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Chapter 11

NEW APPROACHES TO OLIVE MILL WASTES BIOREMEDIATION

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ABSTRACT

Remediation of olive mill wastewater (OMW) is an important issue associated with olive-oil manufacturing, a widespread activity in the Mediterranean area. This high organic loading effluent contains water, organic acids, high-molecular-weight polyphenols such as tannins, antocyanins and catechins, which are considered to be responsible for its brownish black colour and ecotoxic properties. The composition of OMWs is highly variable with respect to each individual component, depending on the process conditions and on the agricultural specificities. Thus, different approaches are applicable concerning to OMW treatment and valorisation , considering the specificities of its production and in particular the oil extraction process. Besides there are several physical, physico-chemical, biological and combined processes to OMW detoxification, each may represent an opportunity for a specific condition. It is important to explore new possibilities that are both environmentally sustainable and economically viable.

Under the biological processes the use of fungi and in particular white-rot fungi present a potential interesting alternative for depollution and biological chemicals production or for protein production for feeding. In this aspect we have been testing the ability of a “white-rot” fungus, *Bjerkandera paranensis*, to use undiluted OMW from a two phase process mill. A chronic ecotoxicity test (*Vibrio fisheri* growth inhibition test) demonstrated that the growth of this fungus contributed for a significant decrease of the OMW ecotoxicity and demonstrating the potential for further studies with this strain for an alternative biological route to OMW treatment and valorization.

Keywords: OMW; biotreatment; *B. paranensis*; ecotoxicity.

INTRODUCTION

The olive oil industry represents one of the most important economic agro-food sectors in the Mediterranean countries that are responsible for the production of more than 98% of the world's olive oil, estimated at over 2.5 million metric tons per year of which about 75% is produced in the European Union (EU). The largest European olive oil producers are: Spain with 36%, Italy with 24%, and Greece with 17%, of the world's total production. The next largest producer is Portugal, with a production of one order of magnitude lower than the three leading countries, followed by France, Cyprus and Croatia (McNamara *et al.*, 2008; Lopes *et al.*, 2009).

During olive oil extraction a process that is conducted by mechanical procedures in olive mills, large amounts of liquid efluents and solid residues are produced, with a high organic load, the nature of which depends on the technology of the extraction process and system employed. Three processes are widely used worldwide for industrial-scale extraction of oil from olives, the traditional discontinuous press-cake system and the continuous three phase decanter system and the modern two-phase centrifugation system. Nowadays, in European countries, two-phase and three-phase centrifugation systems (continuous processes) are the ones most commonly used. The three phase system, introduced in the 1970s to improve extraction yield, produces three streams: pure olive oil, a liquid waste by-product known as olive mill wastewater (OMW) and a solid cake-like by-product called olive cake (bagasse or *orujo* in Spanish*).* From an environmental point of view, OMW is considered the most critical waste emitted by olive mills in terms of both quantity and quality. More recently a two-phase centrifugation system was introduced in the 90´s in Spain as a more ecological approach for olive oil production, yielding the olive oil (liquid phase) and a highly wet solid paste, combining the solid residues from the olives and the process water. This residue is known as *alperujo(*in Spanish) corresponding to the main two-phase olive-mill waste (TPOMW). In this way water consumption during the process is drastically reduced. However a much reduced liquid effluent from the washing operations still has to be disposed.

The quality and quantity of the constituents of olive mill wastewater (OMW) are dependent on many factors: type of olives, type of soil, cultivation system and production process. The OMW contains a majority of the water-soluble chemical species present in the olive fruit, a very high organic load (chemical oxygen demand, COD) typically ranges from 50-150 gl^{-1}, about two orders of magnitude higher than municipal wastewater and has an acidic pH (4-6). Phenolic compounds that are present in olive stones and pulp tend to be more soluble in the water phase than oil, resulting in concentrations ranging from 0.5-25.0 gl^{-1} (McNamara *et al.*, 2008). These phenolic compounds are the main determinants of antimicrobial and phytotoxic olive-mill wastes actions and are responsible for its characteristic black colour (Cabrera *et al.*, 1996).

A common way of dealing with the OMW in many Mediterranean countries was to discharge directly into sewer network an option that is unacceptable without a previous complex and expensive pretreatment; alternatively and when no sewage network is available the favored option it is to store it on artificial lagoons beside the mills where it is left to evaporate until the next season. These ponds are often leaking causing ground water pollution

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and mal odor problems. The use of this water for irrigation is possible but under stringent regulations, in many countries. Since the setting up of more stringent regulations concerning public waste disposal, there is a growing interest in the development of new technologies and procedures for the purification of this wastewater (El-Gohary *et al.*, 2009).

Due to the seasonality of olive oil production the OMW treatment process should be flexible enough to operate in a non-continuous mode. Besides, the olive mills are small enterprises, scattered around the olive production areas, making individual on-site treatment options unaffordable (Paraskeva and Diamadopoulos, 2006; Massadeh and Modallal, 2008; Morillo *et al.*, 2009). The treatment of liquid wastes (OMW) produced from olive oil production is still a major challenge facing this industry and still unsolved in the olive-oil-producing countries. The high recalcitrant organic load and the associated toxicity make the treatment of OMW a challenge.

