Effect of Olive Mill Wastewaters on *Scenedesmus* Sp. Growth, Metabolism and Phenols Removal

Ines Dahmen-Ben Moussa (✉ ines_dahmen@hotmail.fr)

CBS <https://orcid.org/0000-0001-7121-0150>

Mohamed Ali Masmoudi

Centre de Biotechnologie de Sfax

Firas Feki

Centre de Biotechnologie de Sfax

Sirine Choura

Centre de Biotechnologie de Sfax

Sami Sayadi

Center for Sustainable development college of arts and sciences, Qatar

Research

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Abstract

Background The industry of olive oil produces large wastewater quantities. The elimination of these toxic by-products is an important environmental issue that requires a sufficient solution. In this study, *Scenedesmus* sp. was used for the simultaneous lipid and antioxidant molecules production and olive mill wastewater biodegradation.

Results The two-stage olive mill wastewater cultivation did not significantly inhibit the production of microalgal biomass, compared to a single growth stage, when OMW and OMUF were supplied at the beginning of cultivation. OMUF, at 40%, enhanced the Ch *a*, *b* and carotenoids amounts (2.57, 7.4 and 1.69 mg/g dw, respectively), and protein and carbohydrate accumulation (350.96 and 315.62 ng/cell, respectively), after single phase culture. However, the highest OMUF and OMW concentrations increased total lipid content to 639.13 and 679.86 ng/cell, respectively. About 58% of phenol removal was achieved and highest accumulation of polyphenols in algal cells with 40% OMUF condition under one stage strategy. The highest OMUF and OMW concentrations exhibited highest DPPH and ABTS scavenging ability after single and two-phase culture. Moreover, the cytotoxicity of the OMUF against HEK-293 human embryonic kidney cells was evaluated by MTT assay. The MTT assay showed that the biomass of *Scenedesmus* sp. cultured on Tunisian filtered olive mill waste water did not show any toxic effect.

Conclusion Our results indicate that *Scenedesmus* sp. is a promising microalga for the biotreatment of OMW and the production of bioactive metabolites.

Background

The production of olive oil is a main agro-industrial activity in Tunisia. In the ranking by the International Olive Council (IOC), Tunisia was, with 340,000 tons of olive oil produced in 2014/2015, the second principal oil producing country after Spain. This kind of agro-industry generates large quantities of by-products, namely the olive mill wastewater (OMW) which has proven to be a severe environmental problem. Therefore, it's necessary to seek out a suitable and effective management method in order to reduce its harmful effects and meet environmental commitments. The OMW whole annual production in Tunisia is on average 600,000 tons/year and can reach 1 million m³. The organic matter present within the waste includes phenolics, sugars, nitrogenous compounds, fat, organic acids and a substantial amount of suspended solids [1].

Many solutions have been suggested for the recovery of OMW but many factors must be taken into account when choosing the best recovery method, as well as the total quantity of effluents, investment costs, accessible land, industrial or agronomic environment, local laws and the most vital needs. The most used method most nowadays to resolve the issues related to OMW is the direct application as organic fertilizers agricultural soils [1, 2]. These studies revealed OMW positive effects on the fertility of soil and growth of plant. However, recent studies have shown that adding OMW induces significant

changes within the structure and function of microorganisms which in turn cause severe environmental problems and influence soil fertility because of its phytotoxicity and antibacterial effects [1, 3].

Many researchers highlighted the pollutant potential of the OMW residues produced by the three-phase olive oil extraction process and proposed different sorts of treatment methods.

Several purification processes, particularly physico-chemical, are implemented such as thermal, physical and chemical treatment [4], but the antioxidant effect remains the limiting factor in these treatments.

Biological degradation of OMW phenolic compounds in contrast to other processes is considered to be a healthier, more efficient and less costly method of reducing pollutants. Considerable degradation of the two most representative phenolic compounds in OMW (Tyrosol and caffeic acid) has been achieved using microorganisms isolated from the OMW. Several studies have been carried out on aerobic treatment of OMW with microalgae [5]. Other work has proven the effectiveness of aerobic treatment with mixed cultures.

The use of microalgae in wastewater treatment has taken lots of attention in recent years. Prompted by the fact that OMW may be a source of nutrients for the growth of microalgae, mainly phosphorus and organic carbon, some researchers have investigated the possibility. Motivated by the actual fact that OMW may provide nutrients for the growth of microalgae (for example, phosphorus and organic carbon), some reporters have studied the addition of OMW to microalgae cultures [6]. Hodaifa, Martínez et al. and Di Caprio, Altimari et al. [5, 7] suggested using OMW as a growth medium for the biomass production of *Scenedesmus obliquus*. In fact, microalgae and cyanobacteria are characterized by their high metabolic plasticity. They have a metabolic and physiological response to all combinations of environmental factors [8, 9]. The influence of different parameters, including temperature, light intensity, pH, OMW/medium ratio and OMW pretreatment on microalgae growth and accumulation of target microalgal fractions was investigated for the microalgae *Scenedesmus obliquus* [5, 10–12].

The remediation of wastewater using microalgae is an environmentally friendly practice with no pollution detected. Indeed, the biomass produced may be reused and allows sufficient nutrient recycling.

