. edodes* (Berk.) Pegler were obtained from different laboratories (Table 2). These strains were already selected for their ability to colonize agricultural residues. All strains were routinely grown on potato dextrose agar (PDA, Sigma, St Quentin Fallavier, France) plates. Inoculum was prepared by growing the mycelium on agar plates for 2 weeks.

2.3. Solid state culture

Tolerance of *shiitake* mycelium was investigated on PDA containing different concentrations of OMW, ranging from 0 to 100%. The agar medium was sterilized at 121 °C for 20 min, cooled, and used to prepare PDA plates. These plates were inoculated with agar disks (10 mm diameter) from an actively growing colony, and then incubated at 25 °C. Linear growth rates were determined as the average distance covered by the mycelium in two perpendicular directions. After complete colonization of Petri plates (90 mm diameter), the mycelial biomass was assessed using a sterile cellophane disc placed on the agar surface, as described by De Araujo et al. (2000). The mycelium was allowed to grow on the cellophane disc, and then lifted away to be dried at 105 °C.

Table 1
Characteristics of untreated olive mill wastewater (OMW).

Characteristics	OMW
pH	4.9 ± 0.05
Suspended solids (%)	13.84 + 0.7
Total sugar (g l^{-1})	12.8 ± 0.75
Reducing sugar (g l^{-1})	10.08 ± 0.41
Total phenol (g l^{-1})	12.54 ± 0.0.82
Total organic carbon (g l^{-1})	41.66 ± 1.58
Total nitrogen (g l^{-1})	0.66 ± 0.01

Table 2
Origin and source of different strains studied of *Lentinula edodes*.

Strains	Origin	Source
Le118	AMRL[a]	China
Le119	AMRL	China
Le120	AMRL	China
Le121	AMRL	Europe
Le122	AMRL	Europe
Le123	AMRL	Europe
Le124	AMRL	Taiwan
Le125	Commercial strain	Japan, Osaka
Le126	Commercial strain	Japan, Osaka
Le127	Commercial strain	France, Mycelium
Le128	Commercial strain	France, Le Champion
Le129	Commercial strain	Belgium, Belgique Mycelia
Le130	Commercial strain	U.S.A., Lambert spawn
Le131	Commercial strain	Japan
Le132	Hybrid strain	France, INRA
Le133	Homokaryon of commercial strain	France, Le Lion

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2.4. Liquid state culture

The inoculum of strains was prepared by growing the mushroom mycelium on PDA containing 2% (v/v) OMW. Erlenmeyer flasks containing 100 ml of OMW at 10% (v/v) were sterilized (121 °C for 20 min), inoculated with three agar plugs (10 mm diameter), and incubated at 25 ± 2 °C for 30 days, under shaking (120 rpm) conditions. Controls were prepared by OMW without inoculum, and incubated under the same conditions. Experiments were performed in triplicate.

2.5. Analytical assays

Total phenol was quantified by the method of Folin and Ciocalteu (Bärlocher and Graça, 2005). The blue reactive complex formed was determined spectrophotometrically at 760 nm. The phenolic content of the samples was expressed as caffeic acid equivalent. After filtration of OMW from the liquid culture, decolorization of OMW was measured at 395 nm as reported by Sayadi and Ellouz (1993).

2.6. Molecular weight distribution of polyphenols

Gel filtration chromatography using Sephadex G-50 was carried out for the analysis of polyphenolic compounds present in raw and treated OMW (Sayadi and Ellouz, 1993). The column was previously equilibrated with NaOH 0.05 M, LiCl 0.025 M. The flow rate was adjusted to 0.3 ml min^{-1}. The column was calibrated with polymeric standards (blue dextran, 2000 kDa; carbonic anhydrase from bovine, 29 kDa; cytochrome C from horse heart, 12.4 kDa; aprotinin: 6.5 kDa and syringic acid: 195 Da).