Many physical, thermal, physico-chemical and biological management strategies or combined and miscellaneous processes have been proposed for the treatment and valorisation of OMWs but a solution both environmentally friendly and economically viable is not yet widely available.

Physical Treatments

Physical treatments are applicable to cleanse at least as a pre-treatment to OMW. Dilution is an efficient, cheap and simple process to reduce the organic load. However, dilution increases the volume of effluent to treat, which results on bigger needs of storage and, consequently, associated economical costs. Sedimentation/Settling is applied to remove suspended particles in OMW. In fact, most of the organic matter is suspended forms so this process allows reducing COD by separating the supernatant with a low COD of a high COD settled sludge. This is a slow method and usually requires the addition of expensive flocculants in order to enhance efficiency of the process by facilitating the aggregation of small particles. Filtration is another possibility to purify OMW by using a filter that removes suspended particles from the solution. Flotation removes not only solid but also liquid particles, such as oil droplets and suspended solids from OMW, by adding a gas (usually air), which facilitates separation because particles or suspended solids adhere to the air bubbles. Flotation may be applied by using dissolved air flotation or gravity flotation systems. Centrifugation is applicable both in OMW and in 2 phase's olive mil mill wastewater (2POMW) in order to reduce COD and recover oils. Membrane technology is also a physical process based on concentration and separation of OMW specific particles. Separation is done on the basis particle molecular size and shape with the use of pressure and specially designed semi-permeable membranes. There are a few techniques based on membrane technology. Reverse Osmosis membrane separation systems allow removing inorganic salts from OMW. Otherwise, Ultrafiltration separates different fluids or ions while Nanofiltration is based on the pressure difference between the feed (retentate) and the filtrate (permeate) side on a selective separation layer formed by an organic semipermeable membrane. Abdelhafidh *et al* (2006) used ultrafiltration to improve a process consisting in a pre-treatment with *Phanerochaete Chrysosporium* followed by anaerobic digestion. The process has not only decreased COD and heavy metals. Moreover this technology allows decolourise and obtained a less toxic and pathogens free product. This is a self-sustained process in terms of energy requirements. Khoufi *et al* (2009) applied ultrafiltration as a post-treatment after a process of electro-Fenton combined with anaerobic digestion. Ultrafiltration revealed ability to completely detoxify the anaerobic effluent and remove its high molecular mass polyphenols. Furthermore, Microfiltration is a process similar to Ultrafiltration but with larger membrane pore size allowing particles in the range of 0.2 to 2 micrometers to pass. Moreover, Electrodialysis process consists in an electrochemical separation of mineral salts and other ionic species, which are transported by a direct current electrical potential through ion selective membranes from one solution into another. Gas membrane separation allows to achieve two outputs, a permeate and a concentrate, from an initial solution. OMW organic load is highly concentrated on the concentrate phase and the permeate is purified and less pollutant than the original solution. Finally, Pervaporation allows to treats small volumes of OMW, containing volatile organic compounds, by adding electric energy to waste which results on an electric reaction and, consequently, the OMW purification (Roig *et al*, 2006; Niaounakis *et al*, 2006; Arvanitoyannis *et al*, 2007; Weisman, 2009).

Thermal Treatments

Thermal treatments are based on submitting waste to heat. This type of approach allows to stabilize and turn the product usable, reducing its volume and allowing its sanitation before final disposal. This may represent an adequate pre-treatment to landfill disposal of OMW. The process may or may not involve energy recovery. Thermal treatments may be separated in three types of processes: physico-thermal processes, irreversible thermo-chemical processes and lagooning (Niaounakis *et al*, 2006; Arvanitoyannis *et al*, 2007).

Physico-thermal processes are evaporation/distillation and drying. Evaporation/distillation produces a concentrated solution, molasses, plus a volatile stream composed of water vapour and volatile substances. This technique provides a large reduction of Biochemical oxygen demand in a 5-days test period (BOD5) as it may be used as a pre-treatment to OMW. However, before its disposal the concentrate has to be treated, using biological treatments such as aerobic digestion or an activated sludge process, because of its high organic load. Thus, evaporation is a solution only suitable for industrial-scale oil mills because of its high costs and needs of specialized personal.

Drying allows the extraction of the residual oil and to recover energy content from crude olive cake or 2POMW, by decreasing its moisture content to 5-8% (Roig *et al*, 2006; Niaounakis *et al*, 2006; Arvanitoyannis *et al*, 2007; Weisman, 2009).

Biomass briquetting and pelleting may be considered not only a mechanical process but also a thermal process that consists on agro wastes grinding and drying in order to produce uniform particle size and optimum moisture content. Claro *et al* (2008) designed a process for the treatment and reprocessing of olive mill wastes and cork industry wastes (cork dust and powder) and proved to have a clearly feasible and not only environmentally but also economically sustainable process by obtaining a dried solid product with a very high calorific value that can be commercialized has pellets for burning.

Incineration is a complete and controlled combustion of OMW (only applicable to highly concentrated OMWs in order to guarantee process self-sustaining), decomposing or converting it into a less hazardous or less bulky material. It can be applied by means of Circulating Bed Combustor, Fluidized Bed, Infrared Combustion or Rotary Kilns (Niaounakis *et al*, 2006; Arvanitoyannis *et al*, 2007).

