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# Bioremediation of Olive Oil Mill Wastes Through the Production of Fungal Biomass

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ABSTRACT: The environmental problems associated with the olive oil extraction process have a crucial importance, particularly for the Mediterranean countries. The enormous quantities of the black polluting effluents produced are difficult to handle as they are very phytotoxic and possess strong antimicrobial activities. In addition, their particular physiochemical properties hinder considerably treatment with conventional methods. Thus, biological remediation of the olive mill wastes is also attempted with a large array of fungi aiming at neutralizing their heavy pollutant effect, for converting them into new value-added products or for rendering them susceptible to further degradation treatment. White-rot fungi have proven to be particularly efficient at decomposing the phenolic substances which are mainly held responsible for the dark colour and the toxicity of the effluent. Among them, *Pleurotus* species presented the potential to degrade and convert both olive mill waste waters and olive press cake into mushrooms and fodder. In parallel, remediation is achieved through biomass production with a simultaneous decolourization and decrease in phytotoxicity. The distinct properties of certain *Pleurotus* strains could be further exploited for the integrated management of agricultural byproducts.

## 1 INTRODUCTION

One of the most serious environmental problems associated with the agroindustrial sector in the Mediterranean basin is the safe disposal of olive oil mill wastes. According to recent data obtained from the International Olive Oil Council, 98% of cultivated olive trees occur in this particular area, i.e. Spain, Italy, Greece, Turkey, Tunisia, Portugal, Morocco, Syria, Algeria, etc.; while the rest originates mainly from

Argentina, United States, Mexico, Australia, South Africa and Asia (Iran, Afghanistan and China). During the last decade, world olive oil production fluctuated significantly and presented an annual average of 1.7 million metric tons with a mean annual increase of 1.6%. On the other hand, world consumption despite being almost equivalent to production, showed a lower rate (1%) of increase per year. Within the European Union, production and consumption figures of olive oil surmounts three quarters of the respective world totals. Thus, the importance of this particular agricultural activity for South European countries is more than evident.

In the past, olive oil processing was mainly carried out with discontinuous press-type mills but the intensification of production lead to the abandonment of this traditional extraction method and the installation of the more efficient and of higher capacity centrifugal ones. The unfortunate consequence of adopting the latter type is the generation of large amounts of olive oil waste-waters (OMW), and of solid residues known as olive press cake (OPC), that soon created major environmental problems in all olive oil producing countries. As the processing of 100 kg of olives generates roughly 100 l of OMW, 35 kg of OPC and 4 kg of leaves, the annual world olive oil production yields enormous quantities of these wastes: 8.1 million cubic meters, 3.2 million metric tons and 0.3 million metric tons, respectively. Moreover, the total amount of twigs and leaves resulting from pruning each year reaches 15 million metric tons (Nefzaoui 1995). The amount of OMW produced in the Mediterranean area alone could be roughly compared to the pollutant effect of the wastes generated by a population of more than 20 million people. Although OPC and olive-tree leaves do not pose similar environmental hazards, they remain largely unexploited as they are generally used as fuel or incorporated into the soil.

However, both types of olive mill wastes (liquid and solid) have the potential, as recent studies have shown, to form the base for the generation of a large array of products such as plant fertilizers and/or soil conditioners, substrates for the production of biomass and protein (algae and yeasts), biogas, biopharmaceuticals, biopolymers, feed additives, fodder and edible mushrooms (Codounis *et al.* 1983, Ramos-Cormenzana 1986, Chatzipavlides *et al.* 1988, Quevedo-Sarmiento *et al.* 1991, Takashi *et al.* 1994, Balis 1995, Ramos-Cormenzana *et al.* 1995, Zervakis *et al.* 1995).

## 2 OLIVE MILL WASTES: PROPERTIES AND TREATMENT

### 2.1 *Chemical composition, physical and biological characteristics*

The OMW are recalcitrant dark brown-black effluents, with a specific weight of 1.05 g/ml, a distinctive odor and contain high concentrations of