Stress similar to nutritional repletion, OMW addition stress has also been reported to enhance carbohydrates (up to 44%) under two-stage strategy in *Scenedesmus* sp. [13]. Effects of OMW on microalgae are mostly study in *Chlorella vulgaris* with focus to elimination of phenolic compounds and evaluation of the toxicity of final treated effluent by germination seed tests. The effects of OMW on microalgae are mainly studied in *Chlorella vulgaris* to eliminate the phenolic compounds and evaluate the toxicity of the final treated effluent by seed germination tests [14]. They evaluated the potential of *Chlorella vulgaris* in the elimination of phenolic compounds from OMW at different concentrations of inocula evaluated the potential of algal biomass in a second cycle of bioprocessing. The results showed that *C. vulgaris* has the capacity to eliminate phenolic compounds, however a decrease in its effectiveness has been observed with the sight of these compounds in the environment.

Scenedesmus sp., a strain of interest for the production of biofuel [15]; biomass for health food and animal feed [16]; high added value compounds [17]; and the sequestration of CO₂ [18] was selected for this investigation for the purification of OMW. The aim of this work was to analyze the effects of crude OMW and OMW treated by ultrafiltration (OMUF) induced biochemical changes in the freshwater microalgae *Scenedesmus*, during single-stage and two-stage cultivation regimes.

Methods

Scenedesmus cultivation conditions

The freshwater algal strain *Scenedesmus* sp. was isolated from a geothermal water source in Kelibia, Tunisia, and preserved in Petri dishes on solid BG 11 medium. cells were transferred to 250-mL then 1-L Erlenmeyers flasks containing 600 mL BG11 culture medium [19]. Cultures were maintained under continuous illumination of 100 $\mu\text{E m}^{-2}\text{s}^{-1}$, constant agitation at 30 °C.

Procedure and OMW supply strategies

The crude olive mill wastewater (OMW) was taken from a three-phase olive oil extraction located in Sfax (Tunisia). OMUF was obtained after ultrafiltration of OMW through 0.22 μm filters.

To evaluate the effect of OMUF and OMW on biomass and high added value compounds, the following stage cultivation were tested:

- Single stage cultivation (Batch supply strategy) with adding, on day 0, OMUF or OMW (2.5, 5, 10, 20, 40 and 80% v/v) to the medium for 8 days of growth period.
- Two-stage cultivation with OMUF or OMW (40 and 80% v/v) added in the culture after 8 days of cultivation to induce stress in microalgae *Scenedesmus* sp. throughout 4 days of growth period.

Analytical techniques

OMW characterisation

Both crude (OMW) and filtered (OMUF) olive mill wastewater used in this work were characterized (Table 1). All samples were kept at 4 °C until use.

Table 1
Chemical and physical properties of crude (OMW) and filtered (OMUF) olive mill wastewater used in the experiments.

<i>Parameter</i>	<i>Crude OMW</i>	<i>Filtered OMW (OMUF)</i>
pH	5.63 ± 0.01	5.64 ± 0.02
Electrical conductivity (ms/cm)	13.82 ± 0.05	10.71 ± 0.04
COT (g/L)	26.6 ± 1.3	10 ± 0.9
Ash (%)	25.62 ± 0.3	31.97 ± 0.19
Dry matter (%)	4.75 ± 0.05	2.52 ± 0.01
Volatile solids (%)	3.56 ± 0.014	1.72 ± 0.13
Total suspended solids (g/L)	7.36 ± 0.09	-
Volatile suspended solids (%)	1.034 ± 0.06	-
color (m ⁻¹)	249.30	359.00
Phenols (g/L)	4.27 ± 0.05	1.038 ± 0.4
Flavonoids (g/L)	0.84 ± 0.08	0.56 ± 0.12
Tannins (g/L)	1.18 ± 0.05	0.36 ± 0.03
Lipids (g/L)	2.1 ± 0.24	0.7 ± 0.08
N Kjeldahl (g/L)	0.18 ± 0.02	0.70 ± 0.05
P total (g/L)	0.12 ± 0.04	0.08 ± 0.02
NH ₄ ⁺ (g/L)	0.60 ± 0.07	0.51 ± 0.08
NO ₃ ⁻ (g/L)	0.22 ± 0.05	0.16 ± 0.05

Algal biomass characterisation

At the end of experiments, algal cells were harvested by centrifugation (6000 rpm; 3 min) and washed twice. Microalgal biomass was lyophilized, wetted and treated by Sonifier 40 KHz at room temperature for 30 min, before determining the physic-chemical composition. Polysaccharides in the different extracts were measured by the DNS protocol [20]. Protein contents were quantified using the Bradford method [21].

Chlorophyll in fresh microalgal cells was extracted with ethanol and then incubated in the darkness at 4 °C for 1 h. The amount of chlorophyll was quantified by measuring the OD at 710, 663, 645 and 480 nm by UV- spectrometry and then calculated with the Lichtenthaler and wellburn equations [22].

Total lipids were extracted and quantified by the Modified protocol of Bligh and Dyer [23]. Dried algal pellet was mixed with 1.5 mL of chloroform-methanol-water (1:2:0.8, v/v/v) and homogenized for 10 min. Then, 1 mL of chloroform was added to the mixture. Chloroform phase was separated by centrifugation (4000 rpm, 2 min) and was transferred into a weighted glass-tube and 0.5 mL of hexane was added to the lower phase to extract polar lipids.