Pyrolisis is a thermal treatment whereby chemical decomposition is induced in an anaerobic and heated environment. This technique may be processed by using Rotary Kiln, Fluidized Bed Furnace or Molten Salt Destruction. Gasification is a process that converts biomass waste to fuel gas by a thermochemical conversion by means of entrained flow gasifiers, fluidized bed gasifiers or fixed bed gasifiers (Niaounakis *et al*, 2006; Arvanitoyannis *et al*, 2007).

Last but not least, lagooning is the most widespread physical process in what concerns on OMW management. This process consists on using the suns energy and the natural biological route to speed-up the process of evaporation and drying of OMW. It may be done on artificial evaporation ponds or in storage lakes (Niaounakis *et al*, 2006). In fact, by using five serial evaporation open-air multiponds COD, BOD, TS and TSS may be reduced under 40%, 50%, 50% and 75%, respectively (Jarboui *et al*, 2010). Also, the use of stabilisation ponds has been tested to treat a mixture of OMW and perennial (urban) wastewaters. Reducing of phenols, organic load and faecal microorganisms was achieved. OMW phytotoxicity effect was significantly reduced after co-treatment (Jail *et al*, 2010b). The same authors consider that waste stabilisation ponds are technically feasible, low capital investment, simple operation and maintenance, high performance and have no energy requirements to function.

Physico-Chemical Treatments

The physico-chemical treatments are very expensive and/or do not completely solve the problem of the need to dispose the sludge or the by-products that derive from the process (Paredes *et al.*, 2005). The composition of OMW is highly variable with respect to each individual component, mainly because OMW is a natural product, processed from a raw material and subject to varied conditions that are difficult to control, and the traditional biological methods used to treat industrial wastewaters cannot be applied to this type of effluent (Ergül *et al.* 2009).

For instance, precipitation by chemically converting soluble substances into insoluble particles (including metals), which are more easily separable from the original material, allows to purify OMW as well as distillation by heating a liquid until it boils, capturing and cooling the resultant hot vapors and collecting the condensed vapors. Also, coagulation and flocculation consists in the initiation of a chemical reaction that promotes the generation, agglomeration or clumping of OMW particles, facilitating its removal from the initial solution (Arvanitoyannis *et al*, 2007). Usually Flocculation is the process applied after coagulation resulting in the generation of larger particles, which may be more easily removed by a solid-liquid separation system. Examples of inorganic flocculants are ferric chloride ($FeCl_3$), ferric sulphate ($Fe_2 (SO_4)_3$), aluminum sulphate ($Al_2 (SO_4)_3$), sodium silicate (Na_2SiO_3) or lime ($Ca (OH)_2/Mg$ or $Ca (OH)_2/Mg (OH)_2$). Miscellaneous inorganic flocculants are natural clay minerals such as bentonite. Organic flocculants may be, e.g., silicate or chitosan (Niaounakis *et al*, 2006). This type of approach has been tested for several authors. More recently, Khoufi *et al* (2008) applied electrocoagulation to a pre-diluted and centrifuged OMW exhausted fraction before an anaerobic digestion, which improved methanisation of the effluent. Boukhoubza *et al* (2009) combined filtration, lime application and hypocloration of OMW. Results showed an efficient and fast reduction of about 95% of polyphenols, COD, SS contents. Furthermore, this technique is capable of decolourise this highly and recalcitrant effluent. Moreover, Hanafi *et al* (2010) applied an aluminium electrode in an electrocoagulation process applied to OMW after filtered and diluted 5 times. Results showed a significant (70%) of COD, polyphenols and dark colour.

Ion exchange is based on a very simple concept of exchanging of ions between two electrolytes or between an electrolyte solution and a complex. It is used to purify, separate or decontaminate aqueous or other ion-containing solutions, such as OMW, with solid polymeric or mineral "ion exchangers". Neutralisation is another technique that may be used as a pre-treatment of OMW in order to remove suspended and colloidal matter. It consists on the restoration of hydrogen (H_+) or hydroxyl (OH_-) ion balance in solution so that the ionic strength of each is equal. Chemical neutralization methods are often applied to reduce the damage that an effluent may cause upon release to the environment. The pH control may perform a pre-treatment, e.g., by increased OMW pH (by the addition of caustics) before evaporation. This allows maintaining volatile organic compounds in the solid fraction during evaporation, resulting in a distillate with a lower COD (Niaounakis *et al*, 2006.

Adsorption is another technique applicable to OMW cleansing. By using activated carbon, with its strong physical adsorption forces and high volume of adsorbing porosity, it allows an efficient pre-treatment or post-treatment reducing phenols concentration and COD. Furthermore, it is also possible to use olive stones and solvent-extracted olive pulp to produce activated carbon (Azzam Mohammed O.J. *et al*, 2005; Roig *et al*, 2006; Arvanitoyannis *et al*, 2007)

