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AQUEOUS EXTRACT FROM DRY OLIVE MILL RESIDUE AS A POSSIBLE BASAL MEDIUM FOR LACCASE PRODUCTION

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Abstract

Due to its very low moisture content, the solid waste from the two-phase olive oil extraction process (dry olive mill residue, DOR) can be easily stored and used as a readily available basal liquid culture medium *via* its reconstitution with water. Five white-rot fungi (namely *Phanerochaete chrysosporium* NRRL 6361*, Lentinus tigrinus* CBS 577.79*, Pleurotus pulmonarius* CBS 664.97, *Phlebia radiata* DABAC 9 and *Botryosphaeria rhodina* DABAC P82), previously selected in a screening program, were compared for their ability to grow and to produce laccase on non-supplemented aqueous extracts of the dry olive mill residue (ADOR). The most effective strains, namely *L. tigrinus* and *P. radiata*, grown in a 3-l bubble-column reactor on 25% (w/v) ADOR, produced 6602 and 6001 nkatal l^{-1} with respective mean volumetric productivities (MVP) of 68 and 83 nkatal l^{-1} h^{-1} and production yields of 375 and 418 nkatal g^{-1} COD consumed, respectively. These results show that non-supplemented ADOR might constitute the basis of a growth medium allowing promising productions of laccase amenable to further enzyme optimization.

Key words: aqueous extract, dry olive mill residue, laccase, submerged fermentations, white-rot fungi

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1. Introduction

The olive oil extraction industry is greatly increasing its economic and social importance worldwide and, particularly, in the Mediterranean and Middle East countries. Unfortunately, however, it generates large quantities of organic wastes and by-products that cause serious environmental problems mainly due to their strictly temporal production and accumulation and/or incorrect disposal (Morillo et al., 2009; Roig et al., 2006).

The recent new continuous extraction technology, namely two-phase process, has been introduced in many olive-oil producing countries: astonishing is the case of Spain, the biggest olive oil world producer, where it has been adopted by more than 90% of the olive oil factories (Morillo et al., 2009; Roig et al., 2006). The process generates a liquid phase (olive oil) and a water-rich solid organic waste ("alpeorujo"), the latter of which is generally dried and extracted with *n*-hexane to recover residual oil thus yielding a solid waste referred to as dry olive-mill residue (DOR) (Morillo et al., 2009). It has been calculated that the annual production of DOR in Spain amounts to about four million tons (Aranda et al., 2007).

The agronomic use of DOR as an organic fertilizer has been proposed due to its favorable C/N ratio (Alburquerque et al., 2007); however, the presence in this waste of compounds, such as polyphenols, polyalcohols and volatile fatty acids, may exert antimicrobial and phytotoxic action

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(Linares et al., 2003). Consequently, adequate pretreatments prior to its application onto the soil are needed (Alburquerque et al., 2007; Sampedro et al., 2009a). Among them, DOR extraction with water has been shown to be a successful approach leading to a significantly detoxified residual solid waste and an aqueous phase referred to as ADOR (Aranda et al., 2009; Sampedro et al., 2009b). Although a significant fraction of toxic compounds, phenols in particular, is transferred to ADOR upon water extraction (Sampedro et al., 2009b), the concomitant presence of simple sugars, oligosaccharides, organic acids, polyalcohols and inorganic cations makes it suitable as a candidate liquid growth medium for the microbial production of added value commodities, such as extracellular polysaccharides (Morillo et al., 2006, 2009). Unlike other olive-mill wastes (*i.e.*, olive-mill wastewater and alpeorujo), the low moisture content of DOR allows its mid- and long-term storage and thus its utilization/extraction may occur after several months from its production (Morillo et al., 2006); in addition, the low fat content in ADOR should not lead to undesirable effects such as clogging of inlet/outlet lines and foam formation at the reactor level. Like other olive-mill wastes, conversely, ADOR exhibits significant contents of phenols which can act as either stimulators or inducers of laccase (E.C. 1.10.3.2, *para*-diphenol:oxygen oxidoreductase) (Chakroun et al., 2009; Fenice et al., 2003; Pérez et al., 1998), enzyme of commercial interest especially in textile applications (Arora and Sharma, 2010; Conesa et al., 2002; Duran and Esposito, 2000).