Transesterification of algal lipids was performed as described by Dahmen et al. [24]. Briefly, 20 mg of lipid samples and 1 mL H₂SO₄ (5%, v/v) in methanol were added into the glass tube and boiled at 85 °C for 1.5 hours. Afterward, 1 mL Hexane was added, and centrifuged at 4000 rpm for 2 min. After the centrifuge, the upper organic phase was transferred into a clean vial.

Fame profile was carried out using GC-MS spectrometry HP model 5975B inert MSD (Agilent Technologies, Palo Alto, CA, USA). Flame ionization detector (MSD5975B, ionization voltage 70 eV) and Agilent Technologies DB-5MS Capillary Column were used for the analysis. Helium (1 mL min⁻¹) was used as a carrier gas. The injector temperature was 100 °C for 1 min which was increased to 260 °C at a detector temperature gradient of 4 °C min⁻¹. Identification of fatty acid methyl ester was assigned by matching the mass spectra with NIST and Wiley database library Dahmen et al. [24].

Extraction of phenolic compounds

the extraction of phenolic compounds was carried out according to the modified method of Li et al. [25] using an ethanol / water mixture. 200 mg of lyophilized biomass was suspended in the presence of 2 ml of ethanol / water mixture (3/1, v / v). The tubes are placed in a sonicator bath at 40 KHz at room temperature for 30 min and centrifuged at 4000 rpm for 2 min. The process is repeated twice under the same conditions. The ethanolic extract solution were collected and stored at -20 °C until analysis.

The total phenolic content in *Scenedesmus* species extracts were colorimetrically measured using the Folin-ciocalteu reagent as reported by Jimoh et al. [26].

Determination of algal extract antioxidant activity

DPPH scavenging assay

The free radical scavenging activity of ethanolic extracts (1 mg mL⁻¹) was estimated using a stable 2, 2 - diphenyl - 1- picrylhydrazyl radical (DPPH) [27]. The affinity of phenolic molecules for quenching DPPH radicals (% DPPH inhibition) was expressed as a percentage (E%) and calculated using the following equation:

$$\text{Activity (\%)} = (1 - A/A_0) \times 100$$

Where *A* is the reaction mixture absorbance at 517 nm, and *A*₀ is the absorbance of DPPH without sample. The radical scavenging ability of standard Butylated hydroxytoluene (BHT) and ascorbic acid (Vit C) was also determined for comparison.

ABTS radical-scavenging capacity

The ABTS -scavenging capacity of ethanolic extracts (1 mg mL^{-1}) was determined according to Pari and Suresh [28].

Scavenging capacity percentage (E %) has been calculated according to the following formula:

$$\text{Activity (\%)} = ((A_0 - A) / A_0) \times 100$$

Where A is the reaction-mixture absorbance, and A_0 is the absorbance of ABTS without sample. Trolox was used as a standard antioxidant substance.

Cell culture and cytotoxicity assay

Cells of Human Embryonic Kidney (HEK- 293) were obtained from Amerocain Type Culture Collection, Manassan, VA. Cells were cultured at 37°C in an atmosphere contained 5% CO_2 . Modified Eagle medium of Dulbecco (DMEM) was used and supplemented with 10% Fetal Bovine Serum, 2 mM glutamine, and 1% penicillin/streptomycin. The cells were detached with trypsin-ethylenediamine-tetra-acetic acid (trypsin EDTA) when they are confluent. The medium was changed every 2–3 days.

HEK Cells were seed in 96-well microplates at a density of 5×10^4 cells/well and were treated with Sc- BG 11 and 40% Sc-OMUF microalgal biomass (0, 12.5, 50, 100, 200, 400, 800 and 1000 $\mu\text{g/mL}$) for 24 hours. After cultivation, cells viability was assessed by the incubation with MTT (0.5 mg mL^{-1}) at 37°C for 4 hours. The MTT- purple formazan produced by viable cells with active metabolism was dissolved in 100 μL of SDS (10%). Finally, a microplate reader (thermo Scientific Varioskan Flash) was used to measure the optical density (OD) at 570 nm.

Statistical analysis

One-way variance analysis (ANOVA) was applied for each experiment. All data were performed in triplicate and are reported as mean $\pm$ standard error (SE). Statistical significance was determined at $p < 0.05$. Person's correlations among assays were calculated using SPSS 13.0 software.

Results And Discussion

Wastewater characterization

The physic-chemical characteristics of OMUF and OMW which has been used in the culture media in the different experiments are summarized in Table 1. High level of total suspended solids and relatively low pH value can be observed. As was to be expected, OMUF contained values of conductivity, organic matter (COT), and fats much lower than that on OMW. Total phenols (4.27 g/L) are comparable to those found by Di Caprio et al. [5].

Effect of OMW and OMUF on *Scenedesmus* sp. Growth

The growth of *Scenedesmus* sp. exposed to different concentrations of OMW and OMUF was determined after single and two stages of incubation for the selection of the suitable concentration. Growth curves of *Scenedesmus* sp. under different treatments are shown in Fig. 1. Addition of OMUF (Fig. 1A) and OMW (Fig. 1B) to the growth medium significantly decreased the cell density of *Scenedesmus* sp. after 8 days of cultivation in a BG11 medium with different initial concentrations. In all the experiments, the cultures showed a short latency phase and an exponential phase, which extends until day 4, followed by a stationary phase induced by a kind of stress. This stress, often the supplementation of OMUF and OMW, induces a change in the metabolism of microalgae, which can favor the production of some metabolites rather than cell division. The highest cell densities shown by *Scenedesmus* sp. at 3.8 and 3.2×10^6 cell/mL when cultured in 2.5% OMUF and OMW, respectively, is lower than the recorded cell density by Khatoon et al. [29].

