

## Relationship between cultivation mode of white rot fungi and their efficiency for olive oil mill wastewaters treatment

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**Abbreviations:** COD: chemical oxygen demand  
NT: total nitrogen  
OOMW: olive oil mill wastewaters  
TDM: total dry matter  
TSS: total suspended solids  
VM: volatile matter  
WRF: white rot fungi

Four white rot fungi (WRF) strains, *Phanerochaete chrysosporium*, *Trametes versicolor*, *Coriolopsis polyzona* and *Pycnoporus coccineus*, were tested for efficiency of treatment of Olive Oil mill wastewaters (OOMW) in relation with their cultivation mode, *i.e.* under the form of free mycelium, mycelium immobilized in alginate beads and solid state cultivation on Petri dishes. Study of biodegradation of phenolic compounds, chemical oxygen demand (COD) decrease and decolourisation of OOMW have shown that *Coriolopsis polyzona* and *Pycnoporus coccineus* degradation performances were

apparently only slightly affected by the cell cultivation procedures experienced here. In contrast, *Phanerochaete chrysosporium* and *Trametes versicolor* showed respectively marked preferences for solid state and alginate immobilisation procedures. Both mono and polyphenolics were reduced to different extent during incubation depending on the strain, as shown by gel filtration analysis. Final pH obtained after fungal treatment of the OOMW based medium (initial pH of 5.0) was measured in order to evaluate the possibility of releasing friendly the treated wastewater in the

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**environment. Laboratory studies as reported here may be useful for orienting the choice of a strain for treating pollution by OOMW in a particular real situation.**

OOMW generates enormous environmental pollution problems all around the Mediterranean basin, in particular in North Africa. The biological cleaning of this effluent is particularly delicate due to a high concentration of phenolic compounds primarily made up of anthocyanidines (McClure, 1979), tannins (Balice et al. 1982) and lignin derivatives (Diaz, 1983).

Basidiomycetes, mainly of the WRF group, were shown to be able to mineralize lignin (Trovaslet et al. 2007) and phenolic compounds (Pointing, 2001) by using a multienzyme system including Lignin peroxidase (LiP: EC. 1.11.1.1) (Martínez, 2002), Manganese peroxidase (MnP: EC. 1.11.1.13) (Hofrichter, 2002) and Laccase (EC. 1.10.3.2) (Castro et al. 2003). A number of fungal strains, among which the WRF strain *Phanerochaete chrysosporium*, were shown to be active in the treatment of OOMW (Martínez, 2002). To undertake fungal OOMW biotreatment, several procedures were used, such as cultivation on solid and liquid medium, and cultivation under free or immobilized form (Dorado et al. 1999; D' Annibale et al. 2000; Raghukumar et al. 2004; Songulashvili et al. 2007). However, as a consequence of the use of different cultivation procedures by the different authors in the field, data received are often not fully comparable, in particular when considering performances of individual strains. This prompted us to study more

systematically the effects of cell cultivation procedures on the physiological behaviour of some strains used in the treatment OOMW.

Performances of four strains of WRF basidiomycetes previously reported to be able to degrade a broad spectrum of phenolic compounds, *Phanerochaete chrysosporium*, *Trametes versicolor*, *Corioloopsis polyzona* and *Pycnoporus coccineus* (Jaouani et al. 2003) were compared in three cultivation conditions including free mycelium in liquid culture, mycelium immobilized in alginate beads and solid state cultivation on Petri dishes.