Chemical oxidation processes are photochemical approaches for wastewater treatment, particularly to OMW purification. The interest in these techniques has been increasing but it still remains an expensive approach. Examples of tested oxidants for this purposed are oxygen (O_2), hydrogen peroxide (H_2O_2) (Dias *et al*, 1999), ozone (O_3), chlorine (Cl), sodium chlorate (NaCl) and potassium permanganate ($KMnO_4$), between others. Advanced oxidation processes (AOPs) combine hydrogen peroxide with ferrous ions ($H_2O_2/FeSO_4$). It is called the Fenton's reaction and generates highly reactive and oxidizing free radicals with high oxidant power. Generally, phenol compounds degradation and chemical oxygen demand (COD) removal may be improved by submitting OMW to a pre-treatment by the Fenton Oxidation with zero-valent iron and hydrogen peroxide. The reagent components are safe to handle and environmentally benign and results show that total phenol in the OMW is degraded as well as an increase on biodegradability. The Fenton oxidation as a pre-treatment and followed by a classical biological process allows achieving high quality of the effluent water. Another possibility of this pre-treatment is to improve land spreading and the ferti-irrigation by purifying the OMW (Kallel et al, 2009). Moreover, ultrasound irradiation, which allows destroying organic pollutants in waters and wastewaters is a technology capable of photodegradation of phenolic compounds, particularly of cinnamic and/or benzoic acids (Niaounakis *et al*, 2006; Arvanitoyannis *et al*, 2007). Silva *et al* (2007) tested H_2O_2 assisted TiO_2 photocatalytic treatment with promising results. However, technology still has high costs and it's only applicable after separation of solid content and diluted several times. Also, OMW treated by photocatalysis using TiO_2 under UV irradiation showed a decrease on COD, phenols level and coloration at 330 nm (Hajjouji *et al*, 2008). Other possibilities are photocatalysis of Ozone (O_3/UV) and photocatalysis of hydrogen peroxide (H_2O_2/UV). Combinations of hydrogen peroxide and ozone (H_2O_2 /O_3) and also both plus ultrasound irradiation (H_2O_2 /O_3/UV) have already been tested. Wet oxidation and electro-chemical oxidation are also techniques in study for applying to OMW treatment. Finally, Ozonation is a disinfection process used to reduce color intensity, eliminate organic waste, reduce odor and reduce total organic carbon (Niaounakis et al, 2006). Andreozzi *et al* (2008) combined physico-chemical processes, namely, centrifugation-ozonation, centrifugation-solar photolysis, centrifugation solar modified photoFenton and centrifugation-solar modified photoFenton-ozonation. Results showed COD removal until 74, 7%.

Biological Treatments

Biological processes applicable to OMW remediation are divided into anaerobic processes, aerobic processes, aerobic-anaerobic processes, composting, phytodepuration and land spreading. Several studies have reported the biological disposal of this wastewater by anaerobic digestion (Marques, 2001; Fiestas Ros de Ursinos and Borja-Padilla, 1996), being the main interest the production of energy (biogas) and the potential re-use of the effluent in irrigation (Roig *et al.*, 2006). The major limitation of this type of treatment is the inhibition of methanogenic bacteria by the phenolic compounds and the organic acids present in the OMW (D' Annibale *et al.*, 1998), showing that a pre-treatment is necessary to remove undesirable compounds. In this context, a large range of aerobic biological processes, technologies and microorganisms have been tested for OMWs treatment, aiming to reduce organic load, dark colour and toxicity of these effluents. Both aerobic and anaerobic digestion of wastewater using fluidized beds and hybrid reactors has been developed. Digestion is the enzymatic breakdown of large insoluble organic molecules into small soluble organic molecules which can be absorbed and used by either aerobic or anaerobic microorganisms (Arvanitoyannis *et al*, 2007).

Anaerobic digestion (mesophilic digestion or thermophilic digestion, depending on the range of temperatures) is a biological conversion of waste to biogas (a mixture of CO2 and CH_4) in the absence of oxygen. Biogas applications are the heating of the digestion reactors and to generate electricity and/or heat (e.g. with a gas engine) or, after treatment, be fed into the natural gas grid (Fezzani *et al*, 2010).

Anaerobic degradation of OMW may be achieved by means of up-flow anaerobic sludge blanket (UASB) reactor (El-Gohary *et al*, 2009a, El-Gohary *et al*, 2009b), anaerobic baffled reactor (ABR), continuous-flow stirred tank reactor (CSTR) anaerobic contact reactor (Ntaikou *et al*, 2009), anaerobic filter reactor (up-flow and down-flow) or expanded or fluidized bed reactor. Results indicated a good quality final effluent and decreases of COD_{total}, BOD_5, TOC, oil and grease (El-Gohary *et al*, 2009a; El-Gohary *et al*, 2009b).

OMW can also be co-digested with other organic wastes in order to perform a mixture with a composition that enhances high biogas production (Santino, 2007).

Nowadays, landfills are still a very used solution for OMW disposal. However the high cost and the latest regulatory documents on OMW management are reducing its feasibility. Landfills may be considered as anaerobic bioreactors which, in the methanogenic stage, can reduce the pollution load of OMW. Obviously, this type of disposal has very little control by the operators and requires storage of the effluents during the most part of the year. This is a solution that can only be applied to areas with small productions of OMW (Niaounakis *et al*, 2006).

