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Bioremediation of Olive Mill Wastewaters with Fungi

Carla Amaral[1] • Rosário Anjos[1] • Célia Pais[2*]

[1] CITAB (Center for the Research and Technology of Agro-Environment and Biological Sciences), Department of Biology and Environment/School of Life and Environmental Sciences, UTAD (University of Trás-os-Montes e Alto Douro), PO Box 1013, 5001-801 Vila Real Codex, Portugal
[2] CBMA (Center of Molecular and Environmental Biology), Department of Biology/School of Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

Corresponding author: * cpais@bio.uminho.pt

ABSTRACT

Olive Mill Wastewaters (OMW), the residual waters from olive oil extraction industry, are considered the main environmental problem in the Mediterranean area. Due to their high organic loads, and high amounts of organic acids and phenolic compounds, OMW are toxic to several groups of organisms, therefore their reutilization need previous treatment. Decolourization and dephenolization of OMW seem to be the most difficult drawback of the treatments tested so far. However, several fungal species have been successfully used to eliminate colour and phenolics from OMW, reducing toxicity and allowing other types of chemical or physical treatments like anaerobic digestion. This paper presents a brief review of OMW biological treatments by filamentous fungi and yeast like fungi, giving special attention to species able to metabolise some of the recalcitrant compounds.

Keywords: biological treatment, decolourization, dephenolization, filamentous fungi, toxicity, yeasts
Abbreviations: BCB, bubble column bioreactor; **BOD**, biochemical oxygen demand; **COD**, chemical oxygen demand; **Lac**, laccase; **LiP**, lignin peroxidase; **MnP**, manganese-dependent peroxidase; **OMW**, olive mill wastewaters; **STB**, stirred tank bioreactor; **TOC**, total organic carbon; **WRF**, white-rot fungi

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INTRODUCTION

Olive oil consumption has suffered an increase in the last two decades. The highest demand of this natural fat may be related to the use of olive oil as the main fat source in the so-called Mediterranean diet that has been proved to prevent the incidence of cardiovascular and neurodegenerative diseases in humans, and also some types of cancer (Tuck and Hayball 2002; Boudet 2007; De Leonardis *et al.* 2007; Yang *et al.* 2007).

Due to the increase in olive oil consumption the agro-industries responsible for the extraction of this oil intensified their production, improving the extraction mills and consequently the making of residues. The main residue from olive oil agro-industry is a semi-solid wet paste and dark brown coloured water named olive mill wastewater (OMW).

OMW is a complex mixture of water (83-92%), organic matter (4-16%), and minerals (1-2%). The organic matter includes sugars (1-8%), nitrogenous compounds (0.5-2.4%), fatty acids (0.5-1.5%), polyalcohols, polyphenols and pectins (1.0-1.5%), and fats (0.02-1%) (Borja *et al.* 2002, 2006; Sassi *et al.* 2006; Tziotzios *et al.* 2007). These wastewaters present high organic loads, usually indicated as chemical oxygen demand (COD), up to 100-200 gO_2/L (*Asses et al.* 2000), and low biodegradability assessed by the ratio between COD and biochemical oxygen demand (BOD), which varies from 2.05 to 2.35 (Vlyssides *et al.* 2004). Besides these characteristics, OMW has been associated with toxicity towards several groups of organisms like bacteria, seeds and adult plants, aquatic organisms, among others (Paredes *et al.* 1987; Hamdi 1992; Paixão *et al.* 1999; Tsioulpas *et al.* 2002; Aggelis *et al*. 2003; Casa *et al.* 2003; Sassi *et al.* 2006). OMW toxicity has been attributed to the presence of high amounts of phenolic compounds (up to 10 g/L) (Martirani *et al.* 1996; Ramos-Cormenzana *et al.* 1996), and also of other molecules like tannins and lignin like compounds (Capasso *et al.* 1992; Paixão *et al.* 1999), together with heavy metals (Bitton *et al.* 1992; Sassi *et al.* 2006). The high molecular weight phenolic compounds of OMW are usually responsible for its brownish colour and recalcitrant effects, while low molecular weight phenolics have been associated with the phytotoxic and antibacterial effects of these waters (*Asses et al.* 2002; D'Annibale *et al.* 2004).

Spain and Italy are the world's biggest producers of olive oil, followed by other countries of the Mediterranean area, namely Greece, Turkey, Tunisia, Syria, Morocco and Portugal. These countries are responsible for 98% of the olive oil produced worldwide (FAOSTAT 2007; McNamara *et al.* 2008). Consequently, these countries are also the producers of millions of cubic meters of OMW, which represent a serious environmental problem for the Mediterranean region. The disposal of OMW in deep, wide and large open

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ponds is of common use among olive oil producers because Mediterranean climate is beneficial for water evaporation (McNamara *et al.* 2008). These ponds have low implantation and maintenance costs, but can generate bad odours and proliferation of insects (Azbar *et al.* 2004). Because these effluents have high amounts of compounds that may be used as cheap sources of soil nutrients it has been suggested their use for irrigation purposes, especially in arid regions, as a substitute for clean water (Angelakis *et al.* 1999), This type of use has been tested with some success, but the investigations showed some drawbacks, namely the presence of high amounts of minerals, fats and polyphenols (Paredes *et al.* 1999; Sierra *et al.* 2001; Azbar *et al.* 2004) that limit the direct application of OMW to soil without previous treatment (Paixão *et al.* 1999; Vitolo *et al.* 1999; Mekki *et al.* 2006; Sassi *et al.* 2006).

For these reasons, the development of suitable OMW treatments has been a challenge for the researchers. However, until now none of the proposed treatments can be pointed out, both as economically feasible and effective, for treating this type of wastewaters. Proposed treatments include physical, chemical and biological operations, isolated or combined in several ways (Rozzi and Malpei 1996; Azbar *et al.* 2004; Mantzavinos and Kalogerakis 2005; Niaounakis and Halvadakis 2006; Arvanitoyannis *et al.* 2007).

Chemical and physical processes to treat OMW fail to be widespread because they are costly, with low efficiencies, and present serious sludge disposal problems (Mantzavinos and Kalogerakis 2005). Biological processes gained popularity due to their environmental compatibility, lower management costs and equivalent efficiencies compared to physical-chemical ones. Usually, biological methods for OMW treatment include aerobic-activated sludge, co-digestion and anaerobic digestion (Tziotzios *et al.* 2007; McNamara *et al.* 2008).

Although aerobic biological treatments are usually highly efficient, these processes may be limited due to the cost of mechanical aeration that must be continuously provided (Tziotzios *et al.* 2007). Because OMW have high organic loads, anaerobic digestion is the basic biological treatment for this heavily charged effluent. Besides, this type of process produces less waste sludge, may lead to energy generation in the form of biogas, and restarts easily, after several months of shutdown, before seasonal production campaigns (Azbar *et al.* 2004; Mantzavinos and Kalogerakis 2005). Bioremediation of OMW using anaerobic reactors has been done almost exclusively using uncharacterized microbial consortia collected from well established municipal and other wastewater treatment facilities (MacNamara *et al.* 2008). For both types of biological treatments some drawbacks must be overcome. For anaerobic reactors, OMW organic loads do not seem to be a problem. However, the presence in these effluents of compounds like phenolics makes them unsuitable for the treatment, because these compounds present inhibitory activity towards methanogenic bacteria (Hamdi 1992, 1996).

For aerobic processes, besides high aeration requirements, reactors cannot deal with organic loads of OMW without prior dilution. They can only be efficient for low COD feed therefore the use of a pre-treatment aerobic step can reduce some of the phenolics and associated toxicity, enhancing post anaerobic digestion (Mantzavinos and Kalogerakis 2005; McNamara *et al.* 2008).

Microorganisms used for aerobic treatment of OMW vary from aerobic bacteria to filamentous fungi and yeast like fungi. The effectiveness of aerobic bacteria in reducing the phytotoxicity in OMW varies greatly. Some strains seem to be very effective against several compounds, while quite inoffensive towards others, especially the more complex phenolic ones usually responsible for the black colour of these wastewaters.

Fungal bioremediation of OMW has been studied using several strains of white-rot fungi (WRF), non-WRF, and some yeast strains. In this brief review, biological operations using filamentous fungi and yeast like fungi will be discussed considering their effect in COD removal, dephenolization and decolourization of OMW effluents.

BIOREMEDIATION OF OMW WITH FUNGI

Removal of organic matter

It has been pointed out in the introduction that aerobic treatments are not very effective in treating undiluted OMW due to the high organic loads. However, when aerobic microorganisms are used to remove some of the toxic compounds as a pre-treatment, a reduction of COD values can be achieved. The values attained in COD decreasing by growing WRF and other filamentous fungi in OMW are very variable. This variability may be due to several factors, including the strain in test, the degree of dilution of the OMW samples, the different supplements added, and the sterilization processes before incubation with microorganisms.