Within the frame of a previous screening program conducted on large number of fungal strains and based on the assessment of their capability to grow on agar media containing ADOR as the sole growth substrate and to decolorize the anthraquinone-based dye Poly-R 478, four white-rot basiomycetes (*i.e.*, *Phanerochaete chrysosporium* NRRL 6361, *Lentinus tigrinus* CBS 577.79, *Pleurotus pulmonarius* CBS 664.97 and *Phlebia radiata* DABAC 9) and one ascomycete (*i.e.*, *Botryosphaeria rhodina* DABAC P82) were selected (Sampedro, 2008).

Objectives of the present study were: (i) to comparatively assess the ability of these promising strains to grow and to produce extracellular laccase on non-supplemented ADOR under shaken flask conditions and (ii) to perform a preliminary assessment of the feasibility of the process scale transfer at the bench-top lab-scale reactor. To the best of our knowledge, this is the first report aimed to assess the use of non-supplemented ADOR as a possible growth medium for laccase production.

2. Materials and methods

2.1. Materials and microorganisms

DOR, the main physico-chemical characteristics of which were reported elsewhere (Sampedro et al., 2009a), was withdrawn from an olive oil manufacturer (Sierra Sur S.A., Granada, Spain) and stored at –20 °C until used. To obtain ADOR, DOR underwent aqueous extraction for 8 h under orbital shaking (170 rpm) using either 1:4 or 1:8 solid/liquid ratios and subsequent vacuum filtration through Whatman n. 41 filter paper. The recovered water extracts were designated as 25 and 12.5% ADOR, respectively, and stored at -20 °C until used. Their respective compositions were (g l^{-1}): chemical oxygen demand (COD), 45.1±1.0 and 23.0±0.5; total sugars, 7.9±0.6 and 4.0±0.3; total lipids, 0.06±0.01 0.13±0.01; total phenols, 6.1±0.4 and 3.2±0.1; total nitrogen, 0.52±0.02 and 0.31±0.02; their copper and manganese contents were 7.9±0.2 and 4.1±0.1 mg l^{-1}, respectively, and 4.6±0.1 and 2.5±0.0 mg l^{-1}, respectively. The initial color of 25% and 12.5% ADOR amounted to 28600 and 14500 colorimetric units per liter, respectively, while initial pH in both media was 4.48±0.01.

The following fungi were used: *P. chrysosporium* NRRL 6361, *L. tigrinus* CBS 577.79, *P. pulmonarius* CBS 664.97, *B. rhodina* DABAC P82 and *Phlebia radiata* DABAC 9 (formerly *Phlebia* sp.) (D'Annibale et al., 2006b). Taxonomic assignment of the latter strain at the species level was done on the basis of both comparison of its 18S ribosomal RNA gene partial sequence (AY943950) with other available sequences (AY946267 and AF026606) and microscopic features. The strains were maintained and routinely sub-cultured on potato dextrose agar (PDA) slants.

2.2. Inoculum preparation

Ten-d-old PDA slant cultures were suspended in 5 mL of sterile deionized water and used as the inoculum for pre-cultures carried out on potato dextrose broth at 28°C under orbital shaking (180 rpm). After 96 h incubation, the pre-cultures were centrifuged (4,000 g for 10 min) and the pellet washed with sterile deionized water.

The mycelium was homogenized (two subsequent steps of 30 s each, at 7,000 rpm) by Ultra-Turrax (IKA Labortechnik, Staufen, Germany) and added with deionized water to yield a biomass concentration of 5 g l^{-1} which was then used as the inoculum.

2.3. Culture conditions

2.3.1. Shake flask experiments

Non-supplemented ADOR (95 mL of either 12.5 or 25%) was added to 500-mL Erlenmeyer flasks and sterilized in autoclave at 121 °C for 20 min. After cooling, each flask was aseptically added with the inoculum (5.0 mL) and then incubated in an orbital shaker (180 rpm) at 28 °C for 16 d. Samples were withdrawn daily, centrifuged (3800 g for 10 min) and the supernatants used for all tests. All experiments were performed in triplicate.