In all OMW and OMUF treatments, cell density decreased at day 8 and the maximum biomass concentration was lower than that of control culture. Indeed, it has been possible to observe that OMUF and OMW are obviously a nutrient with an optimal concentration of 20 and 10% for the experimental conditions used in the present work. However, from these concentrations the opposite effect was observed, these nutrients would be in excess generating a negative effect at higher concentrations. The negative effects of these compounds seem to be due to the richness polyphenols in the surrounding environment. This may also be due to the dark color which prevents the penetration of light into the culture medium in the tests carried out with the addition of OMUF and OMW compared to that of the control.

Generally, the single stage strategy has been the most studied by previous studies [5, 11, 13]. These effects were studied during 2 stages of culture using the highest OMUF, and OMW concentration. *Scenedesmus* sp. was firstly cultivated in standard BG-11 medium for 8 days and secondly, stress was applied by adding 40 and 80% of OMUF, and OMW after 4 days of culture (Fig. 1C). This result shown in Fig. 1C indicated that there was substrate inhibition at these concentrations.

Compared to one stage, two-phase olive mill wastewater stress did not shown a considerable inhibition of cellular biomass production making it an effective strategy to generate more high value-added metabolites without compromising biomass production.

Overall biomass productivity and specific growth rate obtained for the performed experimental tests are reported in Fig. 2. Larger growth rate and maximum productivity were found when microalgae were cultivated at 5 and 10% OMUF (Fig. 2A) or OMW (Fig. 2B) reaching a maximum value of 0.51 d^{-1} and 1.12 g/L/d , respectively, under 10% OMUF.

For the highest and OMW concentrations used, the variation of μ seems to exhibit an inhibitory effect in *Scenedesmus* sp. growth (Fig. 2). The analysis of variance carried out highlights a significant reduction in biomass productivity in response to the supply of OMUF and OMW. This result was expected since the

OMUF and OMW contain organic acids and phenolic compounds, all known to affect the growth of microalgae [30].

Similar to single stage, two-stage cultivation (adding OMUF or OMW at day 8), growth rate and biomass productivity decreased proportionally with increasing the concentration OMUF or OMW to 40 and 80% compared to control condition. This fact can be explained by the inhibition effect by the substrate for high concentrations which exceeds 40% in culture medium (Fig. 2C).

Effects of OMW and OMUF stress on photosynthetic pigments

To assess the impacts of OMW and OMUF stress on the photosynthetic process content of *Scenedesmus* cells, the chlorophyll and carotenoid contents were measured after during single and two phase culture (Table 2). There was a significant difference in photosynthetic pigments content among *S.* cells cultured at different OMW and OMUF concentrations. Chlorophyll *a*, *b* and total carotenoid contents increased with the increase of OMUF concentration. Highest Ch *a*, *b* and carotenoid contents were found in cells grown under 40% OMUF condition (2.57, 7.4 and 1.69 mg/g dw, respectively). However, during two phase cultivation, Ch *a*, and carotenoid contents of control culture were 5.1 and 0.16 mg/g dw, respectively, while 4 days 80% OMUF stressed culture had noticeable increase in these pigments contents of 42 and 86%, respectively (Table 2). Similarly, ratio of Car/Ch *a* significantly increased with the increase of the OMW and OMUF proportions in the culture medium which is an indirect sign of polyphenols stimulated oxidative stress and inhibited the activity of PSII.

Table 2
Effects of OMW and OMUF on photosynthetic pigments of *Scenedesmus* sp.
during single and two-stage supply strategies.

Treatments	Ch <i>a</i> (mg/g dw)	Ch <i>b</i> (mg/g dw)	Car (mg/g dw)	Car/Ch <i>a</i>
Single-stage cultivation				
Control	0.21	0.21	0.06	0.27
2.5% OMUF	0.38	0.83	0.07	0.19
5% OMUF	0.07	0.40	0.01	0.16
10% OMUF	0.12	0.24	0.07	0.57
20% OMUF	0.38	0.82	0.34	0.89
40% OMUF	2.57	7.40	1.69	0.66
80% OMUF	0.85	0.05	0.16	0.18
2.5% OMW	0.49	0.10	0.13	0.26
5% OMW	0.59	0.96	0.04	0.07
10% OMW	0.43	0.91	0.17	0.39
20% OMW	0.77	1.84	0.16	0.20
40% OMW	0.69	2.39	0.24	0.35
80% OMW	0.80	2.64	0.41	0.51
Two-stage cultivation				
Control	5.102	3.658	0.159	0.031
40% OMUF	3.033	1.708	0.133	0.044
80% OMUF	7.270	0.772	0.296	0.041
40% OMW	4.540	0.051	0.188	0.041
80% OMW	4.127	0.077	0.308	0.099

Our results clearly demonstrate positive effect of OMUF on *Scenedesmus* sp. pigment biosynthesis, including Ch *a*, and total carotenoids production. These effects may be explained by the capability of algae to improve the chlorophyll amount when exposed to low light intensity [31] induced by addition of phenols (brown/dark colour). Little data is available in the literature on OMW influence on photosynthetic pigments synthesized by *Scenedesmus* sp. These results were in line with those of Di Caprio et al. [32], who investigated the influence of phenols extracted from OMW using a resine, on total chlorophyll and total carotenoids produced by *Scenedesmus* sp. In this last study, the mixotrophic and photoautotrophic

culture conditions allow better cell concentration and higher chlorophyll and carotenoid contents compared to the heterotrophic mode, which may mainly attributed to the penetration of light.