Removal of organic matter in sterilized OMW

1. Removal of organic matter in sterilized undiluted OMW: Sterilization of OMW prior to the incubation with the isolates to be tested is performed and has been reported by several authors, justifying this previous step as a measure to ensure that the alterations suffered by the sample are only due to the inoculums, and not to other microorganisms present in the OMW. (Sayadi and Ellouz 1995; Vinciguerra *et al.* 1995; D'Annibale *et al.* 1998; Ben Hamman *et al.* 1999; Asses *et al.* 2000; García-García *et al.* 2000; Tsioulpas *et al.* 2002; Aggelis *et al.* 2003; D'Annibale *et al.* 2004; Kachouri *et al.* 2005; Aissam *et al.* 2007; Martinez-García *et al.* 2009; Ben Sassi *et al.* 2008; Asses *et al.* 2009a; Gonçalves *et al.* 2009). Nevertheless it has been pointed out that the results obtained with sterilized samples should be cautious, since the heating process may lead to physicochemical alterations on some compounds. COD values may decrease, and oxidation of phenolics and quinoids may cause precipitation and darkening of the water (Fontoulakis *et al.* 2002; Aggelis *et al.* 2003; Kachouri *et al.* 2005).

In reports using sterilized samples OMW were tested at several dilutions and with different supplements. The addition of supplements is performed to prevent lack of essential nutrients as nitrogen and phosphorus, which should be in agreement with high C/N ratios for an efficient microbial growth (Fadil *et al.* 2003; Öngen *et al.* 2007; Gonçalves *et al.* 2009). Several microbial strains were tested for their ability to remove organic loads from diluted and undiluted OMW. These strains include the WRF *Phanerochaete chrysosporium*, *P. flavido-alba*, *Pleurotus ostreatus*, *Lentinula edodes*, *Panus tigrinus*, the non-WRF *Aspergillus niger*, *A. terreus*, *A. flavus*, *Penicillium* sp., and several yeast strains. From the last group, the most used were strains of *Geotrichum candidum*, but other yeast species have been already reported as able to remove some organic load from effluents namely, *Candida tropicalis*, *C. rugosa*, *C. cylindracea*, *Trichosporon cutaneum*, *Yarrowia lipolytica*, or others isolated directly from the effluent, that present higher adaptability capacities like *C. holstii*, *C. diddensiae*, *C. ernobii*, *C. boidinii* and *Pichia guilliermondi* (Sayadi and Ellouz 1995; Vinciguerra *et al.* 1995; D'Annibale *et al.* 1998; Hamman *et al.* 1999; Asses *et al.* 2000; García *et al.* 2000; Tsioulpas *et al.* 2002; Aggelis *et al.* 2003; D'Annibale *et al.* 2004; Kachouri *et al.* 2005; Aissam *et al.* 2007; Martinez-García *et al.* 2007; Ben Sassi *et al.* 2008; Asses *et al.* 2009a; Gonçalves *et al.* 2009; Martinez-García *et al.* 2009) (see individual references of each species in **Table 1**).

Mean COD removal efficiencies in cultures of crude OMW treated with a *Panus tigrinus* strain could achieve high performances, removing 79.4% from an initial COD load of 43 gO_2/L (D'Annibale *et al.* 1998). García *et al* (2000) and Sayadi and Ellouz (1995) showed the ability of the well known WRF, *Phanerocahete chrysosporium*, to remove 78% of organic load, but at initial COD values of

Table 1 Resume of the efficiencies of different filamentous and yeast like fungi in the removal of organic matter, phenols and colour from OMW, under different culture conditions, including sterilization, dilution and supplementation of the culture media.

Effects/Goals	% Removal	Culture conditions: - sterilized/n-sterilized (s/n-s) - diluted/n-diluted (d/n-d) - supplemented/ n-supplemented (sup/n-sup)	Strains used	References
Removal of organic matter	79.4%	s, n-d	*Panus tigrinus*	D'Annibale *et al.* 1998
	78%	s, n-d	*Phanerocahete chrysosporium*	Sayadi and Ellouz 1995; Garcia *et al*. 2000
	72%	s, n-d	*Aspergillus niger*	García *et al*. 2000
	63.4%	s, n-d	*A. terreus*	García *et al*. 2000
	50%	s, n-d	*Geotrichum candidum*	García *et al*. 2000
	62.2%	s, n-d	*Candida rugosa PYCC 3238* collection	Ben Sassi *et al*. 2007; Gonçalves *et al*. 2009
	64.8%	s, n-d	*C. diddensiae ymc78 OMW* isolates	Ben Sassi *et al*. 2007; Gonçalves *et al*. 2009
	60%	s, n-d	*C. tropicalis*	Martinez-Garcia *et al*. 2007, 2009
	46%	s, n-d	*A. flavus*	Kachouri *et al*. 2005
	93%	s, d, sup	*A. flavus*	Kachouri *et al*. 2005
	60%	s, d	*G. candidum*	Assas *et al*. 2000
	45%	s, n-d, sup	*C. boidinii*	Aissam *et al*. 2007
	78%	s, n-d, sup	*A. niger*	Aissam *et al*. 2007
	85%	s, d, n-sup	*Lentinula edodes*	Vinciguerra *et al*. 1995
	64%	s,d, n-sup	*G. candidum*	Asses *et al*. 2009a
	57%		*G. candidum*	Asses *et al*. 2009a
	80%	n-s, n-d	*Yarrowia lipolytica*	Scioli and Vollaro 1997
	1.47% - 41.22%	n-s, n-d	*Y. lipolytica*	Lanciotti *et al*. 2005
	5.3%	n-s, n-d, sup	*L. edodes*	Casa *et al*. 2003
	50%	n-s, n-d	*Pleurotus ostreatus*	Olivieri *et al*. 2006
	60.9%	n-s, n-d	*P. tigrinus*	D'Annibale *et al*. 2006
	77%	d, sup	*Ph. chrysosporium*	Kissi *et al*. 2001
	81%	d	*Ph. chrysosporium*	Dhouib *et al*. 2006
	10.7% - 47.5%	d	*G. candidum*	Fadil *et al*. 2003
	12.4% - 3.9%	d	*Aspergillus* sp.	Fadil *et al*. 2003
	18.2% - 50.3%	d	*C. tropicalis*	Fadil *et al*. 2003
	58%	d, sup	*G. candidum*	Asses *et al*. 2009b
	60% Total Sugars	n-s, n-d	*P. tigrinus*	D'Annibale *et al*. 2006
	70% Total Sugars	n-s, n-d	*Trametes versicolor*	Ergun *et al*. 2008
	75% Total Sugars	d, s	*Pl. ostreatus*	Aggelis *et al*. 2003
Dephenolization	92% total phenols	s, n-d	*Ph. chrysosporium (ATCC 64314)*	García *et al*. 2000
	100% orto-diphenols	s, n-d	*Ph. chrysosporium (ATCC 64314)*	García *et al*. 2000
	76% total phenols	s, n-d	*A. niger*	García *et al*. 2000
	64% total phenols	s, n-d	*A. terreus*	García *et al*. 2000
	82% orto-diphenols	s, n-d	*A. niger*	García *et al*. 2000
	76% orto-diphenols	s, n-d	*A. terreus*	García *et al*. 2000
	~ 40% total phenols	s, n-d	*C. diddiensis*	Sassi *et al*. 2008
	~ 40% total phenols	s, n-d	*C. holstii*	Sassi *et al*. 2008
	~ 40% total phenols	s, n-d	*Pichia* sp.	Sassi *et al*. 2008
	51% total phenols	s, n-d	*C. tropicalis*	Martinez-Garcia *et al*. 2007, 2009
	92% total phenols	d	*L. edodes*	Vinciguerra *et al*. 1995
	76% total phenols	d	*Pleurotus* sp.	Tsioulpas *et al*. 2002
	50%	high phenolic content (4,5 g/L)	*Pleurotus*	Aggelis *et al*. 2003
	80%	low phenolic content (0,15 g/L)	*Pleurotus*	Aggelis *et al*. 2003
	78%	s, d, sup	*A. niger*	Aissam *et al*. 2007
	65%	s, d, sup	*Penicillium*	Aissam *et al*. 2007
	50%	s, d, sup	*G. candidum*, *C. boidinii*	Aissam *et al*. 2007
	70%	n-s, n-d, n-sup	*Pl. ostreatus*	Olivieri *et al*. 2007
	97.2%	n-s, n-d, n-sup	*P. tigrinus*	D'Annibale *et al*. 2006
	70%	n-s, n-d, n-sup	*Tr. versicolor*	Ergul *et al*. 2008
	21.6%	n-s, n-d, n-sup	*Y. lipolytica*	Lanciotti *et al*. 2005
	90%	n-s, n-d, n-sup	*Y. lipolytica*	Scioli and Vollaro 1997
	90%	n-s, d, n-sup	*Pl. ostreatus*	Martirani *et al*. 1996
	70%	n-s, d, n-sup	*Ph. chrysosporium*	Kissi *et al*. 2001
	90%	n-s, d, sup	*(Euc 1)*	Dias *et al*. 2004
	85% orto-diphenols	n-s, d, sup	*Ph. chrysosporium*	Dhouib *et al*. 2006
	83% total phenols	n-s, d, sup	*Ph. chrysosporium*	Dhouib *et al*. 2006
	30%	n-s, d, sup	*A. tubingensis*	Ongen *et al*. 2007
	52%	n-s, d, sup	*C. tropicalis*	Fadil *et al*. 2003
	47%	n-s, d, sup	*Geotrichum sp.*	Fadil *et al*. 2003
	44%	n-s, d, sup	*Aspergillus*	Fadil *et al*. 2003
	37% total phenols	n-s, d, sup	*C. tropicalis*	Ettayebii *et al*. 2003
	55% polyphenols	n-s, d, sup	*C tropicalis*	Ettayebii *et al*. 2003
	69% monophenols	n-s, d, sup	*C. tropicalis*	Ettayebii *et al*. 2003

Table 1 (Cont.)