2.3.2. Reactor experiments

At the bench-top level, experiments were conducted in a 3-liter bubble-column reactor (BCR) filled with 2 l of ADOR 25%. The following probes were installed on the column top: dissolved oxygen sensor (Ingold, CH), double reference pH sensor (Phoenix, AZ) and PT 100 temperature sensor. Experiments were performed under the following conditions: inoculum amount 5% (v/v); aeration rate 0.4 vvm; silicon anti-foam, 1 mL L^{-1}; temperature 28°C. Fermentation parameters were monitored by an adaptative/PID digital controller, ADI 1030 (Applikon Dependable Instruments, Schiedam, NL). Sampling was as above. Each condition was tested in duplicate.

2.4. Enzyme assays, analytical and phytotoxicity determinations

Laccase activity was spectrophotometrically determined using 2,6-dimethoxyphenol as the substrate as reported by Fenice et al. (2003). Enzyme activity was expressed as nkatal L^{-1} defined as the amount of enzyme required to oxidize 1 nmol of substrate s^{-1} under the assay conditions. Biomass was gravimetrically determined as previously reported (Fenice et al., 2003).

Extracellular protein was determined by the dye-binding method after trichloroacetic acid precipitation and using bovine serum albumin as the standard (Quaratino et al., 2008). Total phenols (TP) contents in ADOR were determined according to Linares et al. (2003), using syringic acid as the standard. COD, total sugars and nitrogen and color were determined as previously described (D'Annibale et al., 2006a). Phytotoxicity of ADOR culture supernatants towards water-cress (*Lepidium sativum* L.) and lettuce (*Lactuca sativa* L.) was determined as previously reported (D'Annibale et al., 2006b).

2.5. Calculation of kinetic parameters and yields

Biomass ($Y_{x/s}$) and product ($Y_{p/s}$) yields were determined by relating fungal biomass and enzyme activity, respectively, to the extent of COD consumed at the time of their respective maxima. COD consumption and phenol removal rates (CCR and PRR, respectively) were determined by relating their maximal amounts removed to the relative time interval and expressed in terms of mg L^{-1} h^{-1}.

The dimensionless parameter dephenolization selectivity (DS) was calculated by the ratio between maximal amounts of TP removed and COD consumed at that time. Mean volumetric productivity (MVP) of laccase was calculated by relating the activity peak to the respective time interval and was expressed in terms of nkatal L^{-1} h^{-1}.

3. Results and discussion

3.1. Biomass production and growth medium modification

All fungi were able to actively grow on non-supplemented ADOR with a lag phase shorter than 24 h (data not shown). In particular, the amount of fungal biomass produced by the majority of strains did not significantly vary as the ADOR concentration passed from 12.5 to 25.0% with the exceptions of *B. rhodina* and *P. chrysosporium* (Table 1).

Multiple pair-wise comparisons between fungi grown on 25% ADOR, showed that the lowest biomass values were observed in *P. pulmonarius* cultures (Table 1). Marked inhibitory effects on fungal growth were expected in 25% ADOR, the initial TP content of which was significantly higher than in 12.5% (6.1 *vs.* 3.2 g l^{-1} , respectively) (Table 1). Phenols, in fact, are known to exert concentration-dependent toxicity towards fungi (Buswell et al., 1994) and species-specific threshold levels lower than 6.1 g l^{-1} have been suggested for TP in olive-mill wastes (Fenice et al., 2003).

However, the putative inhibitory effect of phenols, due to the increased ADOR concentration, might have been counterbalanced by the enhanced contents of C and N sources (see ADOR composition in Materials and Methods section). In addition, in olive-mill wastewater (OMW), TP concentrations as high as 6 g l^{-1} are generally associated with organic loads significantly higher than that found in 25% ADOR (*i.e.*, 45 g l^{-1}) (Niaounakis and Halvadakis, 2004). With this regard, several screening studies on fungal strains have clearly shown that only few species are able to withstand COD higher than 50 g l^{-1} in olive-mill wastes (Sayadi and Ellouz, 1993; Jaouani et al., 2003).