Aerobic digestion of waste is a biological process of degradation and purification, carried out by aerobic bacteria capable of digest the waste and converting it into carbon dioxide (CO_2), water (H_2O), nitrates, sulphates, and biomass (micro-organisms). Aerobic processes cover attached-growth and suspended-growth systems. Attached-growth (biofilm or fixed-film) may be applied to OMW by using trickling filter, packed-bed reactor or rotating (disk) biological contactor (RBC). Suspended-growth systems are applicable by means of activated sludge and sequencing batch reactor (SBR). Activated sludge reactors, e.g., have shown high COD removal efficiencies as well as full-scale activated sludge plants for the combined treatment of different wastes such as olive-mill effluents and domestic sewage. Ergül *et al* (2009) investigated the possibility of reduce phenolic content by means of a continuously stirred tank reactor (CSTR). Experiments resulted on 70 % of total phenolics removal with white-rot fungi *Trametes versicolor*. Also, Federici *et al* (2006) experimented an aerobic solution consisting in using white-rot fungus *Panus tigrinus* cultivated in mechanical stirred tank reactor (STR) and pneumatically agitated in bubble column bioreactor (BCB). This technique allowed a reduction of COD plus the OMW dephenolization and decolouration. Aerobic bioreactors may also be used as a pre-treatment. Jail *et al* (2009) tested JACTO bioreactor as a pre-treatment to OMW followed by a co-treatment with domestic wastewaters in three consecutive stabilisation pounds. Results showed a reduction of 99, 9% of faecal coliforms, no trace of phenolic compounds and a considerable COD reduction.

Anaerobic processes, used has a pre-treatment, followed by aerobic processes have proved to enhance efficiency of OMW cleansing process. Natikou *et al* (2009) tested an anaerobic CSTR followed by a SBR bioreactor to produce biohydrogen and biopolymers. H_2 and volatile fatty acids were produced during anaerobic fermentation and, subsequently, was used as substrate for aerobic biodegradable polymer production, improving the experiment conditions.

Other aerobic processes are aerated lagoons. Process consists on introducing OMW in aerated stabilization ponds, which naturally are purified by the biological route (Roig *et al*, 2006; Niaounakis *et al*, 2006; Arvanitoyannis *et al*, 2007; Weisman, 2009).

Composting is applicable neither to OMWW nor to 2POMW (Niaounakis *et al*, 2006). It is possible to compost all the organic waste streams, particularly pomaces, pits, pulps, pickling solutions and also lignocellulosic residues from olive trees pruning. OMW or sludge from pond-stored OMW is compostable when mixed with appropriate plant waste materials. Examples are OMW additional to wheat straw mixtures, or a mixture of crude olive cake 2POMW and fresh olive tree leaves inoculated with caw manure. Moreover, when mixing 2POMW with cattle manure or sewage sludge vermicomposting proved to enhance its degradability range (Weisman 2009). Abid *et al* (2006) experimented the co-composting of a mixture of OMW, lawn trimmings and olive husks as bulking agents, which caused a clear decrease in both thermophilic bacteria and thermophilic eumycete counts and a longer persistence of phytotoxicity when compared with a pile with 5% of OMW sludge and with a control pile without OMW. Results showed that the pile with OMW sludge produced compost with high degree of maturity. Other experiments were carried out, namely, the co-composting of sludge issued from OMW evaporation ponds with poultry manure has also been tested showing an ability to maintain moisture at optimum level (40-60%). Compost revealed to have a notable quality, with presence of macronutrients and absence of trace heavy metals, as required by the eco-label standards. Moreover, compost exhibits a substantial richness of stabilised organic matter and absence of phytotoxic suitable for oil amendment as organic fertilizer (Hachicha *et al*, 2009).

Other processes are controlled wetlands and the use of specific aerobic microorganisms to treat either OMW or 2POMW.

Land spreading is one of the most used solutions by OMW managers. Actually this is a cheap and simple solution to OMW management. According to Saadi *et al* (2007) although there is a direct short-term effect of OMW application on soil phytotoxicity, after three months of successive applications there is no further phytotoxic evidence. Thus, when controlled, this may be considered as a safe process but only in lands not associated with sensitive aquifers. Innovative possibilities for the detoxification of OMW mainly from two phase's process may be applied in land application. The application of superabsorbent polymers allows olive mill wastewater to be used as a fertilizer, as it is immobilized, increasing the biological activity that decreases its phytotoxicity, thus making its water, organic matter and mineral content usable for plant nutrition (Davies et al, 2004a; Davies et al, 2004b). Besides land spreading, which may be the irrigation of agricultural soils with OMW (Serio *et al*, 2008; Mahmoud *et al*, 2010), there are other controlled irrigation systems. However, this type of approach intends to cleanse the inserted wastes and it uses not agricultural plants but specific plants, capable of detoxify organic wastes. This process is called Phytoremediation (wetlands) or Phytodepuration plants, which are also an aerobic process applicable to OMW depuration. It may be used a free-water surface (FWS) system or a subsurface flow system (SFS). Possible families of plants to apply to waste phytodepuration are *Betulaceae*, *Platanaceae*, *Magoliaceae*, *Aceraceae*, *Mirtaceae*, *Yuglandaceae*, *Caprifoliaceae*, *Labiateae*, *Tiliaceae*, and *Apocyaceae.* However, there are four families that proved to have higher adaptability and physiological growth when in the presence of organic wastes. Those families are *Salicaceae*, *Pinaceae*, *Fagaceae* and C*upressaceae*, more especially, genus *Pinus*, *Quercus* and *Cupressus* (Niaounakis *et al*, 2006). The OLÈICO project - A new application of phytodepuration as a treatment for the olive mill waste water disposal (LIFE04 ENV/IT/000409) - has developed a phytoremediation technology which proved to be suitable to detoxify OMW. Indeed, the Italian government have introduced this technology in regional legislation.