Effects/Goals	% Removal	Culture conditions: - sterilized/n-sterilized (s/n-s) - diluted/n-diluted (d/n-d) - supplemented/ n-supplemented (sup/n-sup)	Strains used	References
Decolourization	76%	s, n-d	*Ph. chrysosporium*	Sayadi and Ellouz 1995
	90%	s, n-d	*Ph. flavido-alba*	Hamman *et al.* 1999
	70% soluble colour compounds		*Ph. flavido-alba*	Hamman *et al.* 1999
	75 and 72%	s, n-d	*L. edodes*	D'Annibale *et al.* 1998, 2004
	45%	s, n-d, sup	*P. tigrinus*	D'Annibale *et al.* 2004
	40%	s, n-d	*P. guillermondii*, *C. holstii*, *C. diddensiae*, *C. ernobii*	Sassi *et al.* 2008
	8%		*P. guillermondii*, *C. holstii*, *C. diddensiae*, *C. ernobii*	Sassi *et al.* 2008
	75%		*G. candidum*	Assas *et al.* 2002
		s, d, sup	*G. candidum*	Ayed *et al.* 2005
	47%	s, d	*A. flavus*	Kachouri *et al.* 2005
	53%		*A. flavus*	Assas *et al.* 2009a
	65%		*Coriolus versicolor*	Yesilada *et al.* 1998
	81%		*Funalia trogii*	Yesilada *et al.* 1998
	50%	n-s, n-d	*Pleurotus species*	Flouri *et al.* 1996
	72%	n-s, n-d	*P. tigrinus*	D'Annibale *et al.* 2006
	76%	n-s, n-d	*P. tigrinus*	D'Annibale *et al.* 2006
	18%	n-s, n-d	*Tr. versicolor*	Ergul *et al.* 2008
	50%	n-s, d	*Ph. chrysosporium*	Dhouib *et al.* 2006
	60%	n-s, d	*Ph. chrysosporium*	Sayadi *et al.* 2000
	95%	n-s, d, sup	*Ph. chrysosporium*	Kissi *et al.* 2001
	65%	n-s, d, sup	*Pl. ostreatus*	Kissi *et al.* 2001
	73%	n-s, d	*Euc1*	Dias *et al.* 2004
	44%	n-s, d, sup	*A. niger*	Ongen *et al.* 2007
	81%	n-s, d, sup	*A. tubingensis*	Ongen *et al.* 2007
	81%	n-s, d, sup	*A. aculeatus*	Ongen *et al.* 2007
	72%	n-s, d	*Pl. sajor caju*	Jaouani *et al.* 2003
	75%	n-s, d	*Coriolopsis polyzona*	Jaouani *et al.* 2003
	50%	n-s, d	*L. tigrinus*	Jaouani *et al.* 2003
	58%	n-s, d	*Pycnoporus coccineus*	Jaouani *et al.* 2003
	30%	n-s, d, sup	*G. candidum*	Fadil *et al.* 2003
	65%	n-s, d, sup	*G. candidum*	Asses *et al.* 2009b

approximately 80 g/L. In one of the abovementioned reports (García *et al.* 2000), strains of *Aspergillus niger* and *A. terreus* were able to grow on these wastewaters, removing up to 72% and 63.4% of initial COD values, respectively. The yeast *Geotrichum candidum* was not so efficient, removing only about 50% of the initial COD values. The reports of Sassi and coworkers (2007) and Gonçalves and coworkers (2009) also can demonstrate this fact, since the several yeasts tested, either from collections or isolated from natural environments, gave maximum COD removals of 62.2% (*C. rugosa* PYCC 3238, collection strain), and 64.8% (*C. diddensiae* ymc78, OMW isolate). The lowest COD removals were registered for *Y. lipolytica* collection strains.

It is noteworthy to point out that the highest values of COD removal presented by strains of WRF were obtained in supplemented OMW as opposed to the reports that used yeasts isolated from OMW. Comparing the efficiencies obtained it can be concluded that yeasts showed a remarkable ability to remove the organic load from OMW.

The aerobic co-digestion of OMW with other residues has already been used as pre-treatment of OMW for anaerobic digestion (Martinez-García *et al.* 2007, 2009). In these studies isolates of *C. tropicalis* were grown in aerobic digestors with sterilized and undiluted OMW mixed with cheese whey and piggery effluent. In both cases an enhancement of the yeast ability to remove some organic load was observed, especially compounds toxic to methanogenic bacteria, probably due to the improvement of nitrogen intake, rising the C/N ratio. In these reports COD removal was near 60%.

2. Removal of organic matter in sterilized diluted OMW: Some procedures use dilution steps prior to microorganism growth as a way to reduce very high organic loads or high concentrations of phenolic compounds. An isolate of *A. flavus* that could achieve 46% removal of organic load in an OMW sample without dilution improved significantly its degrading abilities when OMW was 10x-diluted and supplemented for adequate N and P levels, being able to remove 93% of the initial COD values (Kachouri *et al.* 2005). The yeast *G. candidum* was grown in medium with low (13.6 g/L) and high (68 g/L) COD values obtained by adequate dilution of sterilized OMW with an initial COD of 136 g/L. The isolate was able to remove 60% of the initial COD in static culture conditions (Asses *et al.* 2000). Aissam and coworkers (2007) reported that isolates of *C. boidinii*, *G. candidum*, *Penicillium* sp. and *A. niger* were not able to grow in diluted OMW with COD values higher than 20 g/L. However the same isolates, when acclimated in media with growing percentages of OMW, could grow on sterilized, undiluted and supplemented OMW, achieving good COD removals after 15 days of incubation. COD removal values varied between 45%, for the *C. boidinii* isolate, and 78% for the *A. niger* isolate (Aissam *et al.* 2007). Other reports used non-suplemented OMW (sterilized/diluted) as growing media for *Pleurotus* sp. and *P. ostreatus* strains, and also *Lentinula edodes* and *G. candidum* strains (Vinciguerra *et al.* 1995; Tsioulpas *et al.* 2002; Aggelis *et al.* 2003; Asses *et al.* 2009a). *L. edodes* isolates growing in 5X-diluted OMW could remove 85% of total organic carbon (TOC) present in the effluent (Vinciguerra *et al.* 1995). The other filamentous fungi used, *P. ostreatus*, showed a good growth profile measured by the uptake of reducing sugars. In this case 75% of

all reducing sugars were metabolised and converted into microbial mass, indicating high organic load removals (Aggelis *et al.* 2003). In a recent work, Asses and colleagues (2009a) used *G. candidum* isolates and obtained 64% COD removal from growth in bubble column, and 57% of the same parameter for cultures in settler.

Removal of organic matter in non-sterilized OMW

1. Removal of organic matter in non-sterilized undiluted OMW: Most of the working teams dealing with treatment of OMW with fungi use non sterilized samples, since no significant alterations in COD values, total phenols concentrations or decolourization were registered when the effluent was kept in the same experience conditions, but without inoculums. On the other hand, considering the possible application of fungal treatment at a large scale, the need for a previous sterilization step in treatment units would make the process economically unfeasible (Olivieri *et al.* 2006).

The literature available shows that the use of raw, non-sterilized OMW as culture media for microbial growth has been performed mainly with filamentous fungi of the *Pleurotus* genera (e.g. *P. ostreatus*, *cornucopiae*, *cystidiosus*, *drnyius*, *eryngii*, *pulmonaris*), and species of WRF, *Panus tigrinus* and *Trametes versicolor* (Flouri *et al.* 1996; D'Annibale *et al.* 2006; Olivieri *et al.* 2006). The exception may be found for yeast cultures with *Yarrowia lipolytica* strains (Scioli and Vollaro 1997; Lanciotti *et al.* 2005). It is also available a study with purified laccase (Lac) extracted from the fungus *Lentinula edodes* (Casa *et al.* 2003). Although dilution decreases the concentration of compounds that can be inhibitory for microbial growth, the use of diluted samples would increase the volumes of waste to be treated, which is not desirable (Ergül *et al.* 2008)

Lanciotti *et al.* (2005) screened several isolates of *Yarrowia* strains from different origins (chilled foods, light butter, superficial water of lagoon, irradiated poultry meat) regarding their capacity to grow on undiluted OMW and reported that the ability of the isolates for COD removal was independent from the isolation source, obtaining rates between 1.47 and 41.22%.