To determine the efficiency of the strains under study to use organic components in the tested media, COD consumption rates (CCR) were comparatively determined for each strain. Table 1 shows that the CCRs of *B. rhodina* and *P. chrysosporium* cultures were higher on 25% than 12.5% ADOR; the respective biomass yields ($Y_{x/s}$) were also found to increase. By contrast, the CCR in *P. pulmonarius* culture decreased as the ADOR concentration increased, albeit the respective $Y_{x/s}$ value was not significantly affected.

The fate of phenols is intrinsically associated with the release of laccase activity, known to be able to bring about their oxidation, and target of this upgrading study. Thus, phenol removal rate (PRR) and delignification selectivity (DS) were also determined (Table 1). Interestingly, dephenolization was invariably higher than 80% with all the strains under study, regardless of ADOR concentration, with the only exception of *P. pulmonarius* (63.6 %). Multiple pair-wise comparison between fungi grown on 25% ADOR in terms of both PRR and DS showed that the most efficient strains were *L. tigrinus* and *P. radiata* (Table 1).

3.2. Laccase production on non-supplemented ADOR

With the only exception of *P. ostreatus*, higher levels of fungal laccase activity were always

observed on 25% ADOR than on 12.5%. In the former medium, laccase production peaks were reached in *L. tigrinus* and *P. radiata* cultures (5384±133 and 7818±517 nkatal l^{-1}, respectively) (Table 2) after 8 and 4 d, respectively. *P. radiata*, in particular, gave rise to the highest values of both laccase mean volumetric productivity (MVP) and product yield ($Y_{p/s}$) on 25% ADOR (81.5 nkatal l^{-1} h^{-1} and 2030 nkatal g^{-1}, respectively) (Table 2).

The ubiquitous presence of laccase activity in all fungal cultures on ADOR was somehow expected since the strains under study had been previously selected on the basis of their Poly R-478 decolorization ability (Sampedro, 2008), a process involving the action of lignin-modifying enzymes (Levin et al., 2004). In addition, laccase is widely known to be either boosted or induced by the presence of phenols, some of which are present in olive-mill wastes (Pérez et al., 1998) or aromatic compounds (Arora and Gill, 2001). With regard to the stimulatory action of OMW phenols on laccase, the addition of this wastewater (5.0%, v/v) or the corresponding amount of its ethyl acetate-extractable TP to the growth medium led to a 10-fold increase in *L. tigrinus* laccase production with respect to the control (Quaratino, 2005). To explain the stimulatory action of phenolic compounds on laccase production, it has been suggested that the enzyme might act as a detoxifying agent able to convert either toxic monomers (Lundell et al., 1990) or degradation intermediates (Yeo et al., 2008) into polymerized products unable to penetrate into the cells.

The same detoxifying response was invoked to explain the increased production of ligninolytic enzymes in *Phlebia* sp. cultures (Rogalski et al., 1991).

3.3. Production of laccase enzyme activities in bubble-column reactor (BCR)

L. tigrinus CBS 577.79 and *P. radiata* DABAC 9 were shown to be the most effective strains in terms of both efficiency of medium utilization and laccase production. Thus, to gain preliminary indications on the feasibility of process transfer, the two fungi were cultivated in a 3-liter BCR. In fact, air-lift bioreactors generally associate higher gas exchange and mass-transfer with less mechanical stress of the microbial culture in comparison with the mechanically-agitated ones (Schugerl, 1985). With this regard, this pneumatically agitated system proved to be more appropriate than a stirred tank reactor (STR) for lignin-modifying enzymes production by *L. tigrinus* on OMW (D'Annibale et al., 2006a; Fenice et al., 2003). Also in the case of other white-rot fungi, the pneumatic agitation favored laccase production due to lower shear stress than STR (Rancano et al., 2003; Rodriguez-Couto and Toca-Herrera, 2007).

Figs. 1 and 2 show the time courses of growth and laccase production by *L. tigrinus* and *P. radiata*, respectively, cultured on 25% ADOR in BCR.