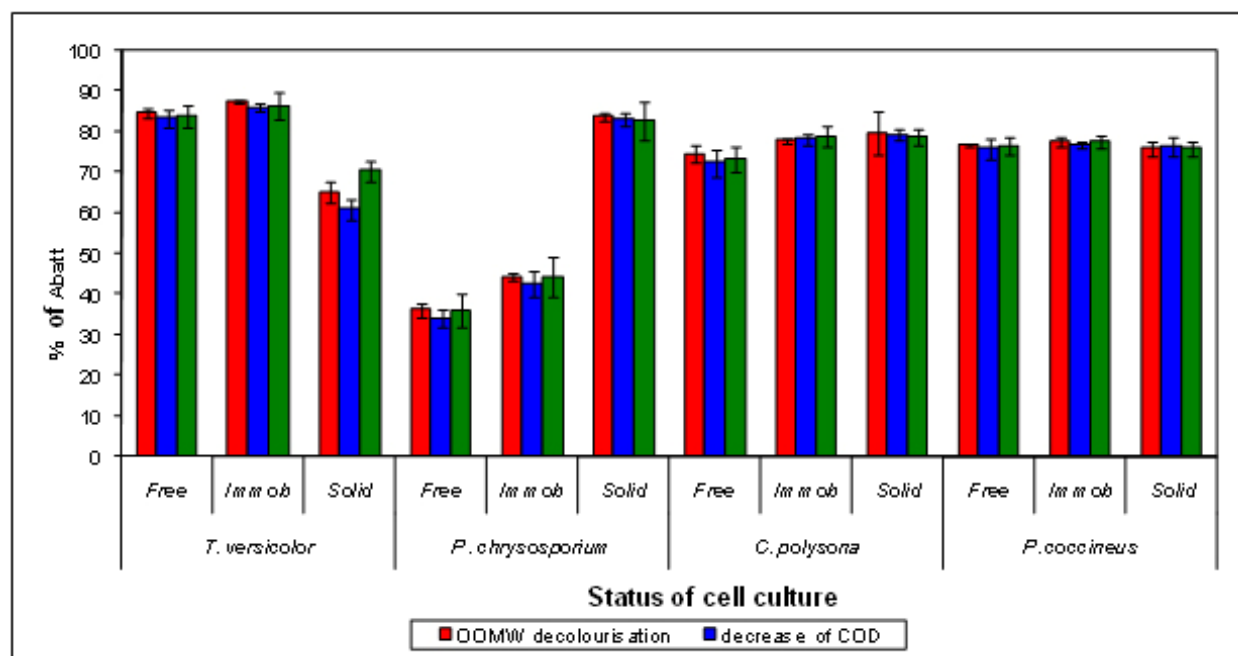
## MATERIALS AND METHODS

### Olive oil mill wastewater

OOMW was obtained from a press extraction system located in a factory close to Fez - Morocco (Table 1). The material was collected in plastic containers and stored at 4°C. Before use, OOMW was diluted with distilled water and sterilized during 15 min at 121°C.

### Strains

The four WRF strains used in this study were from the BCCM/ MUCL (Belgium Coordinated Collections of Micro-organisms / Mycothèque of the Catholic University of Louvain). Before inoculation of the tested media, *Phanerochaete chrysosporium* (MUCL 19343), *Trametes versicolor* (MUCL 30871), *Corioloopsis polyzona* (MUCL



**Figure 1. Effect of cell cultivation procedure on OOMW decolourisation, decrease of COD and phenolics.** Strains were cultivated during 15 days; Free: cultivation under free form; Im mob: immobilized form; Solid: solid state cultivation. All tests were performed in triplicate. Mean values were shown on the graph and error bars were estimated from the 3 values for each result.

38443) and *Pycnoporus coccineus* (MUCL 38527) were maintained at 4°C on Malt extract solid medium (Difco) 2% (w/v).

### Pre cultivation and preparation of the inoculum

Each strain was precultivated three days at 28°C in 25 ml of malt extract medium (20 g/l) contained in 250 ml Erlenmeyer flasks. The mycelium was recovered by centrifugation during 20 min at 6,000 g, and further washed with sterile physiological water. Vortex dispersion of the mycelium was carried out discontinuously in a sterile tube during 5 min in the presence of glass beads (diameter 0.5-0.75mm, Kolo SA, Belgium). The homogenate was centrifuged at 6,000 g for 15 min and washed twice. The recovered pellet was weighed and resuspended in 10 ml of sterile diluted OOMW to be used as inoculums for further experiments. Appropriately diluted inoculums of  $OD_{620nm} = 1.6$  were used for further experiments.