The determination of the enzymatic activity of isolated and purified ligninolytic enzymes in wastewaters is usually used for dephenolization or decolourization purposes. Nevertheless, Casa and collaborators (2003) measured removal rates of organic loads in undiluted and non-sterilized OMW treated with Laccase (Lac) isolated from *L. edodes.* The low values obtained by these authors (5.3%) were explained by the possible poor contribution of OMW aromatic compounds, main targets of Lac activity, to the global organic load of the effluent.

The removal of organic load, colour and phenols, has been the main objective of all the reports mentioned above. Additionally, some authors also tried to improve culture conditions for scaling up of processes, considering economically feasible conditions. Having these goals in mind Olivieri and colleagues (2006) tested the potential of internal loop airlift bioreactors (ILA) using *Pleurotus* cultures. D'Annibale *et al.* (2006) and Ergül *et al.* (2008) tested the growth and bioremediation ability of *Panus tigrinus* and *Trametes versicolor* strains, respectively, in stirred-tank bioreactors (STB). For *P. tigrinus* growth the bubble column bioreactor (BCB) was also tested, and for *T. versicolor* shake-flasks and static culture experiments were performed. The isolates of *T. versicolor* were adapted before inoculation and several aeration regimes were tested for STR experiments. The best results were achieved for 0.25 vvm aeration rate, and under these conditions sugar consumption attained values near 70% (Ergull *et al.* 2009). In similar conditions the *P. tigrinus* strain achieved a COD removal of 60%. The same isolate, in BCB conditions, gave similar results (60.9%) but needing shorter incubation times (6 days for BCB *versus* 9 days in STR). The better results achieved by BCB cultures could be attributed to shear stress in STR conditions (high aeration and high agitation regimes) already reported as responsible for mycelial damage, morphological changes and low enzyme production in WRF (D'Annibale *et al.* 2006).

Despite the promising results obtained regarding organic load removal, when growing in airlift bioreactors the cultures formed pellets which, depending on loading rate conditions, could cause reactor clogging (Olivieri *et al.* 2006).

2. Removal of organic matter in non-sterilized diluted OMW: To achieve higher organic load removal many researchers use diluted OMW and add supplements for microbial growth assays. Examples can be found using species of WRF, other filamentous fungus and yeasts.

Phanerochaete chrysosporium is one of the best-studied ligninolytic fungus, and publications concerning its use for ligninolytic enzyme synthesis or bioremediation of residues with lignin like compounds can be found (Sayadi and Ellouz 1992, 1993, 1995; Kissi *et al.* 2001; Dias *et al.* 2004; Sampedro *et al.* 2004; Dhouib *et al.* 2006; Taccari *et al.* 2009). Kissi *et al.* (2001) compared the performance of *Ph. chrysosporium* and *Pleurotus ostreatus* strains in detoxification and decolourization of diluted OMW in the presence of added nutrients. They observed that the *Ph. chrysosporium* isolate had a better performance achieving 77% COD removals in 20% diluted supplemented OMW. After acclimation, the strain could also grow in a 50% diluted OMW, removing more than 50% of organic load. Dias *et al.* (2004) used a new isolate of *Ph. chrysosporium* named Euc1, which was able to remove 45% of the organic load in 20% diluted OMW. The effect of *Ph. chrysosporium* isolates detoxification in an anaerobic and ultrafiltration coupled treatment, showed that the fungal pre-treatment in a bubble column pilot scale reactor, could compromise with initial COD values up to 100 g/L, and achieved 20-50% COD abatement. This pre-treatment coupled with anaerobic digestion, which was enhanced by the prior aerobic step, could remove 81% of total intake organic matter (as COD) (Dhouib *et al.* 2006).

Several strains from the genus *Aspergillus* were also investigated regarding their ability to detoxify, dephenolize and decolourize olive mill wastewaters. These abilities were first reported several years ago concerning *A. niger* and *A. terreus* isolates (Hamdi *et al.* 1991; Borja *et al.* 1995). These fungi have shorter cultivation times when compared to WRF but recent reports have constrained the use of some *Aspergillus* species due to the production of ochratoxin (Varga *et al.* 2003). As a result, the search was focused on non-ochratoxin fungi producers like *A. aculeatus*, *A. foeditus* var. *pallidu*, *A. niger*, and *A. tubingensis* that, in a screening program for growing in OMW media, gave the best performances (Ongen *et al.* 2007). In a previous report, Fadil *et al.* (2003) compared the performances of fungus from the genus *Aspergillus*, with isolates from the yeasts *G. candidum* and *C. tropicalis* in media with initial COD concentrations, varying from 30 to 120 g/L. They concluded that there is a direct effect of COD initial concentrations on COD and phenols removals. The COD conversion varied between 10.7 and 47.5% in media treated with *G. candidum*, from 12.4 to 39.9% with *Aspergillus* sp., and from 18.2 to 50.3% with *C. tropicalis*. It is also important to point out that the best COD removal for the *C. tropicalis* isolate was for an initial COD value of 60 g/L. Using this yeast the need for several dilutions may be avoided.

Using yeasts to remove the organic load of OMW has been much less common than using filamentous fungi isolates, but several recent studies present promising results.

In a recent assay, Asses and colleagues (2009b) diluted fresh and stored OMW and tested the ability of *G. candidum* strains to grow and remove some of the organic load in those effluents. The authors verified that no fungal growth was observed in stored OMW without sugar addition. When adding glucose as a supplement the isolates could remove 58% of initial COD.

C. tropicalis isolates growing in 7X diluted OMW supplemented with yeast extract, and with co-metabolism indu-

cers achieved 35% COD removal when using non-supplemented OMW and incubation temperature 30°C. Degradation slightly improved with higher temperatures (e.g. 40°C).

The addition of supplements should be adequately pondered so that it will not turn the treatment economically unfeasible. Although some investigation teams have used cheap supplement sources, the use of microorganisms able to grow and bio remediate the waste without addition of external sources of nutrients present obvious advantages (Martirani *et al.* 1996; Sayadi *et al.* 2000; Jaouani *et al.* 2003).

As stated above the WRF *Ph. chrysosporium* has been used as a model microorganism for the removal of aromatics and lignin like compounds from wastes. Nevertheless, the growth of this fungal species in undiluted OMW, or OMW with COD values higher than 50 g/L is severely affected (Sayadi and Ellouz 1993). Sayadi and coworkers (2000) attributed this effect to the presence of phenolic compounds and investigated which fraction of phenolics is responsible for this inhibitory effect. At an initial COD value lower than 40 g/L the best results were obtained in the fraction containing high molecular-mass polyphenols, where the COD content was reduced by approximately 70%. The fraction with low molecular-mass polyphenols was the one with the lowest COD abatement, near 40%, indicating that those were the group with higher toxicity towards microorganisms.

Jaouani and team colleagues (2003) performed a broad screening of fungi to identify decolourizing strains using culture collection isolates including WRF, non-WRF basidiomycetes and ascomycetes. They also tested different initial COD concentrations (from 25 to 100 g/L) and the best results were obtained with isolates from the species *Pl. sajor caju*, *Pycnoporus coccineus*, *Coriolopsis polyzona* and *Lentinus tigrinus*. Their COD removal efficiencies varied from 25%, for high COD concentrations (100 mg/L), to 50% for lower COD concentrations (50 g/L).

Dephenolization processes

Phenolic compounds are secondary metabolites present in several fruits and vegetables, in beverages like beer, wine, black and green tea (Macheix *et al.* 1990), and also in olive oil and derivatives (Servili and Montedoro 2002; De Leonardis *et al.* 2007), resulting mainly from shikimic acid metabolic pathway.

After generation or liberation of the phenolic compounds from olive processing to produce olive oil they solve both in the aqueous and in the oleaginous phases. Generally, only 1 to 2% is in olive oil, 53% in OMW, and 45% in solid residues (Di Giovachino *et al.* 2002; Rodis *et al.* 2002; De Marco *et al.* 2007; Saitta *et al.* 2009). The presence of monomeric phenolic compounds has been related to the phytotoxic and antimicrobial properties of these wastewaters (Ramos-Cormenzana *et al.* 1996; Sayadi *et al.* 2000; Casa *et al.* 2003; Sampedro *et al.* 2004a; Ergull *et al.* 2009). On the other hand, their dark brownish colour, particularly recalcitrant to decolourization, has been attributed to OMW composition in high molecular weight phenolic compounds (*Asses et al.* 2002; D'Annibale *et al.* 2004; Ergull *et al.* 2009).