2.7. Chromatographic analyses

The monomeric composition of the phenolic fraction in OMW was extracted with ethyl acetate as described by De Marco et al. (2007), and it was analyzed by reversed-phase HPLC using binary gradient elution. The analysis was performed on an Agilent HPLC system (USA) equipped with diode array detector. The chromatographic separation was achieved on 5 μm dC18 (4.6 × 250 mm) reversed-phase column (Atlantis, Ireland). The solvent system used was a gradient of solvent A (water:acetic acid, 97:3 v/v) and solvent B (acetonitrile:methanol, 80:20 v/v). The gradient used was previously described by De Marco et al. (2007). Peak detection was

carried out at 280 nm. The monomeric phenols were identified by comparing the retention time of eluted peaks to that of standards.

2.8. Enzyme assays

Laccase (E.C. 1.10.3.2; benzenediol: oxygen oxidoreductase) activity was assayed spectrophotometrically at 30 °C using syringaldazine as substrate, and monitoring the formation of quinine at 525 nm (ε = 65.000 M^{-1} cm^{-1}) [Criquet et al., 1999]. Manganese peroxidase (MnP) and lignin peroxidase (LiP) activities were determined by the oxidation of veratrylic alcohol at 310 nm (ε = 93 000 M^{-1} cm^{-1}) to veratrylic aldehyde. One international unit of enzyme activity (IU) is defined as the amount of enzyme that produces 1 μmol of product per minute. Interferences in enzymatic assays due to OMW were eliminated by adding insoluble polyvinylpolypyrrolidone (PVPP), as described by Sampedro et al. (2007).

2.9. Statistical treatment

One way analysis of variance (ANOVA) was used to analyze the biomass production data and multiple pair-wise comparisons were performed by the Tukey test using Xlstat® software.

3. Results

Strain selection involved two steps. In solid state culture, apical growth and biomass yield were determined for all strains in order to select the best ones for OMW bioremediation. The second step was carried out in liquid state culture, in the presence of OMW. Kinetics of decolorization and the removal of total phenol from OMW were assessed during 30 days with respect to the ligninolytic system. The change of polymeric and monomeric phenol compounds was determined.

3.1. Tolerance of OMW by shiitake strains

All strains of *L. edodes* were capable of growing on PDA with 10% and 20% of OMW (v/v), while no mycelial growth was observed above 40% of OMW. The period of initial growth varied among strains. By contrast, the period for complete colonization of the Petri plate by *L. edodes* strains varied from 10 to 30 days. Apical growth rates were significantly different among strains. They were reduced by 50% as the concentration of OMW increased in PDA (Fig. 1). The apical growth of all strains was higher on PDA without OMW (data not shown). The highest growth rate was recorded for Le118 growing on agar medium containing 10% and 20% of OMW (10.72 and 4.97 mm day^{-1}, respectively), while the lowest growth rate was shown by the strain Le126 (1.38 and 0.46 mm day^{-1}, respectively).

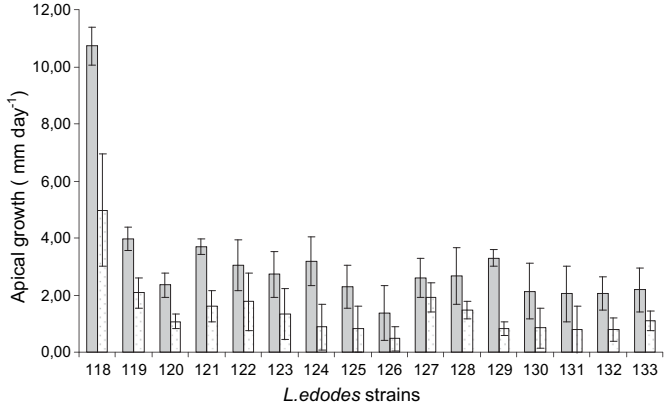

Fig. 1. Growth rate (mm day^{-1}) of sixteen strains of *Lentinula edodes* on PDA containing 10% and 20% of olive mill wastewater (OMW), and incubated at 25 °C until complete colonization of the Petri dish.