Changes in the biochemical composition of *Scenedesmus* sp.

The biochemical composition of *Scenedesmus* sp. biomass in different treatments was evaluated in Fig. 3. Figure 3A shows an increase of carbohydrate and protein content in direct proportion with the increase of concentration of olive mill wastewater in culture medium from 0 to 40% and then decreased when added 80% of OMUF or OMW to the culture medium of *Scenedesmus* sp. indicating that the growth medium was sufficiently stressful. Highest amounts of protein and carbohydrate (350.96 and 315.62 ng/cell, respectively) were found in cells cultivated in 40% OMUF. In the second experimental series, by adding various proportions of OMW, the highest amount of protein (217.71 ng/cell) was found in cells cultivated in 40% OMW. While the highest carbohydrate accumulation (106.48 ng/cell) was found in cells cultivated in 20% OMW followed by 40% OMW (102.41 ng/cell). In this study, the elimination of solid matter from the OMW by ultra-filtration implied an increase in the protein and carbohydrate content. At both single and two-stage culture, carbohydrate and protein contents were largely higher than those determined by Choix et al. [33], but they are lower than those found in some studies [34]. These low protein contents could be attributed to the low intensity of light used in these experiments and the inhibitory effect due to the use of high concentrations of OMW in the culture medium; since our results show that the productivity of biomass increased only for the low concentrations of OMW added in the medium.

During two-stage cultivation, highest protein content was found in 40% OMW (Fig. 3B). However, highest carbohydrate content (138.79 ng/cell) was found in cell cultivated at 80% OMUF, which was significantly higher (5-fold) than the control culture (25.29 ng/cell). Similar results were also found by Di Caprio et al. [13] for carbohydrates content produced by microalgae cultivated with OMW at single and two phase supply strategy.

The lipid content varied between 16.66 and 679.85 ng/cell. Under these conditions, the lipid content of *Scenedesmus* sp. increased significantly ($p < 0.05$). It was 639.13 and 679.86 ng/cell for the highest OMUF and OMW concentrations, respectively, used during single-stage culture.

Similar studies [35] on the bioremediation of OMW by *Yarrowia lipolytica* strain, also revealed that the total lipid content increases with the increase of the OMW concentration (from 10 to 20%) in the culture medium.

During two-stage cultivation strategy, the content of lipid was 17.4 ng/cell under control condition, which increased to 341.27 ng/cell after 3 days of OMUF stress at 80%. The increase in lipid content may be well attributed to the fact that the OMW supply, can suppress cell growth [5], while it increased lipid accumulation probably because of the effect of its toxic phenols.

The energy-rich compounds accumulation by microalgae, such as carbohydrate and lipid, constitutes an essential buffer allowing the cell to survive period of low light intensity mainly the dark color of OMW. These two compounds molecules, source of energy and carbon, contribute not only to cell survival, but also as DNA replation, nuclear cytokinesis and depletion of DNA [36].

Compared to single stage culture, comparatively lower carbohydrate and lipid production during two-stage culture was observed (Fig. 3B). One of possible reasons behind this may be under OMW addition, nuclear and cellular divisions increase those results in the degradation of carbohydrate and lipids of microalgae [37].

The proportional increase in lipid and carbohydrate contents with the enhancement in OMW concentration found in photoautotrophic culture in a single stage can also be explicated by the fact that microalgae accumulate starch as the first compound [37], while to increase the accumulation of lipids, longer periods of stress are necessary.

Based on these results, it is concluded that the best strategy for maximizing the accumulation of biomass and lipid is to cultivate microalgae in a single stage. Regarding the fatty acid content of *Scenedesmus* sp., its profile and data are presented in Table 3. In our study, the fatty acid composition was affected by olive mill waste water addition. Oleic acid (C18:1), the ideal component of biodiesel, reach 62.83 and 68.95% of the total fatty acids of *Scenedesmus* sp. cultivated with 40% OMW and 80% OMW, respectively. For their properties such as viscosity, density and calorific value, fatty acids from 16 to 18 carbon atoms are considering to be necessary ingredients which can ameliorate the biodiesel quality.

