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Preliminary Results of Olive Mill Wastewaters Treatment by Immobilized Microalgae

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Abstract

The Olive Mill Wastewater (OMW) is the principal industry residue extraction of olive oil. Generally is characterized by very high organic load, due to high levels of phenolic compounds and sugars, and have minimum levels of nitrogen compounds and low pH. A variety of biological processes and microorganisms have been tested to treat OMW, including bacteria, yeasts and fungi, where the effectiveness in reducing the toxicity varies greatly.

Taking this into account, the aim of this work is to assess the potential of microalgae in the decrease of phenolic compounds in OMW. For this purpose, the effect of immobilized Chlorella vulgaris in OMW biotreatments has been tested in batch cultures. The effectiveness of the process has also been assessed by phytotoxicity, in germination trials of Lactuca sativa. Biotreatments have been performed with OMW dilutions of 35%, 50% and 60%. Results showed that C. vulgaris has the ability to remove phenolic compounds, achieving a final Phenolic Loss Index (PLI) of 73%. Regarding the phytotoxicity after biotreatment we can conclude that microalgae can reduce the toxicity of olive mill wastewaters also having the ability to degrade the phenolic compounds that inhibit the germination and growth of Lactuca sativa. Furthermore, the algae biomass obtained in this process could be valorized in other applications.

Keywords: Olive mill wastewaters; Bioremediation; Chlorella vulgaris; Phenolic compounds; Phytotoxicity; Lactuca sativa

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Introduction

The Olive Mill Wastewater (OMW) is the principal industry residue extraction of olive oil, characterized by a high organic pollutant and therefore is one of the most serious environmental problems in producers countries of Mediterranean Basin [1-3]. OMW is general characterized by very high organic load, due to high levels of phenolic compounds and sugars, and have minimum levels of nitrogen compounds and low pH [3,4]. OMW disposal practices include their use in irrigation of agricultural land, however directly discharge may impact physic-chemical soil properties, such as pH and porosity and can inhibit plant seed germination, due to high concentration of phenolic compounds [5]. Another possible solution is the use of evaporation ponds, which reduces the volume of waste, however without treating the pollutants or its rejection in the municipal collectors after solid removes pretreatment.

A variety of biological processes and microorganisms have been tested to treat OMW, mainly to remove dark coloration and reduce organic load. A number of different bacteria have been tested in aerobic and anaerobic processes including Azotobacter vinelandii [6], Pseudomonas putida and Ralstonia sp. [7, 8], Lactobacillus plantarum, isolated from the fermentation of olive brine [1], as well as a variety of fungi such as Pleutorus ostreatus [9], Geotrichum candidum [10] and Ganoderma applanatum [11], but in general their effectiveness in reducing the phytotoxicity of OMW varies greatly.

Despite the fact that degradation of olive mill effluents exhibited in the past, they still pose serious problems related to its effective management and safe disposal.

Microalgae are photosynthetic microorganisms that are able to rapidly generate biomass from solar energy, CO_2 and nutrients in bodies of water. Algal biomass and algae-derived compounds are potentially useful in industrial applications and has received increasing interest, particularly in the food, cosmetic and pharmaceutical industries, as well as for biodiesel production [12-16]. Other applications from microalgae are due to their ability to wastewater treatments [17-23]. The photosynthetic mechanism of microalgae, although similar with terrestrial plants, differs in simple cell structure that microalgae exhibit and they shows more efficient exchange of water, nutrients and CO2 that higher plants, which leads to higher rates of conversion of light into biomass [15, 16].

Previous work was shown that Chlorella vulgaris, a single-cell Chlorophyceae, had the ability to grow in medium supplemented with several plants extracts rich in phenolic compounds [24].

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 of final treated effluent.

Material and Methods

Olive Mill Wastewaters (OMW) Sampling

Samples of OMW were collected from a continuous olive mill facility, located in north-eastern Portugal. Samples were collected at the end of oil extraction process, prior to deposition in the basins, in December of 2013. At laboratory arrive the OMW 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 OMW was assessed on agar solid, under axenic conditions. Seven different OMW dilutions on distilled 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.

