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Investigation of olive mill wastewaters treatment by immobilized microalgae.

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1. Introduction – During the extraction process of olive oil a large quantity of liquid and solid residues are produced. Nowadays, two-phase and three-phase centrifugation systems are most commonly used. The two-phase centrifugation system reduces the water consumption during the process and practically integrates the three-phase mill wastewater, nevertheless in smaller quantities. The two-phase olive-mill waste presents a slightly acidic pH and a very high content of organic matter, comprising relatively large amounts of lignin, hemicellulose and cellulose, fats, carbohydrates, as well as phenolic compounds [1, 2]. Despite the fact that degradation of olive mill effluents was exhibited in the past [1-3], two-phase olive-mill waste still pose serious problems related with its effective management and safe disposal.
Biotreatment of wastes water using living organisms is an environmentally friendly, relatively simple and cost-effective alternative to physico-chemical processes. Previous work was shown that *Chlorella vulgaris*, a single-cell Chlorophyceae, was the ability to growth in medium supplemented with several plant extracts rich in phenolic compounds [5].
Therefore, the aim of this work was evaluate the ability of immobilized *C. vulgaris* to remove phenolics compounds from olive mill wastewaters and assess their potential bioremediation by evaluating toxicity on final treated effluent.

2. Experimental – ***Wastewaters Sampling:*** Samples of olive mill wastewaters from two-phases centrifugation (TPOMW) were collected from a continuous olive mill facilities, located in north-eastern Portugal, in December of 2013. At laboratory arrive the wastewaters were filtrated, for removal of suspended solids, acidified until pH 2 (HCl) and finally frozen. For essays samples were thawed and the original pH restored (NaOH).
Microalgae strain: The green algae, *Chlorella vulgaris* (CBSC 15-2075), was used as a test strain to evaluate their bioremediation potential. *C. vulgaris* was growth in 1000 mL flasks with sterilized Bold´s Basal medium in a controlled chamber, under temperature of 22 ± 1 ºC, light intensity of 4500 lux (Gro-Lux fluorescent lamps), 16:8 h light:dark photoperiod, with aeration, until reach exponential growth and to be able to be used for essays. Screen of microalgae growth on TPOMW was assessed on agar solid, under axenic conditions. Seven different TPOMW dilutions on destillated water (10-70% v/v) were prepared on solid agar, prior to inoculation, using Petri dishes. Control growth was prepared with Bold´s Basal medium. All the Petri dishes were inoculated with same *C. vulgaris* concentrations and incubation was in the same controlled chamber, under previous conditions. Degree of algae growth was assessed by comparison with control growth after 7, 12 and 15 days. All the essays were done in triplicate.
Wastewaters biotreatment: Assessment of phenolic compounds removal was made in batch cultures, with *C. vulgaris* immobilized in sodium alginate (1.5% w/v). Batch cultures were developed in 1000 mL flasks with TPOMW diluted on water (v/v). All the batch cultures have been started with same ratio of culture volume to immobilized microalgae volume (beads). Incubation was in the same controlled chamber, under previous conditions, with continuous aeration. Control cultures with same diluted TPOMW were developed with beads of sodium alginate containing no algae. All the essays were done in triplicate.
Polyphenols content: For polyphenols evaluation, samples were washed successively two times, with n-hexane (1:1.25 (v/v)) in order to remove lipid fraction and then a liquid-liquid extraction was carried out with methanol (1:1.25 (v/v)). Total polyphenols content of these extracts was determined colorimetrically at 725 nm, by Folin-Ciocalteau method using gallic acid as a standard. All the analyses were done in duplicate. Removal efficiency was evaluated as Phenolic Loss Index (PLI) and Yield Efficiency (YE), using the following equations:

$$PLI\ (\%) = [(Phn_i - Phn_f) / (Phn_i)] \times 100$$

$$YE\ (\%) = [(Phn_i - Phn_f) / \text{Total days}] \times 100$$

(Phn_i = initial polyphenolics concentration; Phn_f = final polyphenolics concentration).

Germination tests: Phytotoxicity evaluation was performed with lettuce (*Lactuca sativa*) seeds, incubated in a growing chamber, in the dark, at 26°C, for 6 days. Before tests, seeds were washed in diluted commercial NaClO and then washed repeatedly several times with distilled water. Ten lettuce seeds were disposed on each Petri dishes, lined with filter paper and watered with 5 mL of TPOMW 35% after final biotreatment. Controls were prepared in same way, using distilled water for positive control, and using TPOMW from control batch cultures (without algal treatment) for negative control. Samples and controls were performed in quadruplicate. A seed with 0.3 cm radicle was considered germinated. After 6 days, when controls showed good growth, root elongations were measured. Results were expressed as mean root growth (cm). Phytotoxic activity of TPOMW after biotreatments was evaluated by Response Index (RI) using the following equation:

$$RI = (Test/Control) - 1$$

(Test = total number of seeds germinated after 6 days, treated with TPOMW 35% /negative control, after biotreatments; Control = number of seeds germinated after 6 days, treated with water).