### Cultivation medium

OOMW diluted fourfold (v/v) with distilled water, and without any complementation was used as a basal cultivation medium. Initial COD and phenolics content of this medium were respectively  $26 \pm 0.4$  and  $2.6 \pm 0.2 \text{ g l}^{-1}$ . Initial pH of the medium was  $5.0 \pm 0.1$ .

### Cell immobilization in alginate beads

Cell immobilization was carried out by restriction of the cells in a calcium alginate matrix. Two ml of the dispersed mycelium ( $OD_{620nm} = 1.6$ ), were mixed with sodium alginate (Sigma) (2%), and then homogenized in a sterile syringe. The mixture was dropped in a sterile 0.1 M calcium chloride solution to form the beads containing the fungal cells. The formed beads were consolidated at 37°C during 15 min. Thereafter the alginate beads were washed with physiological water (0.9% NaCl) in order to eliminate calcium chloride traces and free cells. The immobilized cells were added to the cultivation medium. In control experiments, sodium alginate beads were prepared without the addition of living mycelium and were further subjected to the same treatment as immobilized cells.

### Cultivation in liquid medium with free and immobilized mycelium.

Twenty five ml of the cultivation medium, contained in 250 ml Erlenmeyer flask, were supplemented with 2 ml of the fungal inoculums ( $OD_{620nm} = 1.6$ ) obtained as described above. Incubation was further conducted at 28°C as described in Results. A corresponding amount of biomass was used for cultivation with alginate immobilized cells.

### Cultivation on solid OOMW based medium

OOMW diluted fourfold (v/v) with distilled water was supplemented with  $15 \text{ g l}^{-1}$  agar (Difco), and further sterilized for 15 min at 121°C. Surface inoculation at the

centre of each Petri dish containing this medium was carried out with one plug of agar sampled with a hollow-punch of 4 mm diameter at the margin of the colony of a seven days malt extract agar culture of the tested strain.

The incubation temperature was maintained at 28°C and radial growth measurements were recorded each day during 15 days. Decrease of COD, colour and phenolic compounds were measured at the end of growth after extraction of the agar bead with distilled water.

### Analytical procedures

Phenolic compounds soluble in OOMW were determined using the colorimetric procedure described in Aissam et al. (2005). Results was expressed as g of tannic acid  $\cdot \text{l}^{-1}$ .

Chemical oxygen demand (COD) evolution expressed in g of  $\text{O}_2 \cdot \text{l}^{-1}$  was estimated according to Aissam et al. (2005).

