OLIVE MILL WASTEWATER BIOREMEDIATION BY *Bjerkandera paranensis*: A SUSTAINABILITY AND TECHNOLOGICAL EVALUATİON

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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 toxic properties. The composition of OMWs is highly variable with respect to each individual component, depending on the process conditions and on the agricultural specificities. In this work, the ability of a "white-rot" fungus, Bjerkandera paranensis, to use undiluted OMW from a two phase process mill (COD = 11.1 gl^{-1}; Phenol Content = 3.9 gl^{-1}; ColourAbs395nm = 7.8) as a substrate was studied. The biodegradation potential of B. paranensis was assessed monitoring several physico-chemical parameters. A chronic ecotoxicity test (Vibrio fisheri growth inhibition test) was carried out to follow the detoxification ability of this fungus. In work, the results demonstrate that OMW was a suitable medium for cultivation of B. paranensis, with corresponding changes in the physico-chemical properties of the OMW. The results showed that B. paranensis removed 93% phenols and 54% COD from the culture medium within 21 days of treatment. In addition, the IC50s values obtained for the different treated samples showed a significant decrease in the effluent chronic toxicity to V. fischeri when the OMW pH was adjusted to 6.0 prior to the treatment (71.8 %), highlighting the OMW detoxification capacity of B. paranensis.

Keywords: *OMW; biological treatment; B. paranensis; ecotoxicity evaluation; detoxification.*

INTRODUCTION

The olive oil industry represents one of the most important economic agro-food sectors in the bordering Mediterranean countries that produce 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 (1, 2).

During olive oil extraction a process that is conducted by mechanical procedures in olive mills, large amount of liquid effluents and solid residues are produced, with a high organic load, the nature of which

depends on the technology of the extraction system employed. Three systems are used worldwide for industrial-scale extraction of oil from olives, the traditional press-cake system, the 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 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 (3).

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 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 (4).

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 (5, 6, 7). 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.

The physico-chemical treatments (coagulation, precipitation or flocculation of OMW organic compounds) 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 (8). 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 (9).

Several studies have reported the biological disposal of this wastewater by anaerobic digestion (10), being the main interest the production of energy (biogas) and the potential re-use of the effluent in

irrigation (11). The major limitation of this type of treatment is the inhibition of metanogenic bacteria by the phenolic compounds and the organic acids present in the OMW (12), 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. Several treatments focused on the degradation of phenolic compounds showed that fungi (13, 14, 15) are more effective than bacteria in OMW detoxification. These fungi appear quite effective achieving removal rates as 40 – 88 % for COD, 60 – 100 % for phenolics, and 45 – 80 % for colouration (7). 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 (16). The main fungal genera described in the available scientific information for OMW dephenolization are: *Aspergillus*, *Coriolus*, *Phanerochaete*, *Lentinula*, *Penicillium* and *Pleurotus*.

The purpose of the present work was to investigate the ability of a "white-rot" fungus, *Bjerkandera paranensis*, to use undiluted OMW (COD = 11.1 mg/l; Phenol Content = 3.9 g/l ; $Colour_{Abs395nm}$ = 7.8) as a substrate. The results obtained proved that OMW is a suitable media for cultivating *Bjerkandera* B33/3, and its growth on OMW cause drastic changes in physical and chemical properties. OMW decolourization was observed during mycelium growth, with a colour reduction since the 7th day (50 %). The results showed that *Bjerkandera* B33/3 removed phenols (90 %) and COD (75 %) from the culture medium after 21 days of treatment.

In addition, the IC_{50}s values obtained during the treatment show a significant decrease in the OMW'chronic toxicity to *V. fischeri* (60.8 %), changing its classification from toxic to lightly toxic.

MATERIALS AND METHODS

OMW Sampling and Characteristics

Sampling of olive mill wastewater (OMW) was carried out from a two phase Portuguese mill farm (Trás-os-Montes, Portugal). Prior to any assay performed with the OMW, this effluent was centrifuged (45 min at 10000 x g) to remove residual solids, and autoclaved at 121 ºC during 20 min. The main properties of this OMW (mean values SD; n=3) were: pH 4.8 ± 0.2; chemical oxygen demand (COD) 11.1 ± 0.4 gl^{-1} ; total phenolic content 3.9 ± 0.1 gl^{-1}, determined as caffeic acid equivalents; colorimetric value (A_{395}) 7.8 ± 0.2.

Microorganism and Culture Conditions

A white-rot basidiomycetes *Bjerkandera paranensis*, a novel fungal strain (17, 18), isolated and identified in our laboratory, exhibiting high decolourisation activities in different dyes (17) was used for the biological treatment of the OMW. This fungus was grown on potato dextrose agar (PDA, Difco, France) slants at 28 ºC and stored at 4ºC. *B. paranensis* was maintained through periodic subculture every 3 weeks on PDA plates.

Liquid cultures were conducted to monitor several parameters, such as phenol concentration, colour and chemical oxygen demand (COD), during the growth of *B. paranensis.* OMW liquid medium was prepared using undiluted OMW set to pH 6.0. The medium was autoclaved at 121 ºC during 15 min. Volumes of 150 ml of the OMW media were used in 500 ml Erlenmeyer flasks and then inoculated with 15 mycelium plugs (7 mm) cut from the front of an actively growing *B. paranensis* fungus in a PDA plate. Incubations were carried out on an orbital shaker at 130 rpm and 28 ºC. The assays were carried out during 21 days. In addition, abiotic controls using no inoculated OMW medium were performed at the same incubation conditions.

