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Evaluation of the microalgae potential as bioremediation agents for olive mill wastewater †

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Abstract: The potential for bioremediation of olive mill wastewaters with different origin - olive washing (OWW) and olive oil extraction (OMW) - by 4 species of microalgae (*Chlorella vulgaris*, *Auxenochlorella protothecoides*, *Scenedesmus obliquus* and *Arthrospira maxima* was evaluated. All microalgae could grow in the wastewaters, but *C. vulgaris* and *C. protothecoides* showed the best performance. The highest biomass productivities of 165.8 mg L^{-1} day^{-1} for OMW and 107.9 mg L^{-1} day^{-1} for OWW were achieved with *C. vulgaris* and *A. protothecoides*, respectively. Moreover, with both species, COD and nitrates content of the two wastewaters were reduced by 60 and more than 50%, respectively. However, a significant removal of polyphenols was verified only in OWW (~ 45%). Overall, these findings demonstrate the potential of *C. vulgaris* and *A. protothecoides* species be used in a biological olive mill wastewater treatment process.

Keywords: microalgae; bioremediation; biomass; olive-mill wastewater

1. Introduction

Olive oil industry is an important sector within the agro-food industries in the Mediterranean countries but constitutes a major environmental problem regarding the disposal of its wastewaters. Olive mill wastewater is a turbid, dark colored, foul-smelling and acidic effluent, which compositions depends on several factors, but especially on the characteristics of the olive oil extraction equipment. The extraction process has evolved over the years from discontinuous (press method) to continuous methods, using centrifugal separators. At first, a process with decanter of three outlets (olive oil, pomace and wastewater) was used, but to reduce the environmental impact generated, the number of outlets was reduced to two, one for olive oil and the other for pomace and vegetable water (and process water). In the two-phase system, wastewaters are produced in less volumes and have less organic load, however, large amounts of semisolid wastes are also produced [1,2].

Nowadays, chemical, biological and integrated technologies are used for the treatment of these wastewaters. As it presents a low biodegradability due to its antibacterial activity, given by the phenolic content, the use of different physicochemical operations is necessary to reduce toxicity. Besides, these processes are also efficient in reducing suspended solids and consequently organic matter content [1]. Bioremediation through microalgae is an interesting option, since it is an environmentally friendly process, as wastewaters can be used as cheap nutrient sources for microalgal biomass production that could be a source of stored chemical bond energy, especially into lipids, carbohydrates and proteins [3,4]. In fact, microalgal cultivation has been successfully used in the

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treatment of two-phase olive mill wastewaters, combined with other physicochemical operations (e.g. [4–6]).

The main objective of this work was to evaluate the potential for bioremediation of two OMW with different origin - olives washing (OWW) and olive oil extraction (OMW) - by microalgae. It was used three species of green microalgae, *Chlorella vulgaris*, *Auxenochlorella protothecoides* and *Scenedesmus obliquus* and the cyanobacterium *Arthrospira maxima*.

2. Material and methods

2.1. Microalgae

The microalgae cultures were obtained from the National Laboratory of Energy and Geology (LNEG) in Lumiar, Lisbon, Portugal. *Chlorella vulgaris* (INETI 58) and *Auxenochlorella protothecoides* (UTEX 25) were maintained in an inorganic medium containing per litre: 1.25 g KNO_3, 1.25 g KH_2PO_4, 1 g $MgSO_4.7H_2O$, 0.11 g $CaCl_2.2H_2O$, 0.5 g $NaHCO_3$, 10 mL Fe-EDTA solution and 10 mL trace elements solution (Chu medium). *Scenedesmus obliquus* (ACOI 204/07) was maintained in Bristol medium containing per litre: 250 mg $NaNO_3$, 75 mg K_2HPO_4, 33 mg $CaCl_2.2H_2O$, 75 mg $MgSO_4.7H_2O$, 175 mg KH_2PO_4, 25 mg NaCl, 60 mg Fe-EDTA, and 10 mL Chu medium. *Arthrospira* (*Spirulina*) *maxima* (Setchell & Gardner, LB 2342) was maintained in a standard inorganic medium for Spirulina containing per liter: 1.25 g $NaNO_3$, 8.4 g $NaHCO_3$, 500 mg NaCl, 500 mg K_2SO_4, 250 mg K_2HPO_4, 40 mg EDTA, 26.5 mg $CaCl_2.2H_2O$, 5 mg $FeSO_4.7H_2O$, 100 mg $MgSO_4.7H_2O$ and 1 mL trace elements solution [7].