In either case, removal of phenolic compounds from olive mill wastewaters, in order to allow their use, is an issue with great relevance in environmental policy.

Removal of phenolic compounds in sterilized OMW

1. Removal of phenolic compounds in sterilized undiluted OMW: The well known phytotoxic and antibacterial effect of OMW has been mainly attributed to the presence of high levels of phenolic compounds (Lanciotti *et al.* 2005; Gonçalves *et al.* 2009). For biological treatments like aerobic or anaerobic digestion the effluent must be diluted several times (10 fold) to prevent inactivation of microorganisms during the process. Dilution increases the treatment costs therefore research on OMW valorisation is focused on the degradation of their phenolic fraction, since the breakdown of phenols is considered one of the limiting steps in OMW bio-treatments (Martirani *et al.* 1996; Fontoulakis *et al.* 2002; Aggelis *et al.* 2003; Peixoto *et al.* 2008). The removal of compounds like monocyclic phenols can be performed by bacteria and fungi (Martinez-García *et al.* 2009). However, the use of filamentous fungi is difficult as compared to that of yeasts because they present slower growth rates and, in liquid media, the generation of floating mycelia can be a problem (Mendonça *et al.* 2004). Despite these difficulty fungi, especially WRF, can be a good source of phenol degrading species, because they grow in wood, where phenolic structures are usually present. In fact, fungal non-specific ligninolytic machinery oxidizes a wide variety of substrates and has been used for bioremediation of environmental pollutants (Sayadi and Ellouz 1995; Hamman *et al.* 1999; D'Annibale *et al.* 2004).

Ph. chrysosporium and *Ph. flavido-alba* are well studied WRF that produce extracelular peroxidases involved in lignin biodegradation. Lignin peroxidase (LiP) and Manganese-dependent peroxidase (MnP) are synthesised during secondary metabolism, and their activities are regulated by levels of nitrogen, carbon and manganese (Sayadi and Ellouz 1995; Hamman *et al.* 1999). Besides, WRF can also produce Laccase (Lac), but extracelular levels are strain dependent and apparently this enzyme has higher activity in *Ph. flavido-alba* strains (Peréz *et al.* 1996).

Several works report the utilization of *Ph. chrysosporium* and *Ph. flavido-alba* in sterilized undiluted OMW, in order to test their ability to remove phenolic fractions and achieve decolourization. The most promising results were reported by García *et al.* (2000) when cultivating *Ph. chrysosporium* (ATCC 64314) aerobically and with stirring. Since high initial COD values have been reported to usually inhibit fungal growth these authors adapted the strain before inoculation and for initial COD values of 83 g/L the fungus was able to remove 92% of total phenols and 100% of orto-diphenols in the medium. In the same assay the activity of non-WRF *A. niger* and *A. terreus*, and the yeast *G. candidum* was tested. The *Aspergillus* strains could also remove high percentages of phenols (76% *A. niger*; 64% *A. terreus*) and orto-diphenols (82% *A. niger*; 76% *A. terreus*) while the yeast was unable to degrade the phenolic fraction.

Regarding the strain of *Ph. flavido-alba* tested, 90% remotion of total phenols from the sample was achieved with initial COD values of about 40 g/L, and with the culture daily flushed with pure oxygen plus veratryl alcohol, an enzyme inducer (Hamman *et al.* 1999). The same enzyme inducer was used by other researchers who also compared the action of LiP and MnP activities towards different molecular-mass phenolic fractions (Sayadi and Ellouz 1995). They concluded that the low molecular-mass fraction was easily degraded while high molecular-mass polyphenols were degraded mainly by LiP activity, and MnP apparently could not depolymerise this fraction.

More recent works have evaluated the ability of yeasts for the treatment of OMW, along with the production of biomass and enzymes (Lanciotti *et al.* 2005). Yeasts are the dominant microorganisms in OMW environment when compared to bacteria and moulds (Sassi *et al.* 2006; Amaral *et al.* 2008). Moreover, several yeasts are adapted to grow on OMW because they can resist to high values of phenolic compounds and can use them as sole carbon and energy sources. Some of the studied species include isolates of *C. tropicalis*, *C. cylindracea*, *C. rugosa*, *Trichosporon cutaneum* and *Yarrowia lipolytica* (Lee *et al.* 2001; Ettayebi *et al.* 2003; Ahuatzi-Chacón *et al.* 2004; Chtourou *et al.* 2004; Páca *et al.* 2007; Gonçalves *et al.* 2009).

Sassi and coworkers (2008) surveyed about 100 yeasts isolated from OMW for their ability to grow and remove phenols from sterilized undiluted OMW. Total phenol removal was around 40% for *C. diddiensis*, *C. holstii* and *Pichia* sp. The same strains were tested for their ability to reduce

phytotoxicity in germination tests, and it was concluded that COD, total phenols and pH were the most important physico-chemical parameters affecting the germination. Depending on the yeast strain and type of treatment one of the parameters may be the key factor for lowering the phytotoxic effect.

C. tropicalis isolates were used for pre-treatment steps before anaerobic digestion. The aerobic step with yeasts used non-diluted OMW samples mixed with cheese whey or with piggery effluent (Martinéz-Garcia *et al.* 2007, 2009) and some COD and phenols removal (51%) were achieved, thus allowing the enhancement of the anaerobic digestion.

Considering reports that used sterilized and undiluted OMW, with supplementation, and the yeasts *C. rugosa*, *C. cylindracea* and *Y. lipolytica* (Gonçalves *et al.* 2009) the authors concluded that the tested yeasts were not very adequate for phenols removal since percentages were low when compared with the ones obtained using WRF in non-supplemented media (Sayadi and Ellouz 1995; Hamman *et al.* 1999), or with WRF in supplemented media (D'Annibale *et al.* 1998, 2004). High initial COD values in the cultures (184, 191, 115 and 179 g/L) could have been the main reason for such low removal measurements obtained when using yeasts.

2. Removal of phenolic compounds in sterilized diluted OMW: Reports using fungal cultures for treatment of sterilized, diluted, supplemented or non-supplemented OMW include strains from the species *A. flavus*, *A. niger*, *C. boidinii*, *G. candidum*, *L. edodes*, *Penicillium* sp. and *Pl. ostreatus* (Vinciguerra *et al.* 1995; Asses *et al.* 2000; Tsioulpas *et al.* 2002; Aggelis *et al.* 2003; Kachouri *et al.* 2005; Aissam *et al.* 2007; Asses *et al.* 2009a).

The strains of *L. edodes* were grown on 5X-diluted OMW and were able to remove 92% of total phenols in 12 days (Vinciguerra *et al.* 1995). Orto-diphenols were the first to be degraded, while the other groups of phenolic compounds were only metabolised after seven days in culture. One of the reasons why these isolates could not degrade the high molecular-mass fraction was, probably, the glucose depletion in the medium. Activity of Lac and MnP enzymes was also detected. Maximum phenolic removal of 76% was reported for *Pleurotus* sp. growing on 75% diluted OMW and this removal ability was accompanied by high Lac release to the culture media (Tsioulpas *et al.* 2002). Germination tests performed using the OMW after treatment showed that phenol removal was not proportional to the reduction in toxicity. On the contrary, treated OMW increased the inhibition of germination, probably because the final products of the enzymatic activities (e.g. phenoxi radicals, quinonoids) can be even more toxic to the seeds. Similar results were reported by Martirani *et al* (1996) and Aggelis *et al* (2003) using *Pleurotus* strains in more diluted OMW (20 and 50%, respectively). In this last study, the authors tested two different initial concentrations of phenols, obtaining different removal rates that varied from 50% for high phenolic content (4.5 g/L), and 80% for low phenolic content (0.15 g/L).

Several fungal strains either commercially available or from collections were studied for decolourization and dephenolization abilities, but fewer contributions have emphasised the degrading properties of strains isolated directly from OMW. From these, the study by Aissam and collaborators (2007) was particularly relevant. Strains of *G. candidum*, *C. boidinii*, *A. niger* and *Penicillium* sp. isolated from OMW were adapted to grow in increasing percentages of OMW supplemented for adequate N and P values. Non-adapted cultures could not remove organic load, phenols or colour, while adapted isolates showed good performance in phenols removals. The best were registered for filamentous fungi, varying from 78% for *A. niger* isolate to 65% for *Penicillium*. The tested yeasts removed almost 50% of phenolic compounds.