3.2. Biomass production

Dried mycelial biomass produced on PDA containing OMW was different among the strains of *L. edodes* (Table 3). The strain Le119 showed the highest biomass production (7.02 mg day^{-1} l^{-1}), followed by Le121, Le122, and Le118 (5.43 mg day^{-1} l^{-1}, 5.26 mg day^{-1} l^{-1}, and 4.75 mg day^{-1} l^{-1}, respectively). The lowest biomass yield was recorded in the strain Le 133 (2.29 mg day^{-1} l^{-1}). In the case of strain Le119, the addition of 10% of OMW to PDA resulted in a threefold increase of biomass production. However, the increase of OMW concentration to 20% reduced the biomass yield of all strains. On the basis of mycelial growth characteristics (apical growth rate and biomass production) on PDA containing with 10% and 20% of OMW, four strains (Le118, Le119, Le121, Le122) were selected for further evaluation.

3.3. Decolorization of OMW

The decolorization of OMW by four selected strains of *L. edodes* is shown in Fig. 2. The maximum amount of color removal was recorded in the strain Le119 (65%), while the strain Le118 exhibited low OMW decolorization during the initial stage of incubation but increased absorbance values with time. The other two strains were found to be moderate decolorizers of OMW. At the end of incubation period, a progressive darkening of the biomass was observed, which could be due to the adsorption of phenolic polymers by the mycelium. Accumulation of colored compounds into the mycelium has been estimated in about 10% of the initial total color.

3.4. Degradation of total phenol

The kinetics of total phenol degradation by *L. edodes* on OMW is shown in Fig. 3. Degradation appeared to be correlated with decolorization (r^2 = 0.867, $p < 0.001$). The maximum reduction of total phenol was recorded in the strain Le119 (75%), while the strain

Table 3
Mycelial biomass (mg day^{-1} l^{-1}) produced in agar culture at 25 °C from sixteen strains of *Lentinula edodes*, using PDA containing 10% and 20% of olive mill wastewater (OMW).

L. edodes strains	PDA with 10% of OMW	PDA with 20% of OMW
Le119	7.02 a[a]	4.49 a
Le121	5.43 b	4.40 a
Le122	5.26 b,c	3.96 b
Le118	4.75 c,d	3.99 b
Le124	4.33 d,e	0.36 g
Le127	4.30 d,e	3.47 c
Le129	4.23 d,e	2.49 d
Le125	4.18 d,e	2.43 d
Le123	4.15 d,e	0.44 g
Le128	4.07 e	3.96 b
Le131	4.05 e	0.35 g
Le130	4.02 e	3.54 c
Le126	3.28 f	3.67 b
Le132	3.13 f	1.42 f
Le120	2.90 f	0.00 h
Le133	2.29 g	1.85 e

[a] Mean values of each parameter within the same column not sharing common letters are significantly different ($p < 0.05$).

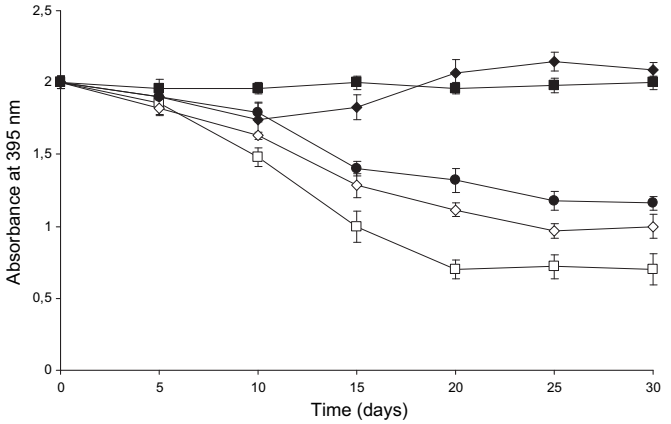

Fig. 2. Color profiles in olive mill wastewater (OMW) incubated at 25 °C, (■) without inoculation (control) and inoculated with four strains of *Lentinula edodes*. (◆) Le118, (□) Le119, (◇) Le121, and (●) Le122.

