Sequential adaptation of *Nannochloropsis gaditana* to table olive processing water

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Abstract

The main objective of this study was to evaluate the suitability of *Nannochloropsis gaditana* to grow by sequential adaptation to TOPW (Table olive processing water) at increased substrate concentrations (10- 80%). Sequential adaptation allows growing *Nannochloropsis gaditana* up to 80% TOPW, although the maximum microalgae biomass productions were achieved for percentages of 20-40%, i.e. 0.308 ± 0.005 g VSS (Volatile Suspended Solids)/L. In all growth experiments, proteins were the majority compound in the grown microalgae biomass (0.44 ± 0.05 g/g VSS), whereas

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phenols were retained up to a mean concentration of 12.1 ± 1.9 mg total phenols/g VSS. The highest microalgae biomass production rate at 80% TOPW took place in the first two days when also took place most nutrients removal. Average removal efficiencies at this percentage of TOPW were 69.1%, 50.9%, 54.3% and 71.8% for total organic carbon, total soluble nitrogen, phosphate and total phenols, respectively. Sequential adaptation can ensure reaching a sustainable microalgae culture as a treatment method for TOPW.

Keywords: Microalgae biomass, phenols, sequential adaptation, table olive processing water, wastewater valorization.

Introduction

Table olives production is an agro-industrial sector mainly concentrated in Mediterranean countries such as Spain, Italy, Greece, Tunisia, and Morocco. Spain is the biggest producer of table olives in the world. The average world production during the last seven years was 2 150 000 tons, with 508 000 tons produced in Spain, i.e. 24% of world production. ^[1] The elaboration of table olives consists in treating the fruits with dilute NaOH solution to hydrolyze the compounds that generate its natural bitterness, i.e. oleuropein, followed by one or two washings to remove the alkali excess and, finally, spontaneous lactic acid fermentation in brine. ^[2]

Water generated in table olive processing industries is characterized by seasonal peaks, high organic matter and phenolic content, as well as high salt concentration.

Unfortunately, this type of water is sometimes discharged untreated into small streams or directly into the rivers with high environmental impacts. In the best-case scenarios, it is transported either to evaporating ponds for water evaporation, where anaerobic

conditions quickly release undesirable smells or to large urban wastewater treatment plants where it is mixed with municipal wastewater. ^[3] However, as environmental regulations and their enforcement have become more stringent, a growing interest in the development of new methods for treatment, exploitation or valorization of TOPW (Table Olive Processing Waters) has emerged.

Many species of microalgae are able to grow efficiently in wastewater conditions due to their ability to utilize the abundant organic and inorganic N and P in the wastewater and to provide a high environmental quality effluent. ^[4-5] All over the world, growth microalgae biomass is gain relevance as an interesting and environmentally friendly process for obtaining a wide range of bioactive compounds such as pigments, foodstuffs, chemicals, pharmaceuticals and nutraceuticals, with high economic interest, or lipids for biodiesel production. ^[6] *Nannochloropsis* is a commonly cultivated organism because of its high growth rate, high lipid productivity, and wide environmental tolerance. ^[7] Previously studies on the valorization of TOPW for microalgae growth were reported by Contreras et al. ^[2], which studied the performance of *Nannochloropsis gaditana* in mediums at different TOPW concentrations. These authors reported that percentages of TOPW higher than 40% resulted in a decrease of the microalgae growth due to the un-optimal conditions of TOPW. Sequential adaptation could be utilized to enhance the metabolic phenotype or special capabilities to tolerant stress conditions in a microalgae strain. ^[8] For example, Tillich et al. ^[9] studied the sequential adaptation of a *Synechocystis* wild-type culture for increasing its temperature tolerance, reporting that the temperature tolerance of the strain was effectively raised by about 2°C. Other authors have employed the sequential adaptation to increase the tolerance to a high variety of stressful conditions, such as salt

concentration or CO₂ concentration; ^[8,10] or to promote the production of some valuable compounds, i.e. lipids or carotenoids. ^[11-12]

Despite all these advances, the optimum adaptation of microalgae to TOPW is still undefined. Therefore, the main objective of this study was to evaluate the suitability of *Nannochloropsis gaditana* as wastewater treatment of TOPW by sequential adaptation. The content of total phenols, proteins, lipids and total carbohydrates were determined in the microalgae biomass after the growth period with a view to its recovery and valorization.

Materials and methods

Microalgae, synthetic medium and Table Olive Processing Water (TOPW)

Nannochloropsis gaditana strain was obtained from the University of Huelva (Spain). A stock culture of *Nannochloropsis gaditana* was maintained photoautotrophically in an 8.0-L glass bottle, containing 5.0-L of culture, by the addition of a synthetic medium. Growth conditions were maintained at an aeration rate of 0.5 v/(v·min) in environmental conditions with a mean temperature of the laboratory maintained at 25 ± 2 °C and average available natural light of 14 hours of light and 10 hours of dark. The synthetic medium used was the f/2 medium for marine algae. The composition and main characteristics of the f/2 medium were previously described by Guillard. ^[13] Its main components and concentrations were: nitrogen source: NaNO₃ (1.125 g/L); phosphorous source: NaH₂PO₄ 2 H₂O (113 mg/L); synthetic seawater: NaCl (27.12 g/L), MgCl₂ 6H₂O (5.23 g/L), MgSO₄ 7H₂O (6.77 g/L), CaCl₂ 2H₂O (1.51 g/L), KCl