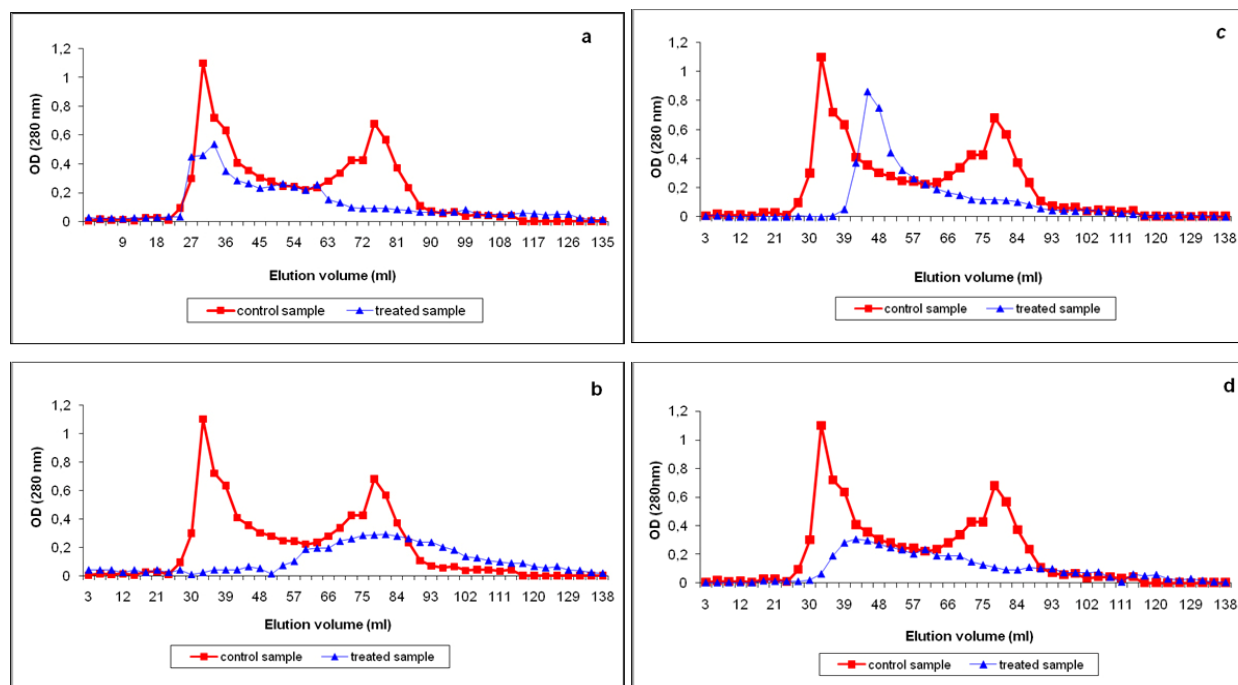
Decolourization of OOMW was evaluated by measurement of absorbance decrease at 395 nm.

Total suspended solids (TSS) in OOMW were determined by centrifugation of a 20 ml sample of the waste at 8,000 x g for 20 min. The centrifugation pellet was transferred quantitatively in a cup of porcelain before weighing and then dried at 105°C during 24 hrs. TSS was expressed in  $\text{g} \cdot \text{l}^{-1}$ .

Total dry matter (TDM) was determined after evaporation of a 10 ml OOMW sample in a porcelain cup at 105°C for 24 hrs. TDM was expressed in  $\text{g} \cdot \text{l}^{-1}$ . Volatile Matter (VM) was determined by the difference between the total dry matter obtained by evaporation at 105°C and the residue resulting from ignition at 600°C for 2 hrs. VM was expressed in  $\text{g} \cdot \text{l}^{-1}$  as compared to dry weight. Chloride content ( $\text{g} \cdot \text{l}^{-1}$ ) of OOMW was determined by the volumetric procedure of Mohr (Rodier, 1996). Total nitrogen (NT) ( $\text{g} \cdot \text{l}^{-1}$ ) was determined by the Kjeldahl procedure (Rodier, 1996). Orthophosphates ( $\text{PO}_4^{3-}$ ) ( $\text{g} \cdot \text{l}^{-1}$ ) were determined by the method of Murphy and Riley (1962).  $\text{Na}^+$  ions ( $\text{g} \cdot \text{l}^{-1}$ ) were measured with a flame spectrophotometer (type PHF 90 D; ISA Biology) in samples of OOMW filtered and appropriately diluted with distilled water. Total sugars were measured by the method of Dubois et al. (1956). For estimating total lipids, seventy five ml of chloroform and 100 ml of methanol were added to 100 ml of OOMW. After shaking and further settling, the mixture was divided into two phases. The upper methanol phase was eliminated, while the lower phase containing chloroform and soluble lipids was evaporated in a Rotavapor device. The amount of lipids recovered was expressed in  $\text{g} \cdot \text{l}^{-1}$  OOMW. All the colorimetric determinations were carried out with a UV-visible spectrophotometer (Brand Jenway 6105).

### Size exclusion chromatography

Molecular weight pattern of phenolics compounds in OOMW before and after 15 days fungal treatment was



**Figure 2. Sephadex G 50 elution pattern of phenolic compounds present in OOMW after treatment with:**  
**(a) immobilized *T. versicolor*.**  
**(b) free mycelium of *P. coccineus*.**  
**(c) solid state cultivated *P. chrysosporium*.**  
**(d) solid state cultivated *C. polyzona*.**

determined by size exclusion chromatography on a Sephadex G 50 column (2.5 cm diameter X 75 cm height) as described by Jaouani et al. (2005). The phenolic fractions were detected at 280 nm. Sterile incubated control was obtained by incubating heat killed biomass (121°C, 15 min) in the presence of the OOMW.