2.2. Wastewaters

The olive mill wastewaters used in this work were obtained from an olive-oil extraction plant in the Douro region, northern Portugal, which uses a continuous centrifugation process with two outlets (olive oil and pomace). It was collected a part of the liquid fraction of the pomace reservoir (hereafter referred to OMW) and washing wastewater from another reservoir (OWW)).

2.3 Experimental setup

Prior to microalgae culture, wastewaters were pre-treated by a 24h sedimentation and a tyndallisation process which consisted of heating at 80°C during 2 h, followed by cooling at room temperature, repeating these process three days in succession. Tyndallised wastewater was stored at 4°C until further use. Then, the culture media were prepared by diluting the OMWs with inorganic media (appropriate for each species): 5% and 50%, v/v, for OMW and OWW, respectively. Finally, it was added 5 % (v/v) of each microalgae inoculum. All experiments were conducted in duplicate in 250 mL Erlenmeyer flasks incubated in an orbital shaker (New Brunswick Scientific) at 23 ± 2 ◦C, under an agitation speed of 100 rpm and kept under continuous illumination (light intensity of 20–25 μmol photons m^{-2} s^{-1} supplied by a white 18 W LED lamp). Wastewaters without inoculum were used as a control.

2.4. Analytical determinations

The following parameters were determined for raw wastewaters: pH, electric conductivity (EC), turbidity, total suspended solids (TSS), total organic carbon (TOC), chemical oxygen demand (COD), biochemical oxygen demand (BOD), polyphenols, orthophosphate ($P\text{-}PO_4$), total nitrogen (TN) and nitrates (NO_3).

pH, EC, turbidity values and TSS were directly measured by using a pH meter (Crison micro pH 2000), a conductivity meter (VWR C030), turbidimeter (2100N IS, HACH) and a UV/VIS-Spectrophotometer (HACH) respectively. TOC and TN were analysed in a Shimadzu TOC–L with a TN unit and an ASI-L autosampler. COD and BOD were measured according to Standard Methods 5220D and 5210D (using a System Oxitop Control), respectively [8]. Polyphenols were determined by spectrophotometry using the Folin-Ciocalteu reagent (Merck) and expressed as equivalent mg gallic acid L^{-1}. P-PO_4 was measured according to Standard Method 4500-P E [8] and NO_3 according to [9].

Microalgae growth was monitored daily, calculating the biomass dry weight (DW) by filtering the samples with a glass microfiber of 1.6 µm pore size and drying overnight at 105 °C. Biomass productivity (P_X, mg L^{-1} day^{-1}) was calculated by:

$$P_X = (DW - DW_0) / (t - t_0), \tag{1}$$

where 'DW, mg/L' is the biomass concentration at any time of the experiment and 'DW_0 g/L' is the biomass concentration at the beginning of the experiment (t = 0 days). After filtration of the culture samples, the filtrate was collected and characterized in terms of pH, COD, polyphenols, P-PO_4 and NO_3, to evaluate the efficiency of the treatment.

3. Results and discussion

3.1. Wastewaters composition

The main physicochemical characteristics of sedimented wastewaters used in this work are summarized in Table 1. It is evident the high turbidity (given by high TSS) and organic matter content, particularly in OMW, which presents excessive TOC, COD and BOD_5 values. From an environmental point of view this is a problem, and it is required an efficient solution for the treatment of these wastewaters. Polyphenols content are also relevant. These compounds are transferred to OMW during olives crushing and olive oil washing phenolic compounds which are toxic to microorganisms and plants [1]. Therefore, to reduce the organic matter, turbidity and toxicity, the effluent was diluted with inorganic media at 5% and 50% (v/v), for OMW and OWW, prior to the microalgal cultivation.