Removal of phenolic compounds in non-sterilized OMW

1. Removal of phenolic compounds in non-sterilized undiluted OMW: The effect of variation of the phenolic content (between 0.11 and 1.4 g/L) in raw OMW was tested against *Pleurotus ostreatus* free cultures, to access the ability of this fungus to remove these compounds. The isolate tested by Olivieri *et al.* (2006) was able to bio-convert about 70% of the phenols over a period of 4 to 7 days. The same authors tested the isolate in airlift bioreactor cultures, but for high COD load mycelia development created clogging problems in the reactor. D'Annibale *et al.* (2006) tested the effect of agitation and aeration on the efficiency in the OMW pollutant removal by *Panus tigrinus* growing on a stirred tank bioreactor (STR) or in a bubble column bioreactor (BCB), for possible up scaling. When growing in BCB, OMW clearing was generally higher than the best results obtained with STR. For BCB *Panus tigrinus* cultures a phenolic removal of 97.2% was recorded, together with maximum dephenolization rates. For similar efficiency in BCB and STR, the first was faster, achieving best results in six days as compared to nine days for the STR. High Lac and MnP enzyme activity were also registered. Shear stress in fungal cells, caused by mechanical movement of stirrers may explain the lower adequacy of STR for bio-treatment of OMW. Adapted *Trametes versicolor* strains were cultured in STR similar conditions (same aeration rate, no dilution of OMW and non-sterilized samples) than that for *P. tigrinus*. Total phenolics removals reached 70%. This strain was very effective in removing all simple phenolic compounds identified, which is very important when an after treatment step as anaerobic digestion must be considered (Ergull *et al.* 2009). The dephenolization process was probably related, as in other reports, with the activity of the extracellular enzymes Lac and MnP, detected in culture media.

Y. lipolytica isolates able to grow on undiluted and non-supplemented OMW effluents have been reported by Scioli and Vollaro (1997) and Lanciotti *et al.* (2005). The strains tested by the last team behaved differently regarding the dephenolization process. From the 20 isolates tested, half showed capacity to remove phenols (21.6%) while the others seamed to cause an increase in total phenols measurements. This fact may be related with metabolic pathway for phenols degradation, which could have caused accumulation and auto-oxidation of intermediate compounds in tyrosine metabolism (Lanciotti *et al.* 2005). In the tests performed by Scioli and Vollaro (1997) the yeast strain removed more than 90% of phenolic content with concomitant production of ethanol and methanol, improving the waste smell. Simultaneously the *Y. lipolytica* isolate produced high amounts of lipase and biomass in few hours.

2. Removal of phenolic compounds in non-sterilized diluted OMW: Several approaches have been also used to remove phenolic compounds from non-sterilized and diluted OMW.

A strain of *Pl. ostreatus* was grown on non-sterilized and very diluted OMW (20% v/v) and was able to remove more than 90% of phenolic compounds from the culture media, after 100h incubation. This high phenolic removal was attributed to phenol oxidase activity whose values reached the maximum in the 13th day. Pure phenol oxidase could not remove toxicity from the effluent, although it removed some phenols. Authors concluded that the transformation of toxic compounds in this strain follows a complex pathway, in which phenol oxidase could play an important, although not exclusive, role. The toxicity of the treated OMW was reduced 7-fold when tested against *Bacillus cereus* (Martirani *et al.* 1996).

Other researchers added several supplements to diluted non-sterilized OMW samples, in order to identify their impact on the fungal enzyme system. Kissi and team colleagues (2001) tested the effect of several supplements in *Ph. chrysosporium* ability for COD, colour and phenols removal.

The best results were obtained with the addition of glycerol (1% v/v) in 20% diluted OMW. In this case the fungus could remove 70% of total phenols content, a value lower than the one achieved by the *Pl. ostreatus* isolate, at the same OMW dilution and without the addition of supplements. The activity of LiP, MnP and Lac were also measured and for LiP the maximum activity was achieved in the first 6 days while for MnP and Lac the enzymatic activity increased from the 6th day onward.

The effect of adding supplements to the medium when using *Ph. chrysosporium* isolates was also tested by several authors. Dias *et al.* (2004) compared the activity of this fungus to that of an isolate from leaves, in a medium enriched with glucose and vitamins. They concluded that their isolate (Euc 1) had better performances than *Ph. Chrysosporium* collection isolates, being able to remove 90% of total phenols from the incubation media. Moreover, they extracted from the fungus and purified the enzyme laccase, which is believed to be responsible for the decolourization process in the studied cultures. Another group reported the pre-treatment with *Ph. chrysosporium*, before anaerobic digestion of diluted OMW, supplemented to have a C/N/P ratio of 100/5/1 (Dhouib *et al.* 2006). The use of the fungal strain enhanced the anaerobic process and at the end of the biological treatment (pre aerobic treatment and anaerobic digestion of *Ph. chrysosporium* treated OMW) the effluent had less 85 and 83% of orto-diphenols and total phenols, respectively. The toxicity of the effluent was tested in the germination of *Lycopersicum esculentum* (tomato) seeds and inhibition of luminescence in the bacteria *Vibrio fischerii* but the treated effluent showed a low decrease in toxicity (26%). In agreement with other reports, the removal of phenols is not always accompanied by a significant decrease in toxicity towards organisms (Martirani *et al.* 1996; Tsioulpas *et al.* 2002; Aggelis *et al.* 2003).

Testing the dephenolization capacity of twenty different *Aspergillus* strains, Ongen and colleagues (2007) demonstrated that supplementation of the culture media with yeast extract was more effective than the supplementation with inorganic salts. From the tested strains, *A. aculeatus*, *A. foeditus*, *A. niger* and *A. tubingensis* presented tannase activity, but no Lac or MnP activities were detected. *A. tubingensis*, the more effective strain in removing phenols, could remove 30% phenols in two-fold diluted and in undiluted OMW. The authors concluded that, due to different performances in the tested media, the aim of the treatment process should be defined previously, since the removal of phenols and decolourization is better achieved in conditions different from those that allow high biomass production and sugar consumption.

Several yeasts have been recently tested for their ability to remove phenols from OMW. Many approaches focused on the use of phenolic compounds as carbon source for yeast growth (Lee *et al.* 2001; Ahuatzi-Chacón *et al.* 2004; Chtourou *et al.* 2004; Páca Jr. *et al.* 2007). Fewer have used yeasts growing directly on entire, even though diluted OMW (Fadil *et al.* Ettayebii *et al.* 2003; Papanikolaou *et al.* 2008).

In shaken cultures *C. tropicalis* isolates showed a better performance than isolates of *Geotrichum* sp. and *Aspergillus* sp. (Fadil *et al.* 2003). In fact, when growing in diluted OMW with 100 g/L COD and supplemented for a final C/N/P ratio of 100/16/1, *C. tropicalis* removed 52% of phenols in a 24 h incubation period, as opposed to *Geotrichum* and *Aspergillus* isolates that, in the same conditions, but in a seven day incubation period removed 47 and 44% of phenols content, respectively. In other experiments, *C. tropicalis* induced by hexadecane as co-metabolite was also able to remove 37% of the phenolic compounds from OMW and cause an alteration in the phenolic profile of the media. This removal resulted from a 69% decrease in monophenol profile and 55% of polyphenol profile. Catalase specific activity increased with higher removal efficiencies, indicating its participation in the metabolism of phenols in yeasts (Ettayebii *et al.* 2003).

Decolourization processes

The phenols present in OMW include compounds from a wide range of molecular masses and their characterization has been done by extraction and separation of compounds by their molecular weight. The better known phenolic fractions are the ones with low (<2 KDa) and high (>40 KDa) molecular-masses. The reason lies in the antimicrobial and phytotoxic properties usually assigned to the low molecular weight fraction, and to the coloured and recalcitrant characteristics that are mainly attributed to the high molecular-mass fraction (Flouri *et al.* 1996; Asses *et al.* 2002; D'Annibale *et al.* 2004; Ongen *et al.* 2007). The complex structure of coloured compounds results from the olive oil extraction process, and from oxidation and polymerization reactions of simple phenolic compounds during stabilization and disposal of these wastewaters (Asses *et al.* 2002; Tziotzios *et al.* 2007).

The measurement of total phenols from OMW samples is usually done after extraction of the phenolic fraction with ethyl acetate by using the colorimetric method of Folin-Ciocalteau. It is common to find, in works dealing with phenols and colour removal of OMW, that the removal of phenols by microbial metabolism is not usually correlated with colour removal. In fact, several reports proved that ethyl acetate extractable compounds are mainly monophenols of low-molecular mass, and colour is usually attributed to high molecular-mass compounds (Peréz *et al.* 1998; Ongen *et al.* 2007). It was also stated that simple phenols could be more or less easily metabolised by bacterial and fungal microorganisms, in opposition to polymerized phenols that are quite recalcitrant to microbial metabolism (Hamdi 1992; Asses *et al.* 2002). The best known group of microorganisms able to degrade the compounds responsible for the blackish colour of OMW is the WRF. Nevertheless, the application of fungal pre-treatments at a large scale is limited by the formation of filamentous pellets and mycelia (Asses *et al.* 2002).