## RESULTS

### Effect of cultivation conditions on general parameters of OOMW fungal biotreatment

The main objective of this investigation was to delimit conditions for high and prolonged activity of WRF for treatment of OOMW, especially to decolourize the waste and eliminate phenolic compounds and other COD contributing substances (sugars, lipids). In order to clarify the metabolic trends of the studied strains, we have studied for each strain, as stated above, the effects of different cell cultivation modes on OOMW decolourisation, decrease of COD and phenolics. Figure 1 synthesizes the data obtained with the different strains cultivated under three modes. Among the four studied strains, *P. chrysosporium* was apparently the strain mainly affected by the cultivation mode. If *P. chrysosporium* was comparable to the other strains in terms of COD, phenolics decrease and

decolourisation, when cultivated under solid state form, this strain exhibited comparatively modest performances under free and alginate immobilized forms. Reduction of the different studied parameters by *T. versicolor*, *C. polyzona* and *P. coccineus* were very comparable, accounting for in most of the cases 85% and more reduction. Yet, lowest significant values (around 65 - 70% decrease) were observed for *T. versicolor* cultivated under solid state form.

### Effect of OOMW biotreatment on final pH of the effluent

Effect of OOMW biotreatment on final pH of the effluent was an interesting parameter to monitor because reduction of acidity of treated OOMW should be a desirable goal to attain before release of the waste in the environment. The pH evolution data (not illustrated) showed that except for *C. polyzona* the strains presented similar behaviour. The pH increased after treatment under solid culture condition and decreased under liquid culture conditions (free and mainly immobilized). On solid medium the pH increased in a very significant way. Initial pH before treatment was  $5.0 \pm 0.1$ . Final value obtained with the free form of *C. polyzona* was  $5.5 \pm 0.1$ , whereas was apparently only slightly affected by other types of cultivation for this particular strain. A significant pH decrease of 0.5 units was observed for *T.*

**Table1. Composition of crude OOMW used in the present investigation.** Analytical procedures used to determine the composition of crude OOMW are described in Material and Methods. All tests were performed in triplicate. The data reported corresponded to mean values with a standard error less than 7%.

pH	5.00
Matter in suspension (g.l <sup>-1</sup> )	32.00
Dry Matter (g.l <sup>-1</sup> )	100.00
Volatile matter (g.l <sup>-1</sup> )	21.00
Lipids (g.l <sup>-1</sup> )	1.00
Chemical Oxygen demand (COD) (g of O <sub>2</sub> .l <sup>-1</sup> )	106.00
Phenolic compounds (g.l <sup>-1</sup> )	12.90
Total sugars (g.l <sup>-1</sup> )	0,41
Sodium (g.l <sup>-1</sup> )	2.10
Chlorides (g.l <sup>-1</sup> )	3.20
Orthophosphates (g.l <sup>-1</sup> )	0,09
Total Nitrogen (g.l <sup>-1</sup> )	0,17

*versicolor*, *P. chrysosporium* and *P. coccineus* under immobilized form. The more important pH drops were observed for *P. coccineus* and *P. chrysosporium* growing under free form, respectively attaining 4.25 and 4.5 values.