aromatic compounds (Table 1). This particular chemical composition in relation with their acidic pH (4.5-5.5), the very complex redox system (conductivity: 8,000-16,000 μs), the high buffer capacity, tension activity and stability as well as the large amounts of suspended solids have hindered considerably the development of an environmentally accepted system for their treatment and disposal (Fiestas Ros de Ursinos and Borja Padilla 1992). In addition, the increased values for biological oxygen demand (BOD: 40,000-100,000 ppm), chemical oxygen demand (COD: 50,000-150,000 ppm) and the $LD_{50}$ toxicity for fish (8.7%) give a clear indication of the degree of the pollution problem (Fiestas Ros de Ursinos 1977). Thus, OMW when fresh are highly phytotoxic (Pérez *et al.* 1986, Flouri *et al.* 1990) and possess strong antimicrobial properties mainly due to their high content in phenolic substances (Juven and Henis 1970, Martinez *et al.* 1986, Moreno *et al.* 1987). On the other hand, as OMW is a natural product rich in organic matter and contains appreciable amounts of sugars, minerals and other substances, many investigators have contemplated its use as a fertilizer and soil conditioner with a direct and indirect effect on plants; OMW can be a source of nutrients and energy for numerous beneficial microbes of the rhizosphere as well (Pérez and Gallardo-Lara 1987, Saiz-Jimenez *et al.* 1986, Flouri *et al.* 1990).

Table 1. Composition of olive mill waste waters (Codounis *et al.* 1981, Balice and Cera 1984, Ramos-Cormenzana 1986, Saiz-Jimenez et al. 1986, Pérez *et al.* 1987).

| Component | Content (%) | Major Constituents |
|---|---|---|
| Water | 83-92 | |
| Fats | 0.03-1.00 | Oil residues |
| Nitrogen compounds | 1.2-2.4 | Glutamine, proline, histidine, glycine, arginine, etc. |
| Sugars | 2.0-8.0 | Raffinose, mannose, sucrose, glucose, arabinose, ramnose, etc. |
| Organic acids | 0.5-1.5 | Acetic, citric, fumaric, lactic, malonic, malic, oxalic, etc. |
| Polyalcohols | 0.5-1.5 | glycerin |
| Pectin | 0.6-1.5 | |
| Minerals | 0.4-1.8 | K, P, Na, Ca, Mg, Fe, Mn, Zn, Cu, Cl, S. |
| Phenolic compounds | 0.3-0.8 | Flavonoids (Luteolin, Quercetin, etc.), phenols (caffeic, cinnamic, 2,6-dihydroxybenzoic, syringic, vanillic, veratric, ferulic, p-coumaric, hydroxytyrosol, pyrocatechine, etc.), oleuropein, etc. |

The OPC is the solid fibrous residue from the olive oil extraction process and contains fragments of skin, pulp and seeds. It is usually treated with solvents to produce at a later stage a lower quality oil used mainly by the cosmetics industry. It is rich in lignocellulose compounds but as it has a low protein content (Table 2) is not suitable for animal feed, and is primarily used as fuel. This type of olive mill waste remains thus largely unexploited although large quantities of it are produced each year (in Greece alone: 250,000 metric tons).

Table 2. Composition of olive press cake (Manios and Balis 1983).

| Component | Content (% d.w.) |
|---|---|
| Moisture | 16-17 |
| Fats | 2.5 |
| Protein | 6.6 |
| Sugars | 2.2 |
| Cellulose | 37.6 |
| Hemicellulose | 13.1 |
| Lignin | 21.6 |
| Minerals | 1.5-2.5 |
| Other compounds | 13.5 |
| Ash | 3.0 |

### 2.2 *Treatment through the use of physiochemical processes*

The usual method of disposal of OMW was directly onto land or into water receptors, i.e. domestic sewage, rivers and sea, without any prior treatment. This practice has created huge environmental problems and was therefore abandoned (at least by the larger olive oil producing mills) and replaced by the system of open evaporation ponds. However, the main drawbacks of such methods were the emission of bad odours, the safe deposition of the remaining sludge, the high maintenance expenses and the waste of the valuable ingredients of OMW. Attempts to improve the technical and economical efficiency of this system led to the adoption of integrated processes (Fiestas Ros de Ursinos and Borja Padilla 1992) based on: i.) intensification of the natural evaporation capacity of the ponds through the use of panels with large interchange surfaces whose function was further enhanced by the use of sprinklers, or by wind powered hydro-pumps. ii.) thermal concentration by means of the single effect forced evaporation of OMW after neutralization with sodium hydroxide followed by aerobic biological treatment, and the double stage forced evaporation followed by inverse osmosis, and iii.) physiochemical methods which are divided into multiple steps and are based in treating the OMW with the appropriate flocculants and coagulants, separating the resulting sludge by decanting or flotation, then neutralizing the pH of the liquid effluent prior to enriching it with nutrients and recycle it through an



aerobic biological filter or driving it into static decanters and finally passing through inverse osmosis or ultrafiltration apparatus. However, the remediation of OMW pollutant loads either through physical or chemical processes alone have failed to come up with sound answers, and is only through their combination with biological approaches that more rational solutions might evolve.