According [6], if $-1 < RI > 0$, then the effect is inhibition; If $0 < RI > 1$, effects is stimulation.
Statistical analysis: Results are expressed as mean values ± standard error. Phenolics data was compared applying Mann-Whitney test and germination tests data was compared applying 2-samples t-test. $P<0.05$.

3. Results and Discussion – In order to evaluate the ability of *C. vulgaris* growth under the presence of high levels of phenolic compounds, several dilutions of TPOMW were prepared on solid agar and inoculated. Results showed that, in generally *C. vulgaris* can growth in tested dilutions. Highest density of cells and fast-grow were observed between 20-40% TPOMW dilutions, being dependent of time to be quite similar to control growth. Lowest density of cells was observed at 10%, concomitant with low nutrients amount, as well as at dilutions up to 50%, suggesting, in this case, some toxic effects. In fact, TPOMW dilutions were performed with distillated water and no other nutrient source was added. In turns, olive mill waste water is characterized by very high levels of phytotoxic and microbial inhibitory compounds, such as phenolics and fatty acids [1]. Several phenolic compounds have showed antialgal activities, including growth inhibition [7-9], as well as some fatty acids [10, 11]. However some algae species could degrade or absorb phenol compounds when their concentrations were lower [12, 13], as we most likely mainly observed at 20-40% TPOMW dilutions.
Therefore, based on these results, batch cultures were carried out with TPOMW 35% diluted on water. These cultures was started with a total polyphenols content of 12.45±0.52 µg/mL and a volume culture:volume of alginate beads ratio always of 20. Beads were prepared with a final alginate solution of 5.35×10^6 cells/mL of *C. vulgaris*. The results obtained showed that immobilized microalgae can remove polyphenols, achieving a final concentration of 3.22 µg/mL ± 0.19, however cultures with beads without algae also decreased slightly the polyphenols content.

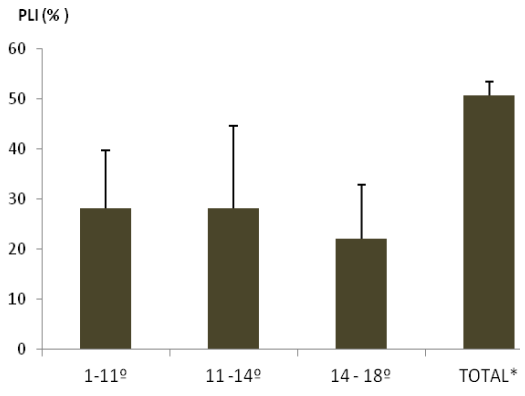

Figure 1. Phenolic Loss Index (%) variation during biotreatments of TPOMW 35% (n=3). *p<0.05.

To ascertain the exact effect of immobilized *C. vulgaris* on polyphenols decreased, values of PLI were taken in to account with control values (Figure 1). It is possible observe that at the end of fermentation a PLI of 51% ± 2.78 was obtained, and during the first 14º days of fermentation PLI was quite constant (around 28%), followed by a slightly decreased. Regarding results obtained until 14 days of fermentation, with a PLI of 46% ± 6.28, not so far from results obtained after 18 days, and also with a Yield Efficiency (YE) of 43% ± 6.10, comparing with a lower final YE of 36% ± 4.91, further fermentations were carried out with short-time treatments.

Subsequent batch cultures were carried out with TPOMW 50% and 60% diluted and preadapted to 10% TPOMW (Bold´s Basal medium/TPOMW), in an attempt to optimize biotreatments. Also, inoculation was with same ratio of alginate beads, but in this case, with higher concentration of *C. vulgaris* (14.6×10^6 cells/mL). Initial polyphenols contents were of 15.27±0.33 µg/mL and 20.37 ± 0.59 µg/mL for biotreatments of TPOMW 50% and 60%, respectively (Table 1). These cultures were incubated during 8 days and results showed that after biotreatment polyphenols content decreased for both essays.

Table 1. Initial and final polyphenols content and parameters evaluated in batch cultures of TPOMW 50% and 60%.

	TPOMW 50%	**TPOMW 60%**
Phn_i (µg/mL)	15.27 ± 0.33	20.37 ± 0.59
Phn_f (µg/mL)	6.71 ± 0.31	11.40 ± 1.18
PLI* (%)	17.27 ± 2.13	14.93 ± 5.11
YE* (%)	40.45 ± 4.96	41.95 ± 13.23

Phn_i = initial polyphenolics concentration; Phn_f = final polyphenolics concentration; * taken in to account cultures control

Although tested dilutions of TPOMW were a slightly different, there were no differences between final PLI and YE, between 50% and 60%. Regardless of preincubation conditions and algae higher concentration in alginate beads, comparing with biotreatments of TPOMW 35% (who achieved a final PLI of 51% ± 2.78), these values were lower, in line with increasing phytotoxic in these essays due to higher TPOMW concentration.