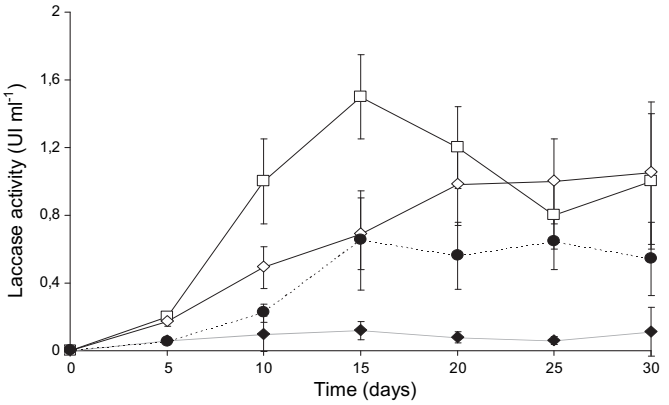

Fig. 4. Laccase activity of four strains of *Lentinula edodes* grown on liquid culture of olive mill wastewater (OMW), and incubated at 25 °C. (◆) Le118, (□) Le119, (◇) Le121, and (●) Le122.

Le118 exhibited lower ability for degradation of total phenol (28%). The highest reduction in total phenol (50%) by the strain Le119 was observed after 20 days of incubation. The strain Le121 showed higher degradation of total phenol than Le122, which started degradation after an initial delay of 10 days.

3.5. Laccase enzyme production

Laccase production was the main enzymatic activity observed during experiments, which involved degradation of the phenolic fraction. Fig. 4 shows significant difference in laccase activity among the four strains studied of *L. edodes*. The onset of laccase occurred after 5 days of incubation, while the maximum enzyme activity was reached by the strain Le119 after 15 days (1.6 ± 0.21 UI ml^{-1}). The strain Le118 produced low and steady titer of laccase (less than 0.2 UI ml^{-1}). The strain Le122 exhibited the maximum enzyme activity (0.65 UI ml^{-1}) after 15 days, while the strain Le121 towards the end of the incubation period (20–30 days).

3.6. Molecular weight distribution of OMW

The evolution of molecular weight distribution from extractable polymeric fraction of OMW during the incubation of selected strains of *L. edodes* is shown in Fig. 5. The untreated OMW showed three groups of aromatic compounds. The first peak had a molecular weight (MW) higher than 30 kDa, the second one less than 12.5 kDa, and the third one apparently less than 5.5 kDa. This clearly indicated that, after 30 days of incubation of strains, the MW distribution of polyphenol was affected. The reduction of the first fraction (MW > 30 kDa) was recorded in the strains Le119, Le121 and Le122, while the same fraction was highly produced by the strain Le118.The strains Le118, Le119, and Le121 produced lower depolymerization of high molecular weight aromatics than the strain Le122.

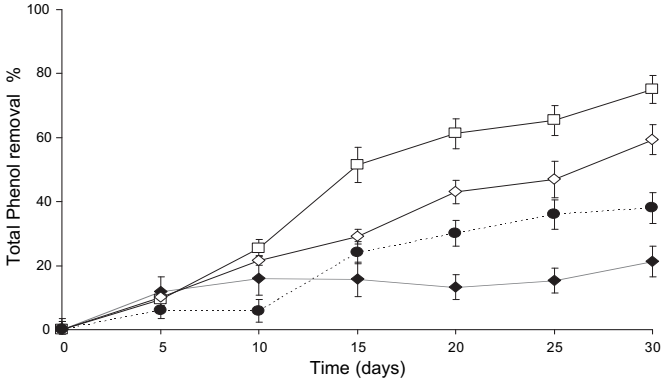

Fig. 3. Kinetics of the removal of total phenol by four strains of *Lentinula edodes* in liquid culture containing 10% of olive mill wastewater (OMW), and incubated at 25 °C. (◆) Le118, (□) Le119, (◇) Le121, and (●) Le122.