Olive mill wastewaters remediation by WRF has been mostly addressed to the identification and characterization of ligninolytic enzymes involved in polyphenols conversion, and also to the improvement of operation conditions to obtain the best enzyme secretion (Sayadi and Ellouz 1995; Hamman *et al.* 1999; Asses *et al.* 2000, 2002; Ayed *et al.* 2005; Olivieri *et al.* 2006; Asses *et al.* 2009b). The WRF group is an efficient extracellular enzyme secretor. These types of enzymes have low substrate specificity, and have been described to be able to degrade aromatic compounds and several xenobiotics, as an adaptation procedure to colonize different habitats. LiP (EC 1.11.1.14) catalyzes the one-electron oxidation of various aromatic compounds with the formation of radicals that are spontaneously decomposed by several pathways. MnP (EC 1.11.1.13) catalyzes the oxidation of manganese from Mn (II) to Mn (III) that can oxidize several phenolic substrates. These enzymes are produced during fungal secondary metabolism in response to nitrogen and carbon starvation (Sayadi and Ellouz 1995).

Several reports have proven the key role of ligninolytic enzymes such as LiP, MnP and Lac (EC 1.10.3.2) in OMW degrading process (Sayadi and Ellouz 1993; Vinciguerra *et al.* 1995; Martirani *et al.* 1996; D'Annibale *et al.* 1998; Peréz *et al.* 1998; Yesilada *et al.* 1998; Sayadi *et al.* 2000; Kissi *et al.* 2001; Tsioulpas *et al.* 2002; Fenice *et al.* 2003; Jaouani *et al.* 2003; Yesilada *et al.* 2003; Dias *et al.* 2004). Although indicated as having key role in decolourization processes of OMW, the expression of these enzymes is different and, apparently, strain/species dependent. That is, for *Ph. chrysosporium* isolates, LiP is the enzyme detected with higher activities, therefore the removal of colour by this fungus has been attributed to the activity of this enzyme (Sayadi and Ellouz 1993, 1995; Sayadi *et al.* 2000; Kissi *et al.* 2001; Aggelis *et al.* 2003). Reports using other fungal species like *Ph. flavido-alba* and *Lentinus edodes* showed that decolourization was achieved without LiP detection, but in the presence of MnP in the culture media (Vinci-

guerra *et al.* 1995; Hamman *et al.* 1999). The authors suggested that decolourization is also a mycelial-binding-dependent process for this strain as also proved for *Ph. chrysosporium* (Hamman *et al.* 1999; Sayadi *et al.* 2000). Martirani *et al.* (1996) and Aggelis *et al* (2003) could not find LiP and MnP activities during OMW bioconversion with *Pl. ostreatus* strains, but high levels of Lac were detected.

Research on OMW decolourization processes has been performed using several different fungal isolates, different culture conditions, and varying OMW characteristics in relation to sterilization, dilution and supplementation of nitrogen and carbon sources. All these factors influence decolourization rates as will be discussed next.

Removal of colour in sterilized OMW

1. Removal of colour in sterilized undiluted OMW: Based on their previous works Sayadi and Ellouz (1995) proved the role of LiP in the decolourization processes during biological treatment with *Ph. chrysosporium*. The authors tested the effect of the addition of veratryl alcohol, a LiP inducer, and obtained a good correlation between production of LiP and decolourization. Although MnP activity could also be detected, the maximum yields of this enzyme in the extracellular medium did not correspond to the decolourization timings. It was also possible to correlate coloured compounds to high molecular-mass compounds and connect decolourization processes to COD removals. The best decolourization percentage was 76% with COD up to 28 g/L. Similar values were reported by Hamman and colleagues (1999) using *Ph. flavido-alba* cultures. In this study, OMW were decolourized in about 70% of soluble coloured compounds, accompanied by overall phenols removal of 90%, in undiluted and non supplemented culture media. Authors attributed the decolourization effect to MnP activity in this fungal species. Although they state that MnP is the most probable ligninolytic enzyme involved in phenolic removal, LiP activity is not excluded in the same type of processes. In fact, LiP activity estimations can be affected due to the inhibition that phenolic compounds can cause to ligninolytic enzymes activity (Sayadi and Ellouz 1992, 1995; Hamman *et al.* 1999).

Strains of *L. edodes* and *Panus tigrinus* were also tested for OMW decolourization abilities (D'Annibale *et al.* 1998, 2004). The assay with *L. edodes* isolates used immobilized cells in polyurethane sponge cubes, considering the promising possibility of biomass recycling (D'Annibale *et al.* 1998). Good removal percentages of 75 and 72% were achieved in the first and second cycles of utilization, respectively. These values decreased with increasing cycle numbers, indicating that the system lifetime was limited to 25 days. Lac and MnP activities were detected in the culture media, revealing some correlation with phenols depolymerization, but not such linear correlations could be made between the presence of enzymes and colour removal.

In an undiluted OMW with COD values up to 82 g/L, high phenolic content (5.5 g/L), supplemented and after removal of total suspended solids, isolates of *Panus tigrinus* were able to achieve a 45% decolourization. This process was accompanied by phenols removal and by Lac and MnP synthesis. The authors referred the long lag time for onset of enzymes detection (9 days) that was ascribed to the time needed by the fungus to adapt its cellular mechanisms to substrates, facing and eliminating their toxic effects (D'Annibale *et al.* 2004).

In a recent survey of yeast naturally occurring in OMW, Sassi and colleagues (2008) selected some isolates able to grow on undiluted OMW and studied their capacity to remove organic matter, phenols and colour from these wastes. The best performing isolates proved to be able to remove some carbon and phenolic compounds at a good extent (40%) but the colour removal was not very efficient. The yeast isolates belonging to the species *Pichia guillermondii*, *C. holstii*, *C. diddensiae* and *C. ernobii* were only able to remove 8% of initial colour, and some of them inclusively caused an increase in medium colour. Moreover, the removal of phenols did not correlate with colour removal. The explanation may reside in the fact that the compounds responsible for colouring the effluent are the most recalcitrant but less toxic high molecular-mass phenols, and the ability to metabolise these compounds has been traditionally attributed to the extracelular ligninolytic enzymes of WRF. The description of yeast ability to produce such type of enzymes is scarce, with the exception of a *G. candidum* isolate (Kim and Shoda 1999; Asses *et al.* 2002; Ayed *et al.* 2005).

2. Removal of colour in sterilized diluted OMW: The former cited *G. candidum* isolate able to decolourize OMW with the synthesis of lignin degrading enzymes, was used in diluted OMW to test the effects of carbon and nitrogen supplementation in decolourization abilities. In these studies the isolate was tested in fresh and stored OMW and the authors verified that the performance of the yeast was improved in fresh OMW, reducing colour in 75% extent, in diluted samples (Asses *et al.* 2002). The stored OMW had a dark colour caused by oxidation of phenols that inhibited the isolate activity. This effect was also attributed to the depletion of sugars, condition necessary to this fungus to exhibit decolourization capacity (Kim and Shoda 1999; Asses *et al.* 2002). Similar results were obtained with *L. edodes* isolates (Vinciguerra *et al.* 1995).

In an attempt to improve the decolourization ability of *G. candidum* in stored OMW Ayed and colleagues (2005) used diluted OMW supplemented with glycerol to give an easily degradable carbon source, and veratryl alcohol to induce LiP synthesis. In these conditions a 65% colour removal was achieved. Measurement of LiP activity allowed the establishment of a direct relation between the presence of the enzyme and the decolourization process.

Also using stored black oxidised OMW, Kachouri and colleagues (2005) isolated several *Aspergillus*, *Geotrichum*, *Penicillium*, *Fusarium* and *Rhizopus* strains. The best OMW growing strain belonged to *A. flavus* and removed about 47% of the black colour. In this experience, neither LiP nor MnP could be detected, and low Lac activity was seen only in the initial days of cultivation. The most active enzyme was tannase, normally a hydrolysable tannins degrading enzyme. The action of this enzyme in decolourization process remained unclear. Latter on, Asses *et al.* (2009a) used the same isolate grown in settler and bubble column conditions, to measure the production of LiP and MnP activities and follow the decolourization processes. They found that considering removal efficiency in both reactors, but due to the difficult maintenance of bubble column reactors, the settler assay could be more adequate to be used for OMW remediation. In this case, 53% colour removal was achieved and this was related with higher productions of LiP and MnP enzymes.

Tsioulpas *et al.* (2002) tested several *Pleurotus* species and although colour removal has not been measured, they reported an alteration of OMW, from black to yellow-bright and brighter, and the enzyme associated with this effect was Lac. Very good results were also reported by Yesilada *et al.* (1998) with *Coriolus versicolor* and *Funalia trogii* isolates. In what colour removal is concerned, the isolates achieved 65 and 81% reduction of colour, respectively, with simultaneous release of high amounts of Lac to the extracellular media. The fungi showed good performances also when immobilized in calcium alginate gels.

Removal of colour in non-sterilized OMW

1. Removal of colour in non-sterilized undiluted OMW: Attempts have been made to compare the decolourizing activity by chemical and biological means. Flouri *et al.* (1996) compared the use of aluminium sulphate, lime and hydrogen peroxide with the use of several *Pleurotus* species to decolourize OMW. Despite the lack of quantitative results they concluded that the use of chemicals, usually

more expensive, is not worthy when compared with the use of fungi for the same purpose. The compounds tested were only able to remove 50% of colour when used in very high and expensive quantities.