The extend time of incubation had not led to an increase in this parameter. A variety of biological processes have been tested to treat olive mill wastewaters, mainly to reduce organic load, such as phenolics compounds [4, 14-16], but in general their effectiveness in reducing the phytotoxicity varies greatly. Algae are sensitive to phenolics, which toxicity depends of number and polarity of aromatic ring substitutes [17]. The most abundant phenolic compounds, generally present in TPOMW, include tyrosol and hydroxytyrosol and *p*-coumaric acid [1] that can be degradete by microalgae [17], howerver this ability depends on phenolic concentration, algae specie, time of exposure and probably of culture conditions. Thus, our results suggested that for higher TPOMW concentration, it will be necessary an optimization of condition of fermentation, such as light regime.

In order to evaluate the TPOMW 35% treatments with *C. vulgaris*, final samples were used for phytotoxicity evaluation. As expected, biotreatment carry out in TPOMW, leads to a higher length root of seeds, comparing with positive control (Figure 2).
Although TPOMW treated with *C. vulgaris* do not affect germination of seeds, compared to water, since Response Index (RI) was zero, for negative control RI was -0.026. In accordance of previous results, higher response of RI for TPOMW treated, reflect a more stimulating activity of these samples, when compared to negative control. These results confirmed that *C. vulgaris* can reduce phytotoxic compounds presents in TPOMW that inhibits germination and growth of *L. sativa*, thus showing a good biotreatment potential.

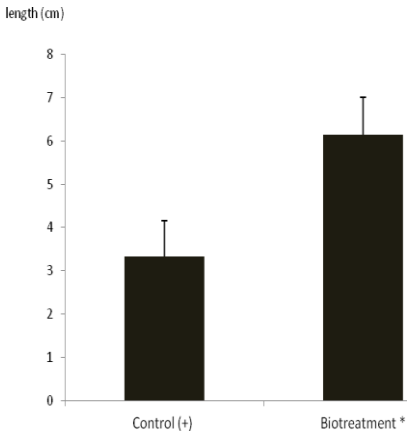

Figure 2. Mean root length of germinated seeds obtained from positive control and biotreatment of TPOMW 35% (M±DP).* $p<0.05$

4. Conclusions – In generally, results from this work showed that immobilized *C. vulgaris* could be an alternative to bioremediation of TPOMW, but more detailed studies are still needed to optimize the process**.** Biotreatment of wastes using living organisms is an environmentally friendly, relatively simple and cost-effective alternative to physico-chemical processes. Furthermore the biotechnology of growing microalgae in waste water is getting importance as biomass production for many other valuable applications.

5. References:

[1] Niaounakis and Halvadakis. Olive Processing Waste Management: Literature review and patent survey. Elsevier, UK, 2006.
[2] McNamara, et al. *International Biodeterioration & Biodegradation*, 61, (2008) p. 127.
[3] Tsagaraki, et al. Olive mill wastewater treatment. *In* Utilization of by-products and treatment of waste in the food industry. Springer, USA, 2007.
[4] Morillo, et al. *Appl Microbiol Biotechnol.*, 82(1), (2009) p. 25.
[5] Monteiro et al. *2nd IWA Symposium on Lake and Reservoir Management: Sustainable Approaches to Enhance Water Quality*, (2011) p. 78.
[6] Williamson, G.B. and Richardson, D. *J. Chem. Ecol.*, 14(1), (1988) p. 181.
[7] Stom, D.I. and Roth, R. *Bull. Envinm. Contam. Toxicol*., 27, (1981) p. 332.
[8] Aruoja, et al. *Chemosphere*, 84(10), (2011) p. 1310.
[9] Shao, et al. *Journal of Environmental Management*, 125, (2013) p. 149.
[10] Ikawa, at al. *Journal of Chemical Ecology*, 20 (9), (1994) p. 2429.
[11] Wu, et al. *Aquatic Toxicology*, 80 (4), (2006) p. 338.
[12] Al-Khalid, T. and El-Naas, M. *Critical Reviews in Environmental Science and Techology*, 42, (2012) p. 1631.
[13] EI-Sheekh, et al. *J. Bioremed. Biodegrad*, 3, (2012) p. 133.
[14] Di Gioia, et al. *Research Microbiology*, 152, (2001) p. 83.
[15] Aggelis, et al. *Water Research*, 37(16), (2003) p. 3897.
[16] Matos, et al. *Letters in Applied Microbiology*, 45(3), (2007) p. 270.
[17] Pinto, et al. *Biotechnology Letters*, (24), (2002) p. 204.