#### Effect of fungal treatment on molecular weight distribution of phenolic compounds

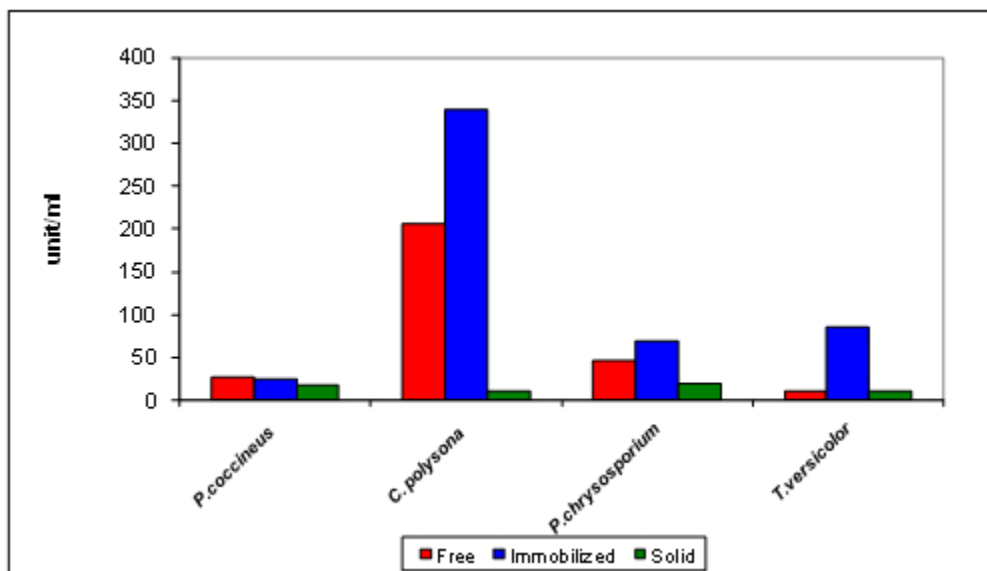
The purpose of this investigation was to evaluate the differential effects of the four studied strains and their cultivation conditions on the low and high molecular weights fractions of phenolics present in OOMW. The twelve situations described in Figure 1 were examined. Four typical situations, summarising the situations encountered during this investigation were illustrated here. Before treatment, a typical elution chromatogram of the OOMW based medium showed two main peaks representing respectively phenolic compounds of more than 30kDa and 2kDa molecular masses (Figure 2a; Figure 2d). The same pattern was obtained when using killed controls (not illustrated); For all the twelve treatments studied, the lower molecular masses phenolics practically disappeared, except for *P. chrysosporium* under solid state where attenuation was only partial (Figure 2c). The amplitude of high molecular masses elution peaks was also systematically lower for all cases studied and complete for *P. chrysosporium* (Figure 2c). For *P. coccineus* growing under free form (Figure 2b), and *C. polyzona* growing in solid state condition (Figure 2d), we observed an apparent shift towards intermediate molecular masses.

#### Studies of laccase production

Laccase was previously identified as a main enzyme involved in the degradation of phenolics in OOMW (Jaouani et al. 2005). The highest laccase content in cultivation supernatant was for *C. polyzona* under free and immobilized forms. In contrast, only low laccase production was generally observed after 15 days incubation.

#### DISCUSSION

The degradation capacities of fungi, in particular white rot strains, were studied for more than twenty years (Field et al. 1993; Pelaez et al. 1995). A lot of data were obtained concerning treatment of pulp and paper, (Kondo et al. 1994; Taspinar and Kolankaya, 1998; De Souza-Cruz et al. 2004; Gamelas et al. 2005), dyes and textile (Lucas et al. 2008), oxidation of polycyclic aromatic hydrocarbons (Zheng and Obbard, 2002) and OOMW (Sayadi and Ellouz, 1995; Jaouani et al. 2005). Yet, the research domain often suffered from difficulty of comparison between the published information, mainly because of the use of different cultivation procedures by different authors, and even in several cases poor description of cultivation mode. The present contribution focused on four WRF strains reputed for their capacity of degrading recalcitrant macromolecules, present in different wastes (Baldrian et al. 2000; Ford et al. 2007). From our results, two major groups apparently emerged, respectively slightly (or not) or



**Figure 3. Laccase production by the strains under various culture conditions.** The enzyme level was measured in cultivation supernatant after 15 days of cultivation.