3.7. Phenolic monomer compound

Ethyl acetate OMW extracts were analyzed with reversed-phase HPLC in order to assess degradation of individual monomeric aromatic components (Table 4). Chromatographic analyses showed that the strain Le119 was capable of completely removing hydroxytyrol, caffeic acid, and *para*-coumaric acid. The removal of other phenolic compounds was of 67.12% and 89.81% for vannilic acid and tyrosol, respectively. The other three strains (Le118, Le121, Le122) completely removed at least one compound, and partially removed 22% of oleuropein (strain Le122) and 93.25% of *para*-coumaric acid (Le118). Non-phenolic compound, such as trans-cinnamic acid, was not degraded.

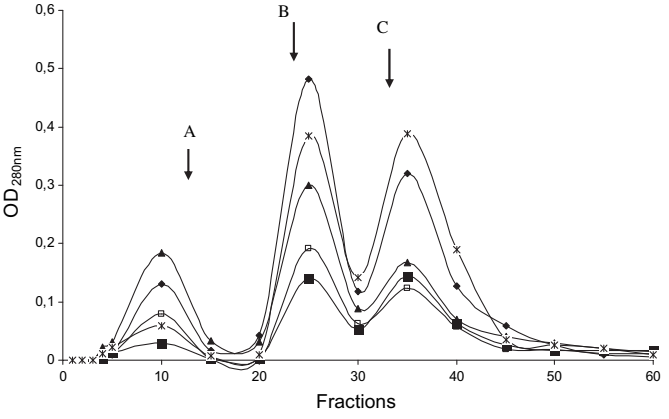

Fig. 5. Evolution of the molecular weight distribution of aromatic polymer present in (■) olive mill wastewater (OMW) without inoculation (control), and after 30 days of incubation at 25 °C with four strains of *Lentinula edodes*. (◆) Le118, (□) Le119, (◇) Le121, and (●) Le122. A: Carbonic anhydrase from bovine, 29 kDa; B: Cytochrome *C* from horse heart, 12.4 kDa; C: Aprotinin, 6.5 kDa.

Table 4
The removal (%) of monomeric aromatic compound by four strains of *Lentinula edodes* (Le118, Le119, Le121, Le122), in liquid culture of olive mill wastewater (OMW) after 30 days of incubation at 25 °C.

OMW component	Le118	Le119	Le121	Le122
3,4-Dihydroxyphenylethanol (hydroxytyrosol)	67	100	57	73.51
4-Hydroxyphenylethanol (tyrosol)	88.83	89.81	79.04	77
(+)-Catechin	73.35	68.98	100	65
Vannilic acid	0	67.12	20.29	11.97
Caffeic acid	100	100	100	100
Para-Coumaric acid	93.25	100	53.07	100
Trans-Cinnamic acid	0	0	0	0
Oleuropein	74.07	77.85	84.14	22

4. Discussion

The use of cellophane membrane allowed the growth of all strains studied of *L. edodes*, as well as the color change of OMW agar medium after incubation (PDA containing 10% and 20% OMW). The initial color was black turning to clear brown after incubation, excepting the strain Le118 which exhibited dark color at the end of colonization. This change of color is an indicator of the action of fungal enzymes on the hydrolysis of phenols during mycelial growth (Sobal et al., 2002). The presence of OMW in the culture medium affected mycelial growth of all strains. Three selected strains (Le119, Le121, Le122) showed mycelial colonies of high density, while the strain Le118 had low density. The use of PDA allowed better assessment of decolorization efficiency in all strains. In fact, it has been shown that the metabolism for decolorization requires the presence of readily available carbon source, such as glucose (D'Annibale et al., 1998). Enzymatic determination using ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)) test (Collins et al., 1998), showed that most selected strains, except Le118, were capable of producing laccase on PDA without OMW (data not shown).