OMW Bio treatments

Based on previous screening results, the assessment of phenolic compounds removal was made in batch cultures, with C. vulgaris immobilized in sodium alginate. Briefly, 100 mL of exponential growth cultures of *C. vulgaris* were mixed with 100 mL of sodium alginate solution 3% (w/v), pH = 7.5, giving a final alginate solution of 1.5%. To ascertain inoculums concentration in batch cultures, cell density was assessed at this stage, by cell counting using a Neubauer hemocytometer. Final alginate solution was dripped into a $CaCl_2$ solution 2% (w/v) with slow stirring. Beads formed were left for 30 min at room temperature and then washed in distillated water. *Before*

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inoculation in batch cultures, beads were incubated overnight in Bold´s Basal medium.

Batch cultures were developed in 1000 mL flasks with OMW diluted on water (v/v). All the batch cultures have 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 OMW, 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 after biotreatments was performed with lettuce (Lactuca sativa) seeds, incubated in a growing chamber, in the dark, at 26°C, for 7 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 dish, lined with filter paper and watered with 5 mL of OMW 35% after final biotreatment. Controls were prepared in same way, using distilled water for positive control, and using OMW without algal treatment for negative control. Samples and controls were performed in quadruplicate. A seed with 0.3 cm radicle was considered germinated. After 7 days, when controls showed good growth, root elongations were measured. Results were expressed as mean root growth (cm). Phytotoxic activity of OMW after biotreatments was evaluated by Response Index (RI) using the following equation:

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

(Test = total number of seeds germinated treated with OMW 35% or treated with negative control; Control = number of seeds germinated treated with water).

According [25] 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 ($p<0.05$).

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 OMW were prepared on solid agar and inoculated. Results showed that, in generally C. vulgaris can grow in tested dilutions. Highest density of cells and fast-grow were observed between 20-40% OMW 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, OMW dilutions were performed with distillated water and no other nutrient source was added. In turns, olive mill wastewater is characterized by very high levels of phytotoxic and microbial inhibitory compounds, such as phenolics and fatty acids [5]. Several phenolic compounds have showed anti algal activities, including growth inhibition [26-28], as well as some fatty acids [29, 30]. However some algae species could degrade or absorb phenol compounds when their concentrations were lower [31, 32], as we observed at 20-40% OMW dilutions. Therefore, based on these results batch cultures were carried out with OMW dilutions higher than 35%.

Batch cultures with OMW 35% dilutions (on distillate water) were started with a total polyphenols content of 6.22 ± 0.26 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 Phenolic Loss Index (PLI) of 73% ± 2.78 and a Yield Efficiency (YE) of 26% ± 2.46. However control cultures with beads without algae also decreased slightly the polyphenols content, probably due to aeration of cultures [33, 34].

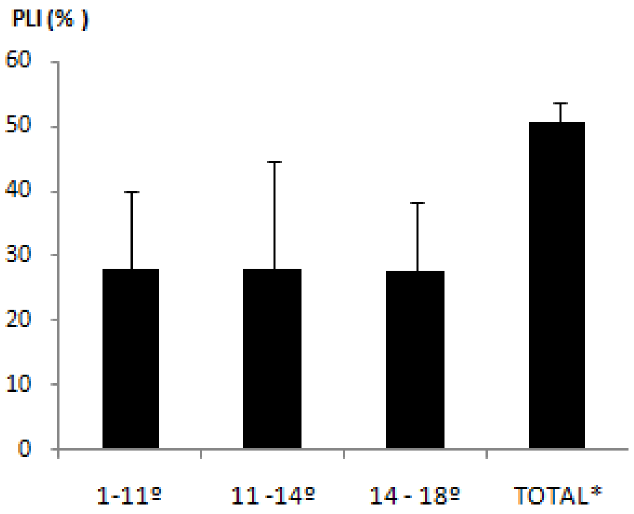

Figure 1. Phenolic Loss Index (%) variation during biotreatments of OMW 35% (n=3). *p<0.05.

Consequently, to ascertain the exact effect of immobilized C. vulgaris on polyphenols decreased, values of PLI were taken into account with control values (Figure 1). It is possible to observe that PLI was quite constant (around 28%), and at the end of fermentation a value of 51% ± 2.78 was obtained, achieving a YE of 18% ± 2.46.

However, regarding results obtained only until 14 days of fermentation, with a PLI of 49% ± 6.28 (Figure 1), and also with a YE of 23% ± 3.05, quite similar to the results obtained after 18 days, further fermentations were carried out with short-time treatments. Furthermore, to process optimization, subsequent biotreatments were carried out with OMW 50% and 60% with C. vulgaris pre-adapted to 10% OMW (Bold´s Basal medium/OMW). 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 content were of 7.64 ± 0.16 µg/mL and 10.18 ± 0.30 µg/mL, for OMW 50% and 60%, respectively (Table 1). These cultures were incubated during 8 days and results showed that after biotreatments polyphenols content decreased for both essays.