## 3 BIOLOGICAL REMEDIATION OF OLIVE MILL WASTES

The strong inhibitory action of OMW against a number of soil bacteria and fungi like *Lactobacillus*, *Bacillus*, *Chaetomium*, *Geotrichum*, *Rhizopus*, *Rhizoctonia* (Saiz-Jimenez and Gomez-Alarcon 1986, Moreno *et al.* 1987, Gonzalez *et al.* 1990) was rather recently correlated with its phenolic content (Pérez *et al.* 1992) and was demonstrated that this detrimental effect is particularly pronounced against sporulating soil bacteria (Paredes *et al.* 1986).

However, many researchers, in the past, attempted to exploit the potential of certain microorganisms to produce their colonies on OMW by detoxifying it. For example, the high amount of nutrients present in olive mill wastes enhanced the growth of the yeast *Torulopsis utilis* (Fiestas Ros de Ursinos 1958). This yeast was shown to grow well on OMW by consuming the sugars and minerals, needing only the addition of nitrogen in order to degrade, through a fermentation process, the phenolic substances of the medium and produce single cell protein (SCP) of high biological value suitable for fodder. Later on, other studies adopted the same approach and by selecting yeast species like *Saccharomyces lipolitica* (Ercoli and Ertola 1983), *S. rouxii*, *S. chevalierie*, *Candida krusei* (Gharsallah 1993), which could tolerate the presence of OMW polyphenols, succeeded in decreasing the COD by 40 to 50% and yield SCP. The growth of the yeasts *S. cerevisiae*, *C. wickerhamii*, *C. molischiana* on the same substrate was also exploited for the production of ethanol (Bambalov *et al.* 1989).

Several species of the genus *Aspergillus* were found to be particularly efficient at degrading OMW, many of them being isolated from this substrate. Thus, strains of *A. niger* yielded large amounts of biomass with the simultaneous elimination of COD and improved the filtration kinetics of the effluent (Raimbult and Mazard 1980, Hamdi *et al.* 1991, Hamdi and Ellouz 1992), while *A. flavus*, *A. versicolor* and *A. terreus* were reported to grow well and decrease the content of OMW in polyphenols (Oka *et al.* 1971, Saiz-Jimenez and Gomez-Alarcon 1986, Martinez-Nieto *et al.* 1991). Similar encouraging results were obtained through the use of

*Giberella fujikuroi* (giberellic acid production, Hernandez and Mendoza 1976), *Cladosporium sphaerospermum*, *Pycnoporus cinnabarinus*, *Penicillium* spp. (SCP and mycelium production, Saiz-Jimenez and Gomez-Alarcon 1986), and *Aureobasidium pullulans* (polysaccharides production, Quevedo-Sarmiento *et al.* 1991).

## 4 CULTIVATION OF EDIBLE MACROFUNGI ON OLIVE MILL WASTES

The use of Basidiomycetes was rather lately adopted for the remediation and exploitation of olive mill wastes (Table 3). The white-rot fungi were found to metabolize efficiently the phenolic compounds present in OMW. Strains of *Phanerochaete chrysosporium*, the most studied lignolytic fungus, have demonstrated they can degrade persistent aromatic pollutants (Hammel 1989) while they grow well in media composed of OMW (Saiz-Jimenez and Gomez-Alarcon 1986). Consequently, *P. chrysosporium* was employed as a decolourizing agent of the black polluting effluent produced by olive oil mills. Decolourization was found to be extensive, occurred during the first phase of growth when glycerol was used as the carbon source or during secondary metabolism in nitrogen-limited cultures, and was due to the depolymerization of high molecular mass aromatics together with the mineralization of a wide range of monoaromatic compounds (Pérez *et al.* 1987, Sayadi and Ellouz 1992). This action was combined with the significant decrease of COD (up to 80%) indicating, thus, the relation of this pollution parameter to coloured substances (Sayadi and Ellouz 1992). Furthermore, efficient lignin peroxidase producer strains of *P. chrysosporium* exhibited the highest rates of OMW colour removal and the most vigorous growth, whereas isolates of *Phlebia radiata*, *Dichomitus squalens*, *Polyporus frondosus* and *Coriolus versicolor* presented to a lesser extent decolourization activity and COD removal because of the incomplete degradation of high molecular weight polyphenols (Sayadi and Ellouz 1993).