Bioremediation comprises a wide range of possibilities to apply into OMW, by using the metabolic potential of microorganisms to clean up contaminated environments. Approaches may be pointed as *In-situ* techniques, when applied in the pollution source and *Ex-situ* techniques, when there is the need of transport the polluted compartment to another place to cleanse it. *In-situ* techniques include bioaugmentation, bioventing, and biosparging. Bioaugmentation consists in removing undesirable chemicals by the addition of organisms or enzymes to the polluted material. Bioventing is a process that stimulates the growth of indigenous aerobic bacteria by increasing soil O_2 concentration, injecting air into contaminated soil, at an optimal rate. Biosparging is based on the increasing of groundwater oxygen concentrations. The injection of air but under pressure below the water table enhances the rate of biological degradation of contaminants by naturally occurring bacteria. *Ex-situ* techniques include slurry-phase bioremediation (bioreactors) and solid-phase bioremediation

(Landfarming, composting, biopiles) (Roig *et al*, 2006; Arvanitoyannis *et al*, 2007; Weisman, 2009).

Micro-Algae have already been found to detoxify OMW, namely *Chlorella Pyrenoidosa* and *Scenedesmus Obliquus*. Otherwise, the main fungal genera described in the available scientific information for OMW dephenolization are: *Aspergillus*, *Coriolus*, *Phanerochaete*, *Lentinula*, *Penicillium* and *Pleurotus*. Namely, typical Fungi used to detoxify OMW are *Aspergillus Niger* (Cereti et al, 2004; Crognale *et al*, 2006), *Aspergillus Terreus*, *Coriolus Versicolor*, *Funalia Trogii*, G*eotrichum Candidum*, *Lentinus Edodes*, *Phanerochaete Chrysosporium* (Dhouib *et al*, 2006), *Phanerochaete Flavido-alba* and *Pleurotus Ostreatus*. *Bacillus Amyloliquifaciens* has also been studied for 2POMW remediation (Niaounakis *et al, 2006)*. Quarantino *et al* (2007) investigated *Panus tigrinus* as well as an innovative sequential combination of commercial laccase and *Panus tigrinus* liquid cultures on OMW purification. Both have markedly reduced OMW phytotoxicity. Non-conventional lipolytic yeasts have also been tested for OMW valorisation revealing COD reduction, reducing sugars consumption and lipase production (Lopes *et al*, 2009; Gonçalves *et al*, 2009).

Several treatments focused on the degradation of phenolic compounds showed that fungi (Asses *et al*., 2009; Sampedro *et al*., 2007; Oliveri *et al*., 2006; Linares *et al*., 2003) are more effective than bacteria in OMW detoxification. These fungi appear quite effective achieving removal rates as 40 – 88 % for COD, 60 – 100 % for phenols, and 45 – 80 % for colouration (Morillo *et al*., 2009). The reason for this lies in the structure of the aromatic compounds present in OMWs that is analogous to that of many lignin monomers and only a few microorganisms, and among this mainly white-rot fungi, which produce a variety of lygninolytic enzymes, are capable of completely oxidize phenols (Hattaka, 1994).

Use of white rot fungi in INCO MEDUSA Water project (contract number ICA3-CT-1999-00010) have proved particularly efficient as an OMW treatment or pretreatment. Several new approaches have been developed supported by this technology (D' Annibale *et al*, 2004; Mekki *et al*, 2006; Federici et al, 2006; Quarantino *et al*, 2006; Ergül et al, 2009; Jail *et al*, 2010a; Jail *et al*, 2010b).

More recently we have been investigating the ability of *Bjerkandera paranensis,* a novel fungal strain (Moreira *et al*, 2001; Moreira *et al*, 2007) for OMW bioremediation under different treatment conditions (pH, nutrients supply) about a chronic bacterial toxicity test (*Vibrio fischeri* growth inhibition test).

COLOUR, POLYPHENOLS AND ECOTOXICITY: RECENT DEVELOPMENTS

The problems arising from OMW are derived from its high organic load and its chemical composition which renders resistant to degradation. The organic fraction contains large amounts of proteins, lipids and polysaccharides, but unfortunately OMW also contains phytotoxic components that inhibit microbial growth, as well as the germination and vegetative growth plants (Morillo *et al*., 2009). Olive oil phenolic compounds are the main determinants of antimicrobial and phytotoxic actions olive-mill wastes. These compounds are either originally synthesised by olive plants as a defence against a variety of pathogens or formed during the olive oil extraction process.

Because olive oil phenols are amphiphilic, only a fraction of the phenolics enters the oil phase, and a large proportion (≥98%) is lost with the waste stream during processing (Rodis *et al.*, 2002). It is estimated that the toxic load of OMW in terms of phenolic compounds is up to thousand times larger than the domestic sewage. Due to their instability, OMW phenols tend to polymerise during the storage into condensed high-molecular-weight polymers that are difficult to degrade. For these reasons, the uncontrolled disposal of OMW has traditionally become a great problem in Mediterranean countries because of their polluting effects on soil and water.