Recently D'Annibale and coworkers (2006) and Olivieri *et al.* (2006) tested the effects of *Panus tigrinus* and *Pl. ostreatus* isolates, respectively, in colour removal from OMW using stirred tank reactors and bubble column reactors. The best results were obtained in bubble column reactors, but the isolate of *Pl. ostreatus* could not remove efficiently the colour of the effluent. Although good phenols removals were achieved (90% after nutrients addition) the colour was not removed probably because OMW was not heat-treated prior to bioconversion as reported previously (Olivieri *et al.* 2006). The assays with *Panus tigrinus* showed that the treatment efficiency was negatively affected in the stirred tank reactor, probably due to the occurrence of shear stress phenomena, causing enzyme denaturation and so lowering the bioconversion and decolourization rates (D'Annibale *et al.* 2006). In the pneumatically agitated system faster and higher colour removals were achieved with the same isolate. Colour removal achieved 72% in 6 days and stabilized until the end of incubation time, as opposed to 76% in STR after 9 days. It was also observed that MnP and Lac activity peaks were obtained earlier in BCB than in STR. Maximum enzyme activities were registered in BCB.

In another experience using STR conditions, but with adapted *T. versicolor* strains, Ergull and colleagues (2009) also registered low colour reduction (18%) with different aeration rates, concluding that aeration had no direct effect on decolourization rates. Lac and MnP activities were monitored and registered, reaching their peaks at the seventh day of culture.

2. Removal of colour in non-sterilized diluted OMW: It was already mentioned that removal of organic matter from OMW is usually more efficient when using diluted samples. The removal of phenols and colour does not deviate from this fact. Dilution lowers the toxic compounds concentration and attenuates the recalcitrant colour of these wastewaters.

As for undiluted OMW samples, also for diluted ones the WRF *Ph. chrysosporium* has been the most used in experimental bioremediation assays. In Dhouib *et al.* (2006) work the fungus was used to pre-treat the olive mill effluent in order to be afterwards treated by anaerobic and ultrafiltration processes. The isolate was inoculated in a bubble column bioreactor and the OMW was diluted whenever COD values exceeded 100 g/L. The authors do not refer the abatement in colour caused by the aerobic treatment, although it is referred that this pre-treatment coupled with anaerobic digestion reduced colour in 50%. It is mentioned that the treatment with the fungus reduced toxicity of the effluent towards *Vibrio fischerii* luminescence in 36%. The authors claim that these results are particularly important because they used non-sterilized waters, where the fungus must compete with other microorganisms, and the system was not controlled for temperature or pH.

Sayadi *et al* (2000) used *Ph. chrysosporium* strains to investigate which OMW phenolic fractions (low <8 KDa, or high >60 KDa) were the most problematic. They concluded that using a culture medium where LiP was abundant the fraction of low molecular mass was extensively decolourized (60%), starting at day three of incubation, and the fraction of medium molecular-masses was decolourized at the same extent, but latter on (9 days). The decolourization of the fraction with high molecular-mass phenols was severely affected. They also tested a culture medium with high MnP production and no or low LiP production, and verified that decolourization in the three fractions was very low. So, they concluded that high molecular-mass compounds do not inhibit *Ph. chrysosporium* growth but affect their degrading system when LiP is not present in the extracellular medium. Nevertheless, high molecular-mass phenolic compounds affect the activity of this enzyme in about 80%.

Kissi *et al.* (2001) compared the decolourization ability of *Ph. chrysosporium* with that of *Pl. ostreatus*, and found that the former was able to remove 95% of colour in 15 days of incubation as opposed to 65% obtained with strains of *Pl. ostreatus* when grown in batch cultures of non-sterilized, 20% diluted and supplemented OMW. Moreover, the authors could identify a correlation between decolourization and removal of COD content and phenols. However, in less diluted OMW (50%) the same *Ph. chrysosporium* strain decreased its ability to only 50% removal of the effluent colour. LiP activity increased during the first 6 days of incubation, declining onwards, while MnP and Lac activities increased from the 6th day. Because the most notable abatement of colour occured during the first 6 days, the authors concluded that LiP was the enzyme playing a key role in dephenolization and decolourization process in *Ph. chrysosporium* isolates, as already mentioned before. Using the same approach Dias *et al.* (2004) compared *Ph. chrysosporium* isolates with their own isolate designated Euc1. Using a rich medium with diluted OMW (20%), the isolate Euc1 removed 73% of colour after a 12 days incubation period. This decolourization was concomitant with high phenols and COD removals, and large Lac production. The authors associated the decolourization to the action of this enzyme, and reinforced the idea that Lac must also play a important role in biodegradation of OMW.

Other fungi belonging to *Aspergillus* species non-ochratoxin producers were tested for their decolourization ability in two fold diluted OMW supplemented with various compounds. The best performances were obtained in the medium with yeast extract as supplement. Decolourization rates varied from 44% for *A. niger*, to 81% for *A. tubingensis* and *A. aculeatus*. Enzymatic activities were measured for Lac, MnP and tannase in a medium with tannic acid. Only tannase activity could be detected but the report does not exclude the role of MnP and Lac in OMW degradation (Ongen *et al.* 2007).

In one of the most extensive experiments 58 fungi belonging to different species were screened for their decolourization abilities using model dyes, and 17 were chosen to grow on OMW-based solid media with COD values ranging from 25 to 100 g/L, and in static and agitated cultures. The best overall results were obtained with *Pl. sajor caju*, *Coriolopsis polyzona*, *L. tigrinus* and *Pycnoporus coccineus*, that removed 72, 75, 50 and 58% of colour, respectively in the more diluted media (25 g/L COD). The same strains could decolourize two model dyes using the extracellular ligninolytic enzymes LiP, MnP and Lac. It is curious to remark that, in the screening tests, LiP production was detected only in two species, *C. polyzona* and *Ph. chrysosporium*, while the other species tested produced preferably MnP and Lac, confirming what was said above about strain dependency of the ligninolytic enzymatic system of fungi (Jaouani *et al.* 2003).

The yeasts *G. candidum*, *Y. lipolytica* and *C. tropicalis* have also been tested for their capacity to remove colour from OMW. Fadil and colleagues (2003) used *G. candidum* isolates and verified that they could remove colour from diluted and nutrient supplemented OMW at a colour removal rate of about 30%. More promising results were published by Asses *et al.* (2009b) that, for fresh diluted OMW (42 g/L COD) supplemented with 1 g/L ammonium sulphate achieved 65% of colour removal. The same authors also tested the capacity of the *G. candidum* isolate to remove colour from stored black OMW, and decolourization was achieved at a rate of 48%, only when the media was supplemented. Both LiP and MnP activities increased during the first 6 days (time of peak) of incubation and then decreased till the end of incubation period (8 days). The decolourization rate was higher in the same period, so confirming the role of these enzymes in decolourization and dephenolization of OMW.

CONCLUSION AND FINAL REMARKS

The wastewater disposal and treatment of OMW from olive oil extraction industries are a problem presently affecting the Mediterranean countries. This type of problems is likely to spread to other world regions, where intensive olive plant cultures are now starting to develop.

Intensive research has been devoted to find the best forms to remediate the pollution caused by these effluents, but the treatment of this type of agro-industrial residue has many drawbacks, resulting from their complex composition and high level of toxicity.

None of the proposed treatments revealed to be adequate for a complete and economically efficient treatment, when used individually, and only the combination of several methods, joining together chemical, physical and biological knowledge might be the key for an economically viable reutilization o these wastewaters.

Bioremediation of OMW is not a novel issue and seems to be a feasible choice. It has been the focus of several studies for the last two decades. Anaerobic digestion is probably the most appropriate biological treatment. Due to the fact that anaerobic digestion can be negatively affected by some compounds research has focused on aerobic treatments, for removal of those toxic compounds. Fungi are a ubiquitous microbial group, which developed ability to resist to several xenobiotic and toxic compounds present in OMW, and present characteristics for effective removal of recalcitrant compounds from these wastewaters. A resume of the literature cited in this review, focusing on treatment with fungi can be found in **Table 1**.

The treatments of OMW with fungi usually do not aim high removals of organic matter, but dephenolization and decolourization. The OMW treated with some of these organisms revealed to be less inhibitory for seed germination and in bacteria toxicity tests.

Although dephenolization can be easily achieved by filamentous fungi and yeasts decolourization is more efficient with WRF. This group of fungi has developed an enzymatic system, characterized by low substrate specificity, but high efficiency in degradation of complex compounds like cellulose and lignin like compounds.

The treatments of OMW with fungi are not a finished issue. Improving what is known so far will enable the scientific community to help olive oil producers to manage their residues in an efficient way. The results reported by several investigation teams encourage the search for new strains and isolates and the development of economically feasible conditions for the utilization of these biological treatments not only at a pilot scale.

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