In an attempt to combine the detoxifying effect which the white-rot fungi exert on OMW with the production of biomass suitable for human food and animal fodder, recent studies were oriented towards the use of *Pleurotus* species. Fungi belonging to this particular genus possess a very efficient lignocellulose degrading system, with the ability to preferentially decompose lignin and yield products of high nutritional value (Platt *et al.* 1983, Zervakis and Balis 1992). Initial experiments with *P. ostreatus* demonstrated the suitability of OMW to serve as a nutrient medium for submerged cultures, and although a lag phase was observed



at the first stages of mycelium growth, biomass was developed and the concentration of phenolics decreased as a result of laccase activity (Galli *et al.* 1988, Tomati *et al.* 1991). Similar results were obtained when *Lentinula edodes* was inoculated into an OMW-based substrate (Grappelli *et al.* 1991).

Table 3. Outcome of the growth of mushroom species on substrates based on olive oil mill liquid wastes.

| Fungus | Outcome | Reference |
|---|---|---|
| *Coriolus versicolor* | Growth supported | Saiz-Jimenez and Gomez-Alarcon 1986 |
| *Phanerochaete chrysosporium* | Good growth, decolourization | Saiz-Jimenez and Gomez-Alarcon 1986 |
| *P. chrysosporium* | Decolourization | Pérez *et al.* 1987, Sayadi and Ellouz 1992 |
| *Pleurotus ostreatus* | Mycelium production | Galli *et al.* 1988 |
| *P. ostreatus* | Degradation of phenolics | Tomati *et al.* 1991 |
| *Pleurotus eryngii, P. ostreatus, P. "sajor-caju", P. "florida"* | Detoxification, Mushroom production | Sanjust *et al.* 1991 |
| *Lentinus edodes* | Good growth, degradation of phenolics | Grapelli *et al.* 1991 |
| *P. chrysosporium, C. versicolor, Polyporus frondosus, Phlebia radiata* | Decolourization | Sayadi and Ellouz 1993 |
| *Pleurotus* spp. | Good growth, decolourization | Flouri *et al.* 1995 |
| *P. eryngii, P. ostreatus,* | Detoxification, mushroom production, | Zervakis *et al.* 1995 |
| *Pleurotus pulmonarius*[1] | Altered morphology of mycelial colonies | |

[1]Olive press cake also served as a substrate for fungal growth.

The adaptation of the *Pleurotus* strains for growing well in such media seems to be an essential prerequisite before any cultivation trials are to be carried out. Sanjust *et al.* (1991), after inoculating a mixture of expanded perlite — OMW with *P. ostreatus*, *P. eryngii*, *P. "sajor-caju"* and *P. "florida"* isolates, have reported the development of normal appearing basidiomata. The yield of the harvested mushrooms varied between 8.4 and 17.5% of the weight of substrate depending on the fungus, while their phenolic and protein content were comparable to those measured in commercial strains. On the other hand, although residual toxicity of OMW was significantly lowered thanks to *Pleurotus* mycelia growing in stationary cultures, the pigmentation of the wastes was not much affected.

However, this was not the case in a recent series of experiments conducted by Flouri *et al.* (1995) and Flouri and Zervakis (unpublished data), where several *Pleurotus* isolates were found to be more efficient at decolourizing both liquid and solidified (with agar) OMW than *P. chrysosporium*. In addition, screening and comparative tests among sixteen strains belonging to six different *Pleurotus* species (i.e. *P. cornucopiae*, *P. cystidiosus*, *P. dryinus*, *P. eryngii*, *P. ostreatus* and *P. pulmonarius*) revealed that *P. ostreatus* (ATCC 38538) and *P. cornucopiae* (ATCC 38547) were particularly effective at degrading the aromatic compounds responsible for the OMW colour. The mycelium growth rates recorded on solid substrate containing 25-100% OMW were comparable to those noted on conventional media (Zervakis and Balis 1996), whereas their lag phase were prolonged especially in heavier concentrations of OMW. Furthermore, there were cases when strains of *P. pulmonarius*, *P. ostreatus* and *P. eryngii* advanced faster on 25% OMW, or even at 75-100% OMW (*P. dryinus*), than on potato dextrose agar or malt extract nutrient media. Of great interest, though needing a further detailed study, was the observation that *P. dryinus* and *P. cystidiosus* isolates growing in Petri dishes with solidified OMW presented altered colony characteristics. Thus, *P. dryinus* ceased to produce chlamydospores on mycelial cultures which in normal conditions are formed in abundance, being a prominent taxonomic character of this species; while the equally distinctive synnematoid fructifications of *P. cystidiosus* appeared in reduced number and with disturbed anatomy (non existent or very scarce and immature conidial masses on the top of coremia).