Table 1. Characterization of the wastewaters used.

Parameter	OMW	OWW
pH	5.1 ± 0.1	4.1 ± 0.1
EC (µS cm^{-1})	270 ± 50	357 ± 12
Turbidity (NTU)	693	138
TSS (mg L^{-1})	699	118
TOC (mg C L^{-1})	67 130	2 382
TN (mg N L^{-1})	809.9	33.3
COD (mg O_2 L^{-1})	206 880 ± 1332	7789 ± 356
BOD_5 (mg O_2 L^{-1})	6050 ± 50	80 ± 10
Polyphenols (mg gallic acid L^{-1})	3875 ± 20	326 ± 69
P-PO_4 (mg P L^{-1})	487 ± 6	18 ± 3
NO_3 (mg L^{-1})	548 ± 21	49 ± 4

It is reported that an optimal C/N/P mass ratio of 46.1/7.7/1 can be deduced for microalgae [10]. It seems that the wastewaters in this work were N-deficient, particularly in OWW as C/N ratios are high (17.8 and 71.5 in OMW and OWW, respectively) whereas N/P ratios are close to optimum (7.7) in OMW and very low (1.9) in OWW.

3.2 Microalgal growth

From the growth curves represented in Fig. 1 it is clear the complexity of the effluents. During the first 3 days, the four species of microalgae showed a similar behavior, with low productivity (lag phase), followed by an abrupt increase of the biomass, in the case of *C. vulgaris* and *A. protothecoides* and finally a deceleration growth phase. The species *S. obliquus* showed the least adaptability to both wastewaters.

Higher productivities (Table 2) were achieved in OMW, as this wastewater has a higher amount of organic matter, which leads to greater availability of nutrients for the growth of microalgae. However, in the case of OMW, it was observed cellular death after 4 days (Figure 1. OMW), that means that, despite the greater availability of nutrients, the toxicity of the effluent is overpowering.

Overall, in both wastewaters, *C. vulgaris* and *A. protothecoides*, showed the highest productivities (Table 2). Using *A. protothecoides* a maximum value of 165.8 mg L^{-1} day^{-1} was achieved for OMW and for OWW was 107.9 mg L^{-1} day^{-1}, using *C. vulgaris.*

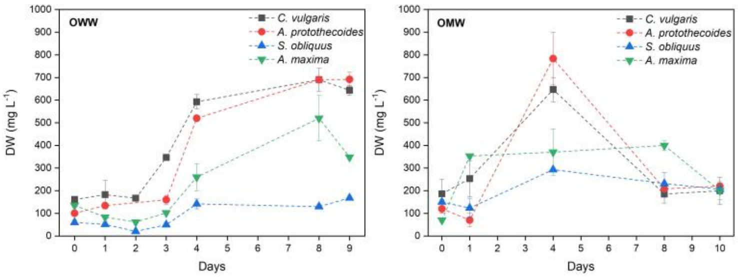

Figure 1. Evolution of the concentration of biomass, given by dry weight (DW), over time in OWW and OMW.

Table 2. Maximal productivities for each species in olives washing wastewater diluted at 50% (OWW) and olive oil extraction diluted at 5% (OMW).