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

	OMW 50%	**OMW 60%**
Phn_i (µg/mL)	7.64 ± 0.16	10.18 ± 0.30
Phn_f (µg/mL)	3.36 ± 0.16	5.18 ± 1.00
PLI^a (%)	17.27 ± 2.13	23.78 ± 7.41
YE^a (%)	20.23 ± 2.48	20.98 ± 6.62

Phn_i = initial polyphenolics concentration; Phn_f = final polyphenolics concentration; [a] taken in to account cultures control

Although tested dilutions of OMW were slightly different, there was no difference between PLI of 50% and 60% cultures, either in YE.

Regardless of pre-adapted inoculums conditions and algae higher concentrations in alginate beads, values of PLI were lower comparing with bio treatments of OMW 35% (who achieved a final PLI of 51% ± 2.78), in line with increasing phytotoxicity, due to higher wastewaters concentration.

The extend time of incubation in these batch cultures did not led to a significant decrease in polyphenols content. As mentioned, a variety of biological processes have been tested to treat olive mill wastewaters, mainly to reduce organic load, such as phenolics compounds 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 [35]. The most abundant phenolic compounds, generally present in OMW, include tyrosol and hydroxytyrosol and p-coumaric acid [5] that can be degradete by microalgae [35], however this ability depends on phenolic concentration, algae specie, time of exposure and probably also on culture conditions. Thus, our results suggested that for higher OMW concentrations, it will be necessary an optimization of fermentation condition, such as light regime.

An important difficulty encountered in commercial micro algal applications is the harvest of biomass, which presents several technological and economic complications. Biomass harvesting requires separation of solid from liquid and is a process covering around 30% of the total production cost [16]. On the other hand, immobilization techniques can allow easy biomass recovery from the treated effluent, as we observed in this work. Also a reduction of the costs can be achieved by combining OMW treatments with other applications. In fact, waste-grown microalgae are a potentially important biomass for biofuel production [16, 20, 23].Therefore, these algae biomass could be used for biodiesel feedstock production, or it can be anaerobically digested to make biogas.

In order to evaluate the OMW biotreatments with C. vulgaris, final samples from OMW 35% were used for phytotoxicity evaluation. As expected, biotreatment carry out in OMW, leads to a higher length root of seeds, comparing with both, positive and negative controls. In turns, OMW without treatment showed lower length root of seeds than positive control (Figure 2).

Although OMW 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 OMW treated, reflect a more stimulating activity of these samples, when compared to negative control.

These results confirmed that C. vulgaris can reduce phytotoxic compounds present in OMW that inhibits germination and growth of L. sativa, thus showing a good biotreatment potential.

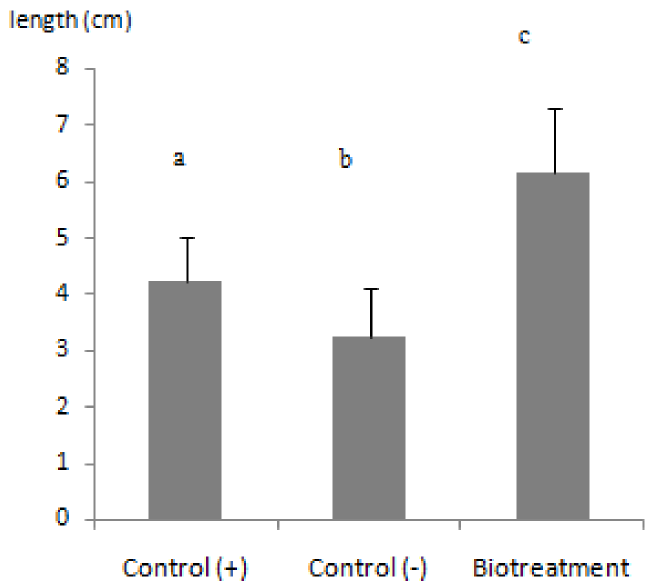

Figure2: Mean root length of germinated seeds obtained from positive and negative controls, and biotreatment of OMW 35% (M±DP). $p<0.05$

Conclusions

In generally, results from this work showed that immobilized C. vulgaris could be an alternative to bioremediation of OMW, but more detailed studies are still needed to optimize the process. Biotreatments 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.

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