Ecotoxicological Evaluation

The ecotoxicological evaluation of OMW samples collected from the liquid culture assays, before and after treatment by *B. paranensis* (T21 days), was carried out using a miniaturized chronic toxicity test: the growth inhibition test using a bioluminescent bacterium culture, *Vibrio fischeri*. This test was performed in 96-well microplates (NUNC^{TM}, Denmark), based in DIN 38412-L37 -1999 (19). The OMW samples were tested without further pH adjustment and after filtration (membrane filter of pore size: 0.45 □m). Toxicity results were expressed in IC_{50}, the concentration responsible for the growth inhibition in 50% of the tested population. IC_{50}-6h values were estimated from the sigmoidal concentration - inhibition curves fitted by the maximum likelihood - logit method using the ToxCalc V5.0.23F (Tidepool Scientific Software, McKinleyville, CA, USA).

Analytical procedures

Total phenolic content (with respect to caffeic acid) was determined according to a modification of the Folin-Ciocalteau method. According to this method, 500 µl Folin-Ciocalteau (4-fold-diluted) phenol reagent was added to 100 µl of 20-fold-diluted samples. After 5 min, 500 µl sodium carbonate (200 gl^{-1}) was added and the absorbance was measured at 725 nm against a blank after being kept at room temperature for 30 min. OMW colour was determined spectrophotometrically in diluted samples (1:20) by measurement of absorbance at 395 nm (9). Analysis of chemical oxygen demand (COD) was carried out following the APHA (2005) (20).

RESULTS AND DISCUSSION

Treatment of undiluted OMW by *B. paranensis*

Biological treatment of undiluted OMW (pH 6.0) was conducted in liquid cultures (batch process in shake-flask on an orbital shaker 120 rpm) with *B. paranensis*. This treatment by *B. paranensis* was monitored following the parameters: phenol concentration, colour, COD and pH.

The results from the treatment are presented in Table 1 as % reduction of the different physico-chemical parameters assessed. To evaluate the inherent degradation, abiotic remediation of OMW was assessed by 21-day incubation of an OMW sample (pH 6) in the absence of *B. paranensis* (Table 1 - Abiotic Control). In this control, COD, phenol content, and colour decreased by 10.3 %, 8.1 %, and 6.3 %, respectively.

Table 1. Reduction (%) of several parameters (COD, phenols content, colour and toxicity) within 21 days of undiluted OMW treatment by *B. paranensis*.

OMW SAMPLES	% REDUCTION			
	COD	**Phenol**	**Colour**	**Toxicity**
Abiotic Control	10.3	8.1	6.3	---
Treated OMW	57.5	93.0	74.0	71.8

The results obtained showed with *B. paranensis* was able to remove a significant part of phenolic content from the OMW - culture medium, under the conditions tested, reaching a maximum of 93% reduction in undiluted OMW medium, without any addition of nutrients but with pH adjustment to 6.0, 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 (9, 21, 22, 23).

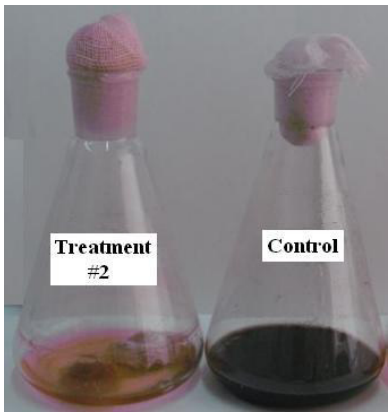

Fig. 1. Decolourization of undiluted OMW (pH 6.0) after 21 days of treatment by *B. paranensis*.

At the end of the treatment of undiluted OMW (21st day), *B. paranensis* was also able to a significant reduction of the pollutant load, 57.5 % in COD, and an extensive decolourization (74%), as can be seen in Fig 1. These results seem greatly satisfactory as real OMW with no dilution was treated. In addition, the results of the ecotoxicological evaluation of the OMW samples, before (untreated) and after treatment by *B. paranensis* (T21 days), using the *V. fischeri* chronic toxicity test (growth inhibition test) were: IC_{50}-6h (%) = 3.4% and IC_{50}-6h (%) = 12.1%, respectively, corresponding to an OMW detoxification of 71.8% (Table 1). This significant decrease in the chronic toxicity of the treated OMW to *V. fischeri*, is probably related to 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) (24) 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 their OMW on the growth of *H. incongruens* was maintained after the treatment, being the IC_{50}-6days values for both untreated and treated OMW 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, in the current study, *V. fisheri* showed a decrease in OMW toxicity when treated by *B. paranensis* (71.8 %). *V. fisheri* and *H. incongruens* presented a similar sensitivity for the initial untreated OMW samples (with an equivalent phenol content): IC50-6h = 3.13 % and IC50-6 days = 3.0%, respectively.

CONCLUSIONS

The OMW treatment results obtained by a white-rot fungus *Bjerkandera paranensis*, highlight 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.

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 colour, when the pH was adjusted to 6.0. In addition, an OMW detoxification of 71.8% was attained by this strain.

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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