Wastewater	Microalgae	$P_{X,\ max}$ (mg L^{-1} day^{-1})
OWW	*C. vulgaris*	107.9 ± 15.3
	A. protothecoides	73.7 ± 3.6
	S. obliquus	20.4 ± 7.6
	A. maxima	48.1 ± 16.8
OMW	*C. vulgaris*	115.1 ± 18.9
	A. protothecoides	165.8 ± 34.1
	S. obliquus	38.3 ± 4.2
	A. maxima	143.3 ± 22.4

3.3 Bioremediation potential

To evaluate the bioremediation potential of the microalgae, it was calculated the removal of the pollutant in terms of COD, polyphenols, P-PO_4 and NO_3. Microalgae can consume organic carbon from wastewaters, using a heterotrophic path if light is absent or a mixotrophic one, combining autotrophic (photosynthesis) and heterotrophic metabolisms [11]. Phenolic compounds are considered toxic to many microalgae but can also be considered as carbon and energy sources. It is suggested that microalgae can remove phenolic compounds by mineralization to carbon dioxide or biochemical modification to other compounds [12]. Nitrogen and phosphorous are the two most important macronutrients in microalgae metabolism. Microalgae can assimilate NO_3, which is one of the most common inorganic nitrogen forms in aquatic environments, by first reducing it to ammonium, and incorporate phosphorous in its orthophosphate forms ($H_2PO_4^-$ and HPO_4^{2-}) through phosphorylation [13].

One can see in Fig. 2 that in control (non-inoculated wastewaters) it was verified some removal of the pollutants, which can be explained by the proliferation of other heterotrophic microorganisms such as bacteria, fungi and protozoa that competes with microalgae. The most easily reduced pollutant by microalgae was nitrate. It was observed a removal of more than 50% in all cultures. Generally, comparing wastewaters, all microalgae present similar performance when removing COD. Although the great availability of organic matter, the best removals were 62% for OMW and 68% for OWW in cultures of *C. protothecoides* and *C. vulgaris*, respectively. The effluents were somewhat recalcitrant to the microalgae treatment. The most significant removals of $P\text{-}PO_4$ were verified with *Arthrospira* (67.0% for OMW and 36.0% for washing wastewater). Since phenolic compounds are toxic for microalgae, it was not expected a significant removal, particularly in OMW. In fact, the removal of polyphenols did not exceed 45 % for OWW, using both *Chlorella* species, and in OMW only *Arthrospira maxima* could consume this pollutant (~40%).

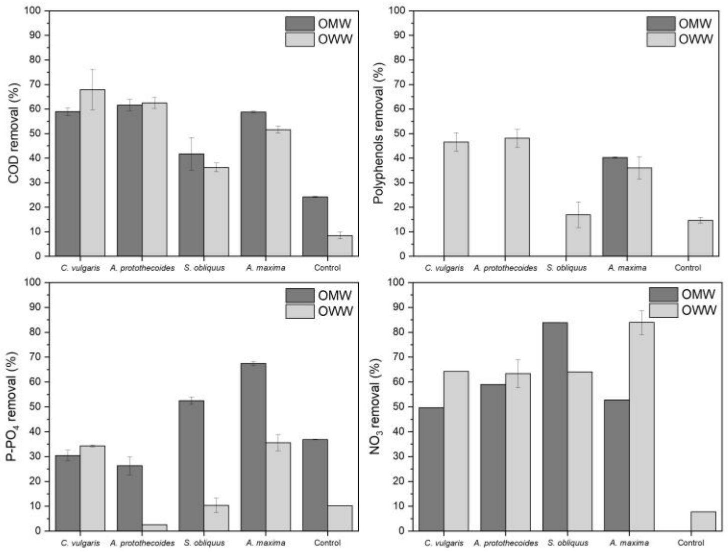

Figure 2. Removals of COD, polyphenols, $P\text{-}PO_4$ and NO_3 by microalgae in OMW and OWW.

4. Conclusions

Although microalgae can grow in these olive mill wastewaters and show potential for its bioremediation, further studies will not be feasible if this effluent is not subjected to a more complex primary treatment, due to its toxicity. Some viable options could be physicochemical methods, such as coagulation-flocculation and chemical oxidation, such as Fenton or photo-Fenton, to reduce organic matter, turbidity and toxicity.

Considering the pollutants removal and biomass productivities, the species *C. vulgaris* and *A. protothecoides* could be employed in the secondary treatment of olive mill wastewaters.

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