At LNEG Duarte and collaborators have carried out different studies that have been utilized a variety of microbial processes (e.g. composting, aerobic bioreactors) to treat OMW to remove the dark coloration, reduce the organic load and remove toxicity (Baeta-Hall *et al.*, 2005; Eusébio et al, 2005; Eusébio *et al.*, 2007; Paixão *et al.*, 2009).

Composting treatment of olive oil husks (from two-phase olive oil milling plant) ((Baeta-Hall *et al.*, 2005) was studied under two different aeration techniques in the husk piles (2.5 – 3.0 ton): forced aeration by air injection and mechanical turning. In both systems were obtained similar results for the phenol content, a reduction of about 83.7 % and 77.6 % in forced aeration pile and mechanical turning pile, respectively, was achieved at the end of the mesophilic phase. A marked decrease was also observed in the lipid content with a reduction of about 75 % in both piles at the end of thermophilic phase. In fact, although there were no significant differences in terms of chemical parameters for both conditions, mechanical turning allowed for higher temperatures (in thermophilic phase), corresponding to a more rapidly developing process and to a higher humidification efficiency. Furthermore, this process is energetically more economic since it requires reduced workmanship and a low initial investment (Baeta-Hall *et al.*, 2005).

The studies reported by Eusébio *et al.*, 2007; and Paixão *et al.*, 2009, used feasible solutions to this environmental problem including aerobic treatments based on bioreactors that use the native effluent (OMW) microbial consortia to degrade the polluting effluent load. In the first work (Eusébio *et al.*, 2005; Eusébio *et al.*, 2007), jet aeration systems were used in the biological treatment of OMW (COD = 80 gl^{-1}; total phenolic content = 75 Folin Index) as a means of combining efficient oxygen transfer with high turbulent mixing. Jet-loop type reactors (JACTO) were a very promising technology developed and scaled-up at LNEG which have been successfully used for biological treatment of OMW. The microbial population developed during these bio-treatments shows high adaptation to the JACTO bioreactor conditions, as well as its efficiency with respect to both COD and phenolic compound degradation and removal. For the 200-dm^3 (scale-up process), the short retention time tested (HRT of 5 days) was found to be the best, and the COD_{out} values varied between 3.98 and 2.4 gl^{-1} during the bio-treatment, corresponding to a reduction of about 87 % and an efficiency between 72 % and 84 % for phenolic compound removal. In the second case study (Paixão *et al.*, 2009), a biological treatment system, a packed-bed batch reactor (60 l) was applied to a Portuguese OMW (COD = 10.400 gl^{-1}; Phenol content = 0.360 gl^{-1} a low phenol content) using its autochthon microbial population and the biodegradation potential of OMW microorganisms was assessed monitoring several physico-chemical parameters along the process. In this treatment, an active microbial community with high degradation ability for the OMW organic load was detected, accounting for 80 %, and 61 % removal of COD and phenols, respectively. In addition, a significant decrease in the chronic toxicity of the treated OMW (62.8 % - *V. fischeri* and 64.3 % - *P. putida*) was also observed after 140 days of treatment, highlighting the detoxification potential of the system studied. More recently we focused our attention on the degradation of phenolic compounds, the main responsible for phytotoxicity, and the black colour removal of the OMW. So, fungal remediation of OMW has been studied using different strains of white-rot fungi. The screening of the ability for OMW decolourization, dephenolization and COD reduction were carried out and the most promising results were those obtained by a novel fungal strain isolated on our laboratory, of *Bjerkandera paranensis*. The use of *Bjerkandera* for the OMW bioremediation has not been as common as other fungi such as *Aspergillus*, *Coriolus*, *Lentinula*, *Penicillium*, including the white-rot *Phanerochaete* and *Pleurotus. Bjerkandera paranensis* grew in all OMW dilutions tested including on 100% OMW, and the grown mycelium had high-density hyphae. Similar results were also obtained with *Pleurotus sajor-caju* (Massadeh and Modallal, 2008), a basidiomycete Euc-1 and *Phanerorochaete chrysosporium* (Dias *et al.*, 2004), the best studied ligninolytic fungus. Results from the study conducted in an OMW with a COD = 78.5 gl^{-1} and phenols = 7.7 gl^{-1}, before being thermally processed (at 100 ºC for 1 h), using *P. sajor-caju* showed that this fungus presented a faster growth rate when 25 and 50 % OMW dilutions were tested but an inhibition of mycelium growth was observed for the higher concentrations (75 and 100% OMW). Results described for Euc-1 and *P. chrysosporium* showed that these fungi do not grew at levels above 60% of an OMW with a COD = 130.5 gl^{-1} and phenols = 4.0 gl^{-1}. In contrast with these results, the data obtained with the white-rot *B. paranensis* only presented a slightly growth inhibition (7%), for 100% OMW although the two.phase OMW was significantly lower organic load (COD = 11.1 gl^{-1} and phenols = 3.9 gl^{-1}). In addition, *B. paranensis* was capable of completely decolourize the OMW (see figure 1). Comparing these decolourization results for *B. paranensis* with those described in similar studies using the basidiomycete Euc-1 and the *P. chrysosporium* (Dias *et al.*, 2004), where no decolourization was observed, it can be highlighted the good decolourization ability of *B. paranensis*. In overall, the results obtained showed with *B. paranensis* was able to remove a significant part of phenolic content from the OMW - reaching a maximum of 93% reduction for undiluted OMW and without any addition of nutrients, in contrast with other fungi previous studied that need a prior dilution of OMW to dilute its initial phenol content values to ≤ 3 gl^{-1}, with or without additional nutrient (Benitez *et al.*,1997; Blánquez *et al.*, 2002; Ongen *et al.*, 2007; Ergul *et al.*, 2009).