FA (%)	Single stage				
	Control	40% OMUF	80% OMUF	40% OMW	80% OMW
C10:0	0	0	0	4.16	0
C11:0	0	0	0.61	0	0
C12:0	0	1.09	0	0	0
C13:0	0	0	0	0	0
C14:0	1.25	1.15	0.91	1.06	0
C16:0	22.35	14.85	11.60	14.81	17.73
C17:0	0	0.37	0	0	0.04
C18:0	4.55	2.49	6.46	5.75	5.50
C20:0	1.78	0	0	0	1.84
C22:0	0.39	0	0.12	0.35	0.32
C23:0	0	0	0	0.09	0.06
C24:0	0.14	0	0.07	0.23	0.20
C26:0	0	0	0.20	0.149	0.09
C28:0	0	0	0	0	0.13
SFA	30.46	19.95	19.98	26.60	25.93
C16:1	7.55	15.99	14.81	6.68	2.13
C18:1	33.62	29.63	20.74	62.83	68.95
C20:1	1.68	0	1.65	1.45	1.26
MUFA	42.86	45.62	37.20	70.96	72.34
C16:2	0	0	0.21	0	0
C18:2	26.67	34.43	42.60	2.43	0.89
C18:3	0	0	0	0	0.82
C20:5	0	0	0	0	0
PUFA	26.68	34.43	42.82	2.43	1.72

Table 3

Effect of OMW and OMUF on variation in the fatty acid content of the lipid fraction of the biomass of *Scenedesmus* sp. during single supply strategy.

As recorded in this Table 3, the dominant fatty acids extracted from *Scenedesmus* cells grown under 40% OMW and 80% OMW conditions, respectively, are those of the C16 and C18 series (70.96 and 72.34% of the total fatty acids), respectively, which can allow to have a good quality biodiesel [38]. There is no clear effect in the fatty acid profile as OMW concentration increased, whereas it showed a slightly lower saturated fatty acid content (19.98%) and higher polyunsaturated content (42.82%) under 80% OMUF addition as compared with 80% OMW addition (25.93 and 1.72%, respectively), the best concentration for lipid production. Clearly, the Fatty acid profile depends on the light received by *Scenedesmus* sp.

Residual polyphenols in the medium

The removal of polyphenols, which are contained in OMW was investigated. Figure 4 shows the residual phenol in the culture medium and the percentage of removal of these phenols by *Scenedesmus* sp. grown at different concentrations of OMUF and OMW during single and two-stage supply strategies.

Under single stage strategy, polyphenols were efficiently removed by *Scenedesmus* sp. in 40% OMUF condition which reach 45% at day 4 then increased to 58% at day 8 (Fig. 4D). At this dilution, biotreatment have been started with polyphenols content of 0.41 g/L and after fermentation decreased to 0.17 g/L (Fig. 4A). In the case of treatment of unfiltered OMW, the maximum of elimination of phenol of approximately 75% is reached by the microalgae cells cultivated in the medium BG 11 + 20% on day 4 followed by 45% for the test + 40% OMW (Fig. 4E). While, the polyphenols do not changed, since it was started with 1.7 g/L and at final of the single stage of culture it was measured a value of 1.1 g/L (Fig. 4B).

Under two stage cultures, biotreatment in 40% OMW dilution have been started with polyphenols content of 1.7 g/L and after 3 days of culture it decreased to 0.9 g/L. Instead, for higher concentration used, 80% OMW the polyphenols do not changed, since it was started with 4.3 g/L and at final of the experiment it was measured a value of 3.3 g/L (Fig. 4C) with a lowest removal phenol of 2% (Fig. 4F). Results also showed a higher decrease of polyphenols content by *Scenedesmus* sp. grown under single stage than two-stage.

Most of the literatures refer that the phytotoxicity of OMW is attributed to phenolic compounds [39]. Microalgae sensitivity to phenolics is frequently related to the number and to the polarity of hydroxyl group substituents in the aromatic ring [40].

Several authors have reported that biological treatments have shown a decrease in the phenolic amount of OMW. Ramos-Cormenzana, Juarez-Jimenez [41] reported that a 50% response of the phenol content of *Bacillus pumilus* and Piperidou et al. [42] demonstrated an effective reduction in the phytotoxicity of MOW (> 90%) by *Azotobacter vinelandii*. Other studies using *Azotobacter chroococcum* [43] have shown a significant abatement in COD and in phenolic compounds concentration. The cultivation of two species together, *Ralstonia* sp. and *Pseudomonas putida*, is effective in degrading several phenolic compounds

present in OMWs [44, 45]. McNamara, Anastasiou [46] reported that the use of consortia of aerobic bacteria for the bioremediation of OMW had been successful, leading to a significant reduction in COD and the complete elimination of some simple phenolic compounds. Fungi, like *Geotrichum candidum*, also appear to be very effective at removing phenolic compounds, thus allowing elimination of more than 90% [47].

TOC removal

Apart from metabolites and biofuel production, the *Scenedesmus* sp.- culture process in the presence of OMUF and OMW during single and two stages of cultivation allows simultaneous treatment of these toxic wastewater. The efficiency of treatment was estimated by measuring the concentration of TOC after separation of microalgae biomass (Table 4). During single stage, the maximum values of TOC removal under OMUF and OMW addition were achieved in the culture is however not statistically significant. The toxic effect in treated OMW could not with 20% OMUF and 10% OMW, in which 64% and 93%, respectively. During two-stage, TOC reduction reach 92 and 94% at 80 and 40% OMW, respectively. Polyphenols are pointed out as the main responsible for OMW phytotoxicity, but other substances in their composition may have also some toxic characteristics.