L. tigrinus reached the maximal growth (10.9 g l^{-1} of biomass) after 6 d leading to a rapid decline of both COD and TP contents. At the end of the process, both parameters were reduced by 56.8 and 91.9%, respectively (Fig. 1).

L. tigrinus laccase activity peak increased markedly passing from shaken flasks to the bioreactor scale (5384±133 *vs.* 6602±200 nkatal l^{-1}) (Fig. 1) and the time required for its attainment in BCR was halved as compared to shaken flasks, thus increasing the average volumetric productivity (Table 3). A second significantly higher laccase activity peak (8768 ±150 nkatal L^{-1}) was observed in BCR after 10 d and the respective productivity was higher than in shaken flasks (36.7±1.7 *vs.* 28.0±0.6 nkatal l^{-1} h^{-1}) (Table 3). With regard to the physiological regulation of laccase production in *L. tigrinus*, the attainment of high activity levels has been shown to require either nitrogen-sufficient or nitrogen-rich conditions (Chernykh et al., 2005; Quaratino et al., 2008); the combined use of two inducers, namely 2,4-dimethylphenol and $CuSO_4$, further boosted laccase production by this fungus (Chernykh et al., 2005). These conditions are far from those encountered in 25% ADOR which was deliberately used in this first study without either nutritional supplements or inducer addition. As a consequence, laccase volumetric activities and related productivities and yields were lower than those reported with the same strain grown on either chemically defined media (Quaratino et al., 2007, 2008) or on olive mill wastewater supplemented with both sucrose and yeast extract (Fenice et al., 2003) (Table 3). Similarly, laccase production levels by another *L. tigrinus* strain, namely 8/18, were higher than those obtained in the present study although either induction with copper (Chernyk et al., 2005) or mycelium immobilization (Leontievsky et al., 1994) were required to boost enzyme production (Table 3).

It is worth noting that 25% ADOR was highly conducive to biomass production as compared to other media (Quaratino et al., 2007, 2008), thus suggesting that laccase production is not a tightly growth-associated event in *L. tigrinus*.

With regard to *P. radiata*, the rapid biomass growth in BCR (8.1 g l^{-1} after 6 d incubation) was associated with a quick COD consumption and phenol removal (Fig. 2). Although the time required to attain the laccase activity peak in *P. radiata* cultures in BCR was shortened with respect to shaken flasks (Fig. 2), a very steep decline in activity was observed thereafter. In this respect, both the maximal activity levels and the time course of enzyme production were similar to those reported for *P. radiata* ATCC 64658 on N-limited media (Lundell et al., 1990).

In the present study, production levels and average laccase productivity by *P. radiata* DABAC 9 were significantly higher than those observed with the same strain that had been grown on a rich medium, such as potato dextrose broth (D'Annibale et al., 2006b) (Table 3).

Table 1. Biomass production, biomass yield ($Y_{x/s}$), COD removal rate (CRR), phenol removal rate (PRR) and dephenolization selectivity (DS) in fungal cultures conducted on liquid media containing either 12.5 or 25% (w/v) of non-supplemented ADOR

Fungus	*Biomass (g l^{-1})*		*$Y_{x/s}$ (g g^{-1})*		*CRR (mg l^{-1} h^{-1})*		*PRR (mg l^{-1} h^{-1})*		*DS*	
	12.5%	*25%*	*12.5%*	*25%*	*12.5%*	*25%*	*12.5%*	*25%*	*12.5%*	*25%*
B. rhodina	4.7±0.6bA	9.7±1.1dB	0.38±0.03aA	0.76±0.09bB	42.8±0.2aA	66.3±8.5cB	9.9±0.1aA	26.0±3.5cB	0.23±0.01aA	0.39±0.05abB
L. tigrinus	4.7±0.8bA	5.1±0.6cA	0.63±0.09abA	0.78±0.06bB	80.3±6.8bA	71.5±1.1cA	31.8±1.0cA	56.8±1.9dB	0.36±0.01bA	0.80±0.03dB
P. chrysosporium	3.5±0.9abA	10.3±1.0dB	0.40±0.08aA	0.57±0.04abB	45.5±6.4aA	63.1±1.7cB	15.5±1.0bA	19.6±0.2bB	0.34±0.04bA	0.31±0.01aA
P. pulmonarius	2.2±0.4aA	1.8±0.2aA	0.39±0.06aA	0.47±0.09aA	29.4±3.7aB	13.4±3.9aA	15.3±0.7bB	7.8±0.2aA	0.52±0.05cA	0.56±0.01cA
P. radiata	3.1±0.5abA	2.9±0.4abA	0.65±0.11bA	0.76±0.16bA	47.9±6.8aA	42.0±4.4bA	31.2±1.3cA	29.2±1.5cA	0.62±0.05dA	0.70±0.06dA