Ecotoxicological Evaluation

The results of the ecotoxicological evaluation of the OMW samples by *B. paranensis* (T21 days), using the *V. fischeri,* a bioluminescent bacterium culture chronic toxicity test (growth inhibition test) showed a significant decrease in the chronic toxicity of the treated OMW (34.1 to 71.8%), being the highest detoxification potential achieved (IC_{50}-6h = 12.1%), coincident with a reduction of 93% in phenol content, from 3.9 gl^{-1} to 0.3 gl^{-1}, that is considered the main factor responsible by OMW toxicity.

Aggelis *et al.* (2003) carried out a chronic toxicity test using *Heterocypris incongruens* a freshwater ostracoda (growth inhibition test), to evaluate the detoxification ability of *Pleurotus ostreatus* in OMW treatment. *P. ostreatus* have reduced the OMW phenol content from 4.18 gl^{-1} to 1.13 gl^{-1} (73% reduction) during its treatment, however this reduction did not corresponded to a significant detoxification. The inhibitory effect of this OMW on the growth of *H. incongruens* was not affected by the treatment. The IC_{50}-6days values obtained for both untreated and treated OMW were identical (IC_{50s} = 3% OMW). This was probably due to the fact that the remaining phenolics or oxidation products in OMW were more toxic for *H. incongruens* than the initial phenolics. In contrast treatment *with B. paranensis*, even when lowest reduction of phenols (51%) was obtained, with a remaining 1.9 gl-1 phenols in the treated OMW, a significant decrease in OMW toxicity was still observed.

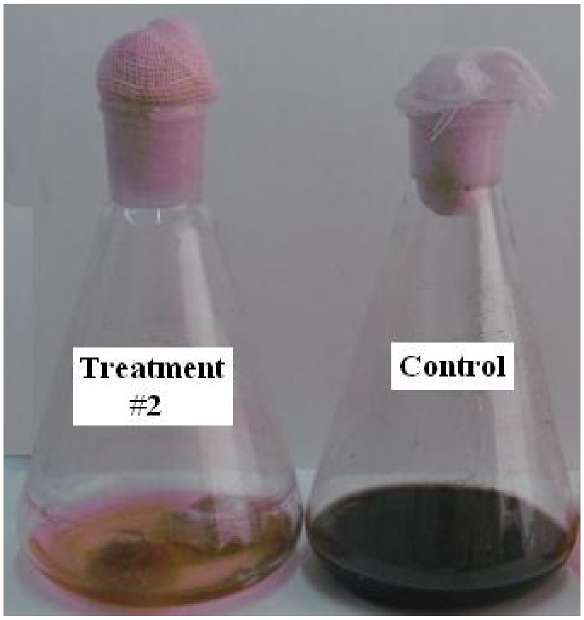

Fig. 1. Decolourization of undiluted OMW after 21 days of treatment by *Bjerkandera paranensis* (Treatment #2 – OMW pH 6; Abiotic control).

CONCLUSION

The OMW treatment still represents a great technological and environmental challenge. There is not a universal methodology that can be applied to the different industries and a solution is dependent on size, extraction process and other specific local conditions. Our recent work with a white-rot fungus *Bjerkandera paranensis*, highlights the potential of this novel strain to be used as a good alternative strain for OMW bioremediation in comparison with the fungal strains already described in studies of OMW treatments and other physicochemical processes due to the simple energy and technology requirements and the possibility to overproduce enzymes and /or protein enriched biomass for a number of applications.

In the undiluted OMW treatment by *B. paranensis* it was achieved a reduction of 57.5 % in COD, 93% in phenol content and 74% in color without addition of nutrients. In addition, a detoxification of 71.8% was attained by this strain. However the OMW is still considered slightly toxic to microorganisms (IC_{50}-6h = 12.1%) to be discharged directly in the environment. Moreover, the maximum emission limit values ELV for COD and phenols, legislated for the disposal of effluents in watercourses in Portugal, are: 150mgl^{-1} and 0.5mgl^{-1}, respectively. The values obtained for the best treatment are still above these required ELVs. Therefore this has to be considered at the moment as a pre-treatment for complementing with other type of treatments (eg. for biogas plants)

These are promising results for further research combining an eco-efficient aerobic-anaerobic technology for the bioremediation of this agro-industrial effluent, using *B. paranensis* as the microorganism responsible by the aerobic step, due to its potential to remove OMW polluting load (COD, phenol content and colour).

Moreover, studies based on the production of bioproducts (enzymes and biopolymers) by *B. paranensis* can be a useful tool to a possible OMW valorisation and further scale-up of OMW bioremediation technology, which together will form a pillar for future developments within this field.

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