Treatments	TOC (g/L) _{initial}	TOC (g/L) _{end}
Single stage		
Control	0	0.04
2.5% OMUF	0.26	0.25
5% OMUF	0.62	0.5
10% OMUF	1	0.48
20% OMUF	2	0.67
40% OMUF	4	1.43
80% OMUF	8.001	3.56
2.5% OMW	0.66	0.082
5% OMW	1.33	0.12
10% OMW	2.66	0.17
20% OMW	5.32	0.36
40% OMW	10.65	3.71
80% OMW	21.31	8.15
Two-stage		
Control	0.04	0.028
40% OMUF	12	1.25
80% OMUF	24	3.89
40% OMW	42.63	2.42
80% OMW	85.27	6.65

Table 4

Total organic carbon (TOC) in the culture supernatant before and after treatment with *Scenedesmus* sp. during single and two-stage supply strategies.

Recently, Chiavola et al. [48] studied the effectiveness of biological treatment of OMW previously diluted to 25×, 32×, 16 × and 10×, which was carried out in a batch reactor. The results indicated that there was a suppression of the biodegradable organic content in all the used concentrations, with yields around 90 and 60% for chemical oxygen demand (COD) and phenolics compounds, respectively.

Potential biosorption and accumulation of phenols in algal cells was monitored during culture of the microalgae sc in a single stage and two stages after addition of OMUF and OMW, demonstrating the

greatest accumulation of polyphenols in the 40% OMUF culture followed by 80% OMUF, 20% OMUF (Fig. 5A) and finally 20% OMW (Fig. 5B). However, a very high accumulation of polyphenols was estimated with 80% OMUF dilution after two stages cultivation (Fig. 5C). These preliminary data suggest that phenolics might be metabolized by microalgal cells. Although the search for the enzymes involved phenol biodegradation of these strains is necessary to understand if the mechanisms of intracellular degradation are the same as those described for *Ochromonas danica* [49].

High performance liquid chromatography (HPLC) was utilized for the quantification of phenol residue in the culture biomass extracts (Table 5) and supernatant. Wastewater from oil mills (OMW) constitutes a prospective store of molecules or precursor of precious molecules, in particular orthodiphenols (very high amount of aromatic compounds ranged from 2.5–20 g/L) [50]. The removal of the majority of low molecular-mass phenolic compounds has been confirmed by Chromatography analysis. The most abundant component in OMW and OMUF was Hydroxytyrosol (317.5 and 258.2 mg/L, respectively) (data not shown). Results illustrated in the Table 5 have shown increased concentrations of vanillic and caffeic acids and the appearance of new compounds (hydroxytyrosol, tyrosol, Oleuropeinaglycone and Gallic acid) in microalgae biomass. These phenolic compounds show a powerful antioxidant activities and exhibit anti-inflammatory and anticancer properties [51–53].

Compounds	Sc alone	SC-Treated 40% OMW	Sc-Treated 40% OMUF
Hydroxytyrosol	-	17.5	16
Tyrosol	-	23.9	15.2
Gallic acid	-	1.4	1.1
Vanillic acid	0.05	2.2	3.7
Caffeic acid	0.01	0.02	0.01
Oleuropeinaglycone	-	11.1	9.5

Table 5
Main phenolic compounds present in Sc-OMW and OMUF sample at beginning and treated OMW by *Scenedesmus* sp. after single-stage culture (mg/g).

Effect of OMW and OMUF on antioxidant activities of *Scenedesmus* sp.

High added value compound is effective to protect cells against damaging effect of reactive oxygen species (ROS) in organisms. In this study, the antioxidant capacity of *Scenedesmus* sp.-phenolic extracts (S-PE) were evaluate by DPPH and ABTS^{•+} assays.

In the present study, DPPH percent scavenging activities of S-PE were measured in different at 1 mg/mL (Table 6) and compared with standard compounds (BHT and Viamin C). Based on our findings, the inhibition percentage increases with the raise of the initial OMUF and OMW added to the growth medium of *Scenedesmus* sp. Different antioxidant extracts have their own mechanism to assess antioxidant activity in samples.

Table 6
Antioxidant capacity of *Scenedesmus* sp.
ethanol extracts OMW, OMUF determined by
DPPH and ABTS assays during single and
two-stage supply strategies.

Treatments	DPPH (%)	ABTS (%)
Control	23.76	6.3
2.5% OMUF	26.25	5.73
5% OMUF	35.57	7.5
10% OMUF	29.95	8.25
20% OMUF	44.21	10.24
40% OMUF	53.3	35.21
80% OMUF	73.49	38.36
2.5% OMW	33.45	13.87
5% OMW	23.81	3.67
10% OMW	70.58	9.87
20% OMW	76.3	28.4
40% OMW	76.76	83.85
80% OMW	26.75	44.7
Control	67.27	62.19
40% OMUF	97.10	74.39
80% OMUF	93.44	97.10
40% OMW	70.86	72.74
80% OMW	75.12	71.66
BHT(1 mg/ml)	83	-
Vit C (1 mg/ml)	86	-
Trolox (1 mg/ml)	-	86.26

Result reported in table 3 demonstrated that S-PE from 40% OMW (76.7%) showed high free radical scavenging activity followed by 20% OMW (76.3%) and 80% OMUF (73.5%). In fact, the S-PE showed higher capacity of DPPH radical scavenging compared with BHT (83%) and Vit C (86%), when microalgae were grown with both 40 and 80% OMUF (97.1 and 93.44%, respectively) in two-stage strategy (Table 6), due to the contribution of the phenolic compounds that represent the dominant antioxidants in these extracts [54].