Data are the mean of three experiments. Means were subjected to multiple pair-wise comparisons by the Tukey test ($P \leq 0.05$): same lowercase and uppercase letters denote absence of statistical significance between strains grown on the same ADOR concentration (column means) and within the same strain grown on different ADOR concentrations, respectively

Table 2. Laccase productions, specific activities, yields ($Y_{p/s}$) and mean volumetric productivities (MVP) in fungal cultures conducted on liquid media containing either 12.5 or 25% (w/v) of non-supplemented ADOR

Fungus	*Volumetric activity (nkatal l^{-1})*		*Specific activity (nkatal mg^{-1} protein)*		*$Y_{p/s}$ (nkatal g^{-1})*		*MVP (nkatal l^{-1} h^{-1})*	
	12.5%	**25%**	**12.5%**	**25%**	**12.5%**	**25%**	**12.5%**	**25%**
B. rhodina	150±1aA	150±2aA	7±0aB	2±0aA	12±0aA	12±0aA	1.5±0.0abA	1.5±0.0aA
L. tigrinus	2117±33cA	5384±133bB	108±1cA	123±3cB	1268±15dA	792±15bB	11.0±0.1cA	28.0±0.6bB
P. chrysosporium	117±0aA	150±1aB	7±0aB	3±0aA	13±1aB	17±0aA	1.2±0.0aA	1.5±0.0aB
P. pulmonarius	1117±133bB	150±2aA	38±5bB	5±0aA	198±25bB	38±4aA	2.8±0.3bB	1.5±0.0aA
P. radiata	1900±33cA	7818±517cB	47±0bA	98±6bB	407±56cA	2030±245cB	19.8±0.3dA	81.5±5.3cB

Data are the mean of three experiments. Means were subjected to multiple pair-wise comparisons by the Tukey test ($P \leq 0.05$): same lowercase and uppercase letters denote absence of statistical significance between strains grown on the same ADOR concentration (column means) and within the same strain grown on different ADOR concentrations, respectively

Table 3. Laccase volumetric activities (VA), mean volumetric productivities (MVP), specific activities and product yields ($Y_{p/s}$,) in *L. tigrinus* CBS 577.79 and *P. radiata* DABAC 9 cultures grown on 25% ADOR in bubble-column reactor. For sake of comparison, other production data obtained with either the same or other strains belonging to the same species are reported.

Fungus	*Laccase*					*Notes*	*References*
	VA (nkatal l^{-1})	*MVP[a] (nkatal l^{-1} h^{-1})*	*Specific activity (nkatal g^{-1} biomass)*	*Specific activity (nkatal mg^{-1} protein)*	*$Y_{p/s}$ (nkatal g^{-1})*		
L. tigrinus CBS 577.79	6602 8768	68.3 [4] 36.7 [10]	600 781	45 128	375 320	ADOR[e]/BCR[f]	Present study
L. tigrinus CBS 577.79	28506	118.8 [10]	25914	323	2741	CDM[g]/BCR	Quaratino et al., 2007
L. tigrinus CBS 577.79	36674	169.8 [9]	33340	445.1	3595	CDM/STR[h]	Quaratino et al., 2008
L. tigrinus CBS 577.79	76682	245.8 [13]	30672	n.a.[d]	n.a.[d]	OMW[i]/STR	Fenice et al., 2003
L. tigrinus 8/18[b]	18000	92.5 [12]	n.a.[d]	n.a.	n.a.	CDM/STR	Leontievsky et al., 1994
L. tigrinus 8/18	11002	28.7 [16]	n.a.	n.a.	n.a.	CDM/SH[l]	Chernykh et al., 2005
P. radiata. DABAC9	6001	83.3 [3]	983	52	418	ADOR/BCR	Present study
P. radiata. DABAC9	1820	10.8 [7]	n.a.	n.a.	n.a.	CDM/SH	D'Annibale et al., 2006b
P. radiata ATCC 64658	6000	83.3 [3]	n.a.	1714	n.a.	CDM/BCR	Kantelinen et al., 1989
P. radiata ATCC 64658[c]	16000	22.2 [3]	n.a.	n.a.	n.a.	CDM/BCR	Kantelinen et al., 1989
P. radiata ATCC 64658[c]	13000	n.a.[d]	n.a.	n.a.	n.a.	CDM/BCR	Gayazov and Rodakiewicz-Nowak, 1996