strongly affected by the cultivation mode. Data were obtained after 15 days of incubation, corresponding to a period of treatment often used by several authors, and corresponding to maximal decrease of COD and phenolics (Kissi et al. 2001; Jaouani et al. 2003). The first group included *C. polyzona* and *P. coccineus*. Therefore, strains of this first group could be selected for bioremediation in context permitting only restricted cultivation modes. An example is the *in situ* cleaning of an OOMW contaminated soil, where bioaugmentation with an active strain is a possible technique. This particular strategy is obviously related to solid state cultivation. When treating a liquid effluent, a procedure using immobilized cells of *C. polyzona* and *P. coccineus* should be appropriate. The use of fungi belonging to the second group, including here *P. chrysosporium* and *T. versicolor*, should be more restricted to particular cases. For example, if *P. chrysosporium* could be used for solid state procedures, the strain has only limited use for liquid effluents as compared to WRF of the first group. An interesting feature shared by all the studied fungi is their capacity to decolourize and degrade phenolics without any supplementation of the OOMW based medium. This was previously reported for certain strains; in particular *P. chrysosporium* (Kissi et al. 2001; Sayadi and Ellouz, 1995). This could however result from the origin of the strain and/or the OOMW. In fact, qualitative and quantitative differences in carbon, nitrogen, phosphorus composition and nature of phenolic compounds contents between different OOMW have been reported (El Hadrami et al. 2004).

Gel filtration experiments have shown that both high and low Molecular Masses phenolics are degraded and assimilated by the WRF. This corroborates previous

observations (Sayadi and Ellouz, 1995) for *P. chrysosporium* and for *C. polyzona* (Jaouani et al. 2005). In the case of *P. coccineus*, growing in the form of free mycelium, and *C. polyzona* in solid state form, we have observed new peaks corresponding to intermediate molecular masses, possibly originating from partial depolymerisation of the heavy polyphenols fraction and/or laccase mediated partial repolymerisation of low molecular mass phenolics (Jaouani et al. 2005). However, a study of laccase production by the strains (Figure 3) did not reveal clear correlation between laccase production and degradation/ repolymerisation capacities. Yet, it should be mentioned that laccase levels were determined here after 15 days cultivation. Intermediate values would be useful to determine in order to establish if any correlation exists between laccase activity and phenolics degradation. It was for example shown that ligninolytic enzymes may be sensitive to proteases produced by different WRF strains during their growth cycle (Jimenez-Tobon et al. 2003). Although, apparently not detected in the present investigation (not illustrated), the role of other enzymes, including Lignin peroxidase, Manganese peroxidase (Kondo et al. 1994; Belinky et al. 2003; Songulashvili et al. 2007) and cellobiose dehydrogenase (Vanhulle et al. 2007) should be also evaluated in more details. For example, Sayadi and Ellouz (1995) reported a possible role for Lignin Peroxydase and Manganese Peroxydase from *Phanerochaete chrysosporium* in the decolourization of OOMW. Aissam et al. (2005) have detected a Manganese peroxidase activity produced by a *Aspergillus niger* strain degrading polyphenols in OOMW.

Another important point related to a successful biotreatment is to attain an environmentally friendly final

wastewater, in particular characterized by a reduction of acidity. This should be also taken more carefully into account. As shown here, this was achieved at least partially with *C. polyzona* and *T. versicolor* under certain cultivation conditions but not with most of the immobilized forms of other strains. As another example, El Asli et al. (2005) have shown that immobilization of a bacterial strain may in fact result in acidification of a growing medium containing OOMW.

In conclusion, we think that laboratory studies as reported here may be useful for orienting the choice of a strain for treating pollution by OOMW. Improvement of experiments in microcosms reproducing at best the different situations that could be encountered in real situations should be a desirable future research axis. A search for the hidden reasons explaining the different behaviours of strains should be also investigated in order to provide a rationale in the use of WRF for bioremediation of pollution generated by OOMW. In this particular context we think that it would be fruitful to assess the individual sensitivities of the strains to constraints imposed by the different forms of growth to diffusion of oxygen and nutrients towards the cells.

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