Indeed, reduction of DPPH solution by hydrogen donation is shown by the free radical scavenging method (DPPH) [55]. It results in a decrease in the absorbance of the DPPH radical caused by antioxidants which was visually obvious as a color deviation from purple to yellow.

The capability of S-PE extract and Trolox to quench ABTS. radicals is shown in Table 6. ABTS test is generally classified as a single electron transfer.

The results demonstrate that all samples exhibited slightly lower ABTS⁺-radical scavenging activity (84%) compared to Trolox (86%) in single stage strategy. However, S-PE extracted from microalgae grown at 80% OMUF (97%) in two-stage culture showed the highest ABTS⁺-radical scavenging power, which is higher than Trolox (86%) followed by 40% OMUF (74%) and 40% OMW (73%) (Table 6). The ABTS assay usually measures its neutralization by phenolic antioxidants which quench hydrogen atom transfer or transfer a single electron by direct reduction [56]. This result could be attributed to the higher level of residual polyphenols in the microalgal biomass cultivated under a two-stage culture regime.

HEK-293 cell viability

The cytotoxicity effects of various concentration of *Scenedesmus* sp.-biomass, which has been harvested from 40% OMUF cultures (Sc-OMUF), on viability was assessed by MTT assay in the human HEK-293 cell line. As shown in Fig. 6, incubation with microalgal biomass at 12.5, 25, 50, 100, 200, 400, 600, 800 and 1000 µg/mL, resulted in a dose dependent inhibition of cell proliferation, and this reduction was significant relative to control cells ($p < 0.05$).

However, incubation with Sc-OMUF restored viability, and lower concentrations (12.5, 25 and 50 µg/mL) induced the proliferation of HEK cells in comparison with control cells ($p < 0.05$). The highest cell inhibition 33% was observed at 1000 µg/mL concentration of *Scenedesmus* sp.-OMUF, on HEK-293 cells (Fig. 6). The present paper is the first to estimate the effect of Sc-OMUF on HEK-293 cell lines viability. The MTT assay showed that the biomass of *Scenedesmus* sp. cultured on Tunisian filtered olive mill waste water did not shown any toxic effect. This result showed lower cytotoxicity in HEK-293 at a concentration ranging from 100 to 500 µg/mL (6–22%) when compared to previous reports that the cytotoxicity of *Spirulina maxima* and *Spirulina platensis* ethanolic extract were approximately 24.5 and 26%, respectively [57, 58].

Conclusion

These findings indicate that biological treatment by the green algae *Scenedesmus* sp. could be an effective solution used for bioremediation of OMW polyphenols. *Scenedesmus* sp. grown under single stage with 40% OMUF stress is the best approach to accumulate the lipid, protein and carbohydrate amounts, but results in a decrease in biomass. After our proposed two stage cultivation, 4 days of 40% OMUF stress increased the pigments content and antioxidant activity. Greater elimination of COT in cultures with higher stress seems to come from a phenol and toxicity reduction. This study demonstrates for the first time that exposure of the Human Kidney Cell line (HEK 293), as an *in vitro* model to Sc-OMUF did not show cytotoxicity for the first time. This data suggest that SC-OMUF was non toxic for human kidneys. In our study, the regulation of the duration of single and two stage cultivation would be necessary to achieve the process objectives: Production of biomass and bioactive metabolites and elimination of phenols.

Declarations

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Contributions

IDB conceived the study, executed microalgae cultures, prepared and analyzed extracts, performed the statistical analysis, and write the manuscript. MM, FF and SS participated in the study design, provided technical advices, and reviewed the manuscript. SC performed HPLC analysis. All authors read and approved the manuscript.

Corresponding author

Correspondence to Ines DAHMEN-BEN MOUSSA;
E-mail: ines_dahmen@hotmail.fr ;

Ethics Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing of interests.

Abbreviations

OMW: olive mill wastewater

OMUF: ultrafiltrated olive mill wastewater

DPPH : 2 , 2 -diphenyl -1- picrylhydrazyl radical

TOC: organic matter

Ch: Chlorophyll

Car: Carotenoid

S-PE: *Scenedesmus* sp.-phenolic extracts

HEK: Cells of Human Embryonic Kidney

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Figures

Figure 1

Variation of the cell density of *Scenedesmus* sp. with the initial concentration of OMUF (A) and OMW (B) under single and two-stage (C) supply strategies. Values are mean $\pm$ SE.

Figure 2

Variation of the maximum specific growth rate and volumetric biomass productivity values with the initial concentration of OMUF (A) and OMW (B) under single and two-stage (C) supply strategies. Values are mean $\pm$ SE.

Figure 3

Changes in the biochemical composition of *Scenedesmus* sp. with different OMUF and OMW concentrations under single (A) and two-stage (B) supply strategies. Values are mean $\pm$ SE.

Figure 4

Phenol concentrations in the medium (A, B and C) and phenol removal (%) (D, E and F) by *Scenedesmus* sp. cultivated with different OMUF and OMW concentrations during single and two-stage supply strategies.

Figure 5

Intracellular amount of polyphenol accumulated in *Scenedesmus* sp. cultivated with different concentration of OMUF (A) and OMW (B) during single and two-stage (C) supply strategies.

Figure 6

Effect of extract of *Scenedesmus* sp. cultivated under control condition (Sc) sp. and with 40% OMUF during single supply strategy, on HEK-293 cell viability using MTT assay. Data are expressed by means $\pm$ SD (n = 3).