[a]Data between square bracket express the time (days) required to attain the activity peak; [b]mycelium immobilized on polycaproamide fibers; [c]mycelium immobilized on polypropylene and grown under semi-continuous cultivation conditions; [d]n.a., not available; [e]ADOR, aqueous dry olive; [f]BCR: bubble column reactor; [g]CDM: chemically defined medium; [h]STR: stirred tank reactor; [i]OMW, olive mill wastewater; [l]SH: shaken flasks.

In the present study, although laccase production in *L. tigrinus* cultures was higher than that of *P. radiata*, the MVP of the latter was significantly higher (83.3±2.0 *vs*. 68.3±1.7 nkatal l^{-1} h^{-1}, respectively) (Table 3). Similarly, both specific activity and $Y_{p/s}$ value clearly suggested that *P. radiata* was a more efficient laccase producer than *L. tigrinus*.

However, the steep activity decline observed after the attainment of the enzyme peak in *P. radiata* cultures requires an accurate definition of the process shut-down and explains why semi-continuous approaches have been used with this species to optimize the process (Gayazov and Rodakiewicz-Nowak, 1996; Kantelinen et al., 1989;).

3.4. Residual phytotoxicity in BCR culture supernatants

Assessment of residual toxicity of a spent growth medium is among the feasibility criteria for a given bioprocess. Thus, the time-dependent evolution of residual phytoxicity of culture supernatants from BCR was determined; olive-mill wastes, in fact, are known to exert noxious effects on higher plants (Aranda et al., 2007; Roig et al., 2006;). With this regard, Fig. 3 shows that the culture supernatants from both fungal cultures grown in BCR on 25% ADOR were significantly detoxified but the detoxification level with *P. radiata* was higher and was obtained in a shorter time.

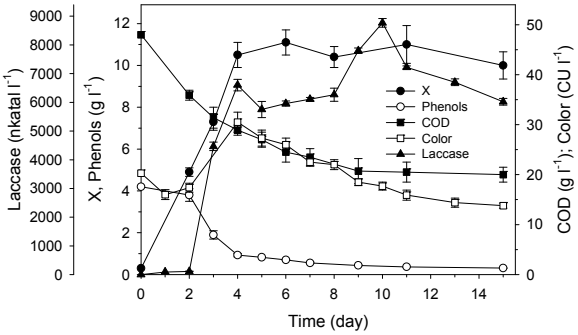

Fig. 1. Time course of growth (X) and laccase production by *L. tigrinus* CBS 577.79 cultured on 25% ADOR in a 3-l bubble-column reactor and the corresponding evolution of COD, total phenols and color removal. Values are the means of two independent experiments and error bars indicate standard deviations

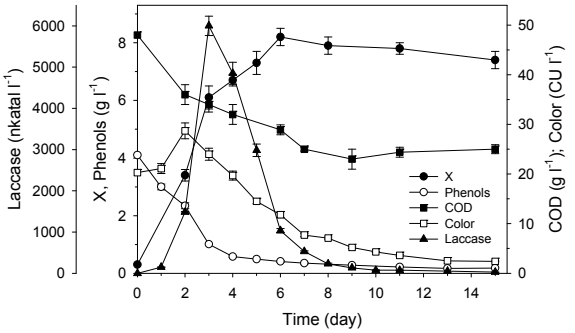

Fig. 2. Time course of growth (X) and laccase production by *P. radiata* DABAC 9 cultured on 25% ADOR in a 3-l bubble-column reactor and the corresponding evolution of COD, total phenols and color removal. Values are the means of two independent experiments and error bars indicate standard deviations