On the other hand, *P. eryngii* produced high biomass yields in various concentrations of both pretreated and non treated OMW outperforming *P. ostreatus* and *P. pulmonarius* strains (Zervakis et al. 1995). In particular, liquid cultures containing 25-50% OMW supported heavy mycelium production, thus demonstrating an efficiently operating lignin-degrading system induced by the phenolics present in the medium. The degradation of phytotoxic substances present in OMW media for *Pleurotus* growth were assessed by plant seeds germination tests. The germination indices were significantly increased when spent OMW substrates were measured versus raw OMW. In this case, *P. ostreatus* decomposed better the compounds responsible for the effluent's toxicity and thus germinability maintained increased values (80-90% of the control) even in low OMW dilutions (up to 50%).

In the past, OPC was only once reported to serve as a substrate for the production of fungal biomass (Karapinar and Worgan 1983). Strains of *Aspergillus oryzae*, *A. niger*, *Sporotrichum pulverulentum* and *Trichoderma viride* were found to hydrolyze this medium by halving its cellulose content and increasing the protein content by 75%. Much more recently though the OPC was tested for the cultivation of *Pleurotus* mushrooms (Zervakis *et al.* 1995). Three *Pleurotus* isolates (i.e. *P. ostreatus*, *P. eryngii* and *P. pulmonarius*) were inoculated by means of mycelium-agar plugs on sterilized nutrient media composed of plain OPC or OPC supplemented with various dilutions of raw OMW (12.5%-50%), which filled 11 plastic beakers. Colonization of the different types of substrates was completed within one to two months (faster for the plain OPC, no growth at all in the OPC supplemented with 50% OMW), and fruitbodies primordia appeared sooner for the *P. pulmonarius* at low OMW concentrations and considerably later for the *P. eryngii* strain. Earliness values were in general comparable to those quoted either for other commercial *Pleurotus* strains or for isolates fructifying on conventional substrates (Royse *et al.* 1991, Zervakis and Balis 1991). On the other hand, yields and biological efficiencies were also adversely affected by the OMW supplementation; the plain OPC presented the higher values for both cultural parameters, while *P. eryngii* produced the most encouraging results as far as quantity and quality of the harvested mushrooms were concerned. It is important to state here that no malformed basidiomes were observed for this particular species despite the fact that production flushes were rather irregular, whereas the composition of the substrate influenced the yield and the appearance of *P. pulmonarius* fruitbodies. In general, although those media (OPC and OPC plus OMW) were not in any way pretreated or supplemented with nutrients in order to diminish their growth-inhibitory effect or enhancing their nutritive properties, *Pleurotus* cultivation was achieved with both biological efficiency values and mushroom quality being very promising, especially in the case of *P. eryngii*.



## 5 PROSPECTS AND ORIENTATION OF RELEVANT FUTURE STUDIES

Despite the severe problems encountered up till now at coming up with the appropriate solution for the disposal of notorious agricultural pollutants like the OMW, a rational and economically feasible answer could be the adoption of an integrated scheme of activities within the frame of sustainable agriculture, which efficiently combines physiochemical and primarily biological approaches with the minimal environmental impact and capital outlay. The application of biotechnological methods conforming to fundamental agro-ecological principles can be realized along the production process of olive oil through the use of pheromones and insecticide traps for pest control, elimination of volatile emissions of the mills with biofilters, bioremediation and recycling of OMW with the aid of nitrogen-fixing bacteria for being used as fertilizers, co-composting of solid residues and effluents for soil amendments and conditioners, and valorization of OPC and OMW for producing edible mushrooms and fodder (Balis 1995, Flouri *et al.* 1990, Haniotakis 1993, Zervakis *et al.* 1995).

With regard to mushrooms in particular, the outcome of preliminary studies demonstrated that OPC alone or supplemented with OMW might well serve for *Pleurotus* cultivation, provided that the suitable growing techniques are developed together with the careful selection of the most efficient strains/species and the amelioration of the substrate itself with the appropriate pretreatment. The spent OPC could then be integrated into animal feed, as the increase in the protein content together with the improved digestibility due to the preferential lignin degradation, contribute to its nutritional value.

Last but not least, the detoxification of OMW achieved through the degradation of phenolic substances with *Pleurotus* mycelium, is evident by the decolourization of the effluent and the significant decrease of its detrimental properties on plants. The fungal biomass could be further exploited as liquid spawn, fertilizer and fodder enrichment. These findings again indicate the need for further research towards the exploitation of the biological potential of certain edible white-rot fungi at remediating agricultural wastes.

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