Comparative assessment, based on apical growth rate and biomass yield on OMW agar medium, showed that the strains Le118, Le119, Le121 and Le122 were the most efficient, and accordingly selected for further evaluation of decolorization and the removal of total phenol from OMW. The inhibitory effects of OMW on fungi have already been reported (Fountoulakis et al., 2002; Alaoui et al., 2008). Considering this fact, dilutions of OMW were prepared to allow mycelial growth of *L. edodes*. In this study, the use of mycelium previously grown on PDA containing 2% of OMW resulted in enhanced tolerance of strains to inhibitory compounds from liquid OMW. This positive effect of pre-adaptation to OMW, previously reported for several white-rot fungi (Leontievsky et al., 2002; D'Annibale et al., 2004), has been attributed to the triggering effect of phenolic compounds for inducing the production of ligninolytic enzymes (Fenice et al., 2003; Farnet et al., 2004).

Table 5
Comparative analysis of parameters studied in four strains of *Lentinula edodes* (Le118, Le119, Le121, and Le122) tested in liquid culture of olive mill wastewater (OMW) at 25 °C.

L. edodes strains	Decolorization (%) 30th day	Total phenol removal (%) 30th day	Laccase activity 15th day (UI ml^{-1})	Removal of monomeric phenols (%)[a]
Le119	65	75	1.50	75
Le121	50	59	0.69	61
Le122	42	38	0.65	56
Le118	–	21	0.12	62

[a] Average of degradation of the eight monomeric compounds tested.

Laccase was detected as the main lignin-degrading enzyme in strains studied, which was similar to previous reports (D'Annibale et al., 2004; Olivieri et al., 2006). It has also been reported that LiP and MnP are capable of decolorizing and removing phenolics from OMW (Sayadi and Ellouz, 1993, 1995). The molecular weight of phenolic compound from OMW was modified by all strains at different levels. The strains Le119 and Le121 were able to reduce all fractions obtained from OMW extract. This was in agreement with previous results from Jaouani et al. (2003) and D'Annibale et al. (2004). However, Casa et al. (2003) reported the appearance of a novel phenolic fraction in OMW after treatment with laccase.

The difference of distribution patterns from aromatic compounds was also observed in OMW incubated with the fungus *Phanerochaete chrysosporium* in liquid culture, which was associated to the production of LiP, and with purified LiP (Sayadi and Ellouz, 1993, 1995). This interpretation is based on the assumption that other auxiliary degradative mechanisms involving enzymes and small molecular weight agents, such as hydrogen peroxide and oxalic acid (D'Annibale et al., 1998), are implicated in the breakdown of aromatic substances, which could serve as substrates brought about by white-rot fungi (Durán and Esposito, 2000). The fact that low molecular weight aromatics remained could be explained by the limitation of incubation time or the enzymatic system (Sayadi and Ellouz, 1993).

In relation to the effect of mycelial culture on OMW phenolic compounds, the strain Le119 was found to be more efficient than the other strains for completely removing hydroxytyrosol, caffeic acid and *para*-coumaric acid. The monomeric phenol compounds have been shown to be particularly phytotoxic, and this toxicity increases with the synergistic effect of monomers (Boukhoubza et al., 2007). Therefore, Isidori et al. (2005) showed that the most toxic compounds were catechol (EC50s ranging from 0.40 mmol l^{-1} for *Sorghum bicolor* to 1.09 mmol l^{-1} for *Cucumis sativus*) and hydroxytyrosol (EC50s ranging from 0.47 mmol l^{-1} for *S. bicolor* to 1.55 mmol l^{-1} for *C. sativus*). The non-degradation of trans-cinnamic could be due to the lack of appropriate substituent in the aromatic ring to enable laccase action (D'Annibale et al., 2004).

Considering all screening parameters studied (Table 5), the strain Le119 of *L. edodes* showed the highest performance for treatment of OMW. Accordingly, it has been selected for further experimental work in solid state cultures, using a mixture of olive biomass, including olive tree wood and olive cake, as substrate, where OMW acts as the wetting/moistening agent.

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