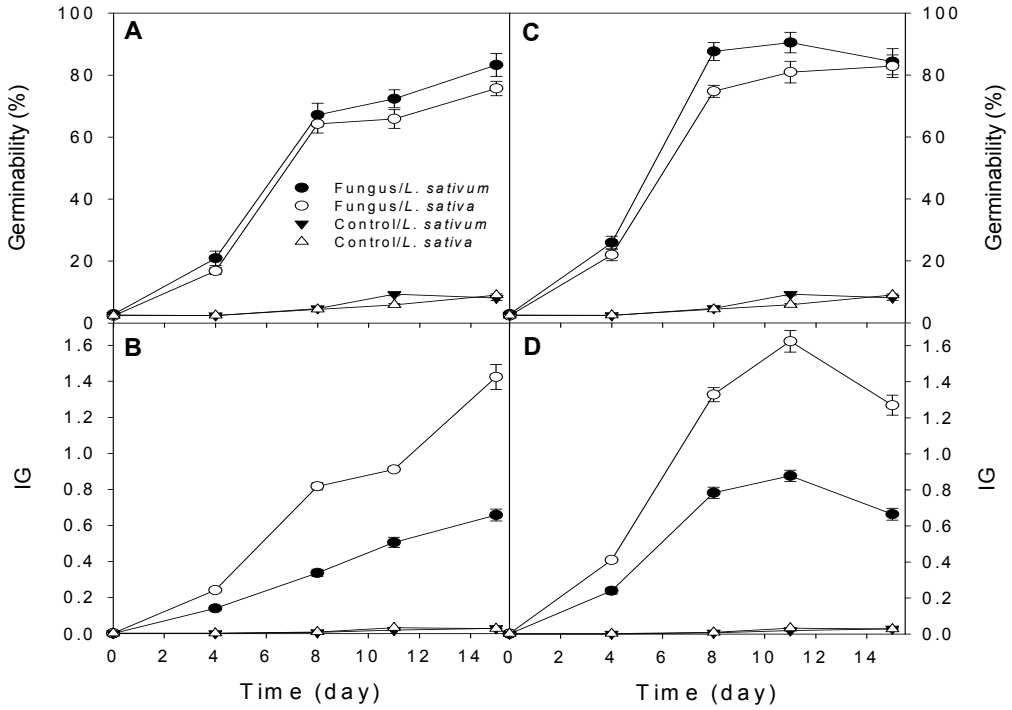

Fig. 3. Time course of percent germinability and index of germination (IG) in *L. tigrinus* CBC 577.79 (A and C, respectively) and *P. radiata* DABAC 9 (B and D, respectively) culture supernatants derived from 3-l bubble-column reactor experiments on 25% ADOR at 28 °C. Values are the means of four replicates and error bars indicate standard deviations

These results suggest the possibility of using the discarded fractions of ADOR after the earliest downstream processing steps (*e.g.*, filtration, centrifugation and ultrafiltration), in order to recover the enzyme, for agronomic purposes (Aranda et al., 2009).

4. Conclusions

One of the main bottlenecks of the possible valorization of agro-industrial wastes, including olive mill wastes, is their seasonality. In this context, DOR represents an exception since its low moisture content allows mid- and long-term storage, good standardization through mixing of different batches, continuous supply over time and possible ready reconstitution with water to obtain ADOR.

This study demonstrates the suitability of ADOR as a basal medium for laccase production by both *L. tigrinus* and *P. radiata*, used as model fungi. In fact, enzyme productions can be deemed to be promising taking into account that, at this phase, ADOR was non-supplemented and the culture conditions non-optimized. Moreover, the productivities and yields obtained at the lab-scale bioreactor, higher than in shaken flasks, provide a first positive indication regarding the possible scale-up of the process. Hence, wide perspectives of production improvements appear realistic with further scaling up studies.

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