(0.73 g/L), NaHCO₃ (0.20 g/L); Trace metals: EDTA Na₂ (4.36 mg/L), FeCl₃ 6H₂O (3.15 mg/L), Cu SO₄ 5H₂O (10 µg/L), Zn SO₄ 7H₂O (22 µg/L), Co Cl₂ 6H₂O (10 µg/L), Mn Cl₂ 4H₂O (180 µg/L), Na₂ MoO₄ 2H₂O (6 µg/L); and Vitamins: Vitamin B1 (100 µg/L), Vitamin B12 (2 µg/L), Vitamin H (1 µg/L). For the preparation of the synthetic medium, the sodium nitrate and phosphate source were adjusted to 13.2 mM and 0.7 mM, respectively. Finally, the vitamins solution was filtered through a 0.20 µm filter (GVS CA/Sterile 25mm, Indianapolis, USA).

Table olive processing water (TOPW) was taken from a local industry placed in Arahal, Seville (Spain). TOPW was collected in the months of spring when washing process was produced. Therefore, TOPW came mainly from washing tanks immediately after NaOH treatment. For culture experiments, TOPW was centrifuged to eliminate suspended solids at 4000 rpm for 15 min and vacuum filtered through Millipore 0.45 µm filter (Albet GF 47mm, Dassel, Germany). Physicochemical characterization of table olives processing water and synthetic medium were shown in Table 1.

Experimental design

Growth experiments were carried out in open 1.0-L glass bottles in duplicate. The culture medium for the growth experiments were different mixtures of synthetic medium and TOPW. Proportion of TOPW in the culture medium was increased at different percentages 0%, 10%, 20%, 40% and 80%, v/v. The growth experiments at 0% TOPW were inoculated with the solid fraction which remained after the centrifugation of 100 mL of stock culture at 4000 rpm for 15 min. The subsequent growth experiments were inoculated with the microalgae biomass which remained after the centrifugation of 100 mL of the previous growth experiment. The growth experiments were placed inside

of the laboratory operating with an aeration rate of 0.5 v/(v·min) in environmental conditions from July to October, with a mean temperature of the laboratory maintained at 25 ± 2 °C and average available natural light of 14 hours of light and 10 hours of dark. The experiment duration was fixed to ensure the adaptation of the microalgae biomass to the experimental conditions.^[7,14] Test monitoring stopped when the TOC concentration remained constant for at least two consecutive samplings.

Analytical techniques

The following chemical analyses were carried out for the characterization of the synthetic medium, the TOPW as well as for the effluents from each microalgae culture test at the end of the process. The concentration of VSS (volatile suspended solids; g/L), Phosphate (mg/L) by the vanadomolybdophosphoric acid method, Conductivity (mS/cm) and pH were carried out according to the recommendations of the Standard Methods of APHA (2005).^[15] Additionally, given that flocs were not observed in the culture medium, volatile suspended solids concentration was also estimated by correlation with turbidity by measuring the absorbance at 750 nm (Genesys 10 VIS spectrophotometer, Thermo Scientific, Horsham, England) and expressed as gVSS_t/L.^[16] TOC (Total organic carbon; mg/L) and TN (total soluble nitrogen; mg/L) were determined using a Rosemount analytical Dohrmann DC-190 carbon analyser. The TOC analyser was calibrated with a standard solution of potassium phthalate prior to the TOC analyses. Total phenols concentration was quantified by spectrophotometry through the Folin-Ciocalteu method.^[17]

For microalgae biomass, the concentrations of total phenols, proteins, lipids and total carbohydrates were determined according to the procedures described by Contreras et

al. [2] The determinations were carried out after an ultrasound pre-treatment in order to liberate the compounds inside the cell wall and allow a correct quantification. Total phenols concentration was quantified through the Folin-Ciocalteu method, [17] using a standard curve based on acid gallic. The concentration of proteins in the microalgae biomass was measured in accordance with Lowry et al. [18] The concentration of proteins was determined by a standard curve based on albumin concentration. Lipids concentration was determined by optical density at 530 nm, according to Wang et al. [19] Total carbohydrate analysis was determined according to the colorimetric method described by Bellou and Aggelis. [20] The amount of total carbohydrate was determined by a standard curve based on glucose concentration.

Softwares

Sigma-Plot software (version 11.0) and Microsoft Excel 2010 were used to create graphs, perform the statistical analysis (mean values and standard deviations) and fit the experimental data to the tendency lines presented in this work.

Results and discussion

Sequential microalgae growth

The feasibility of growing *Nannochloropsis gaditana* was evaluated through the monitoring of turbidity and VSS at the different percentages of TOPW. Figure 1 shows the variation from the initial point of the cultivation period to the final of turbidity (g VSS_t/L) and VSS (g VSS/L) against the used percentages of TOPW (%) at the end of the cultivation period. In most assays, the pairs of values of turbidity (g VSS_t/L) and g

VSS/L were similar, indicating that both parameters could be used to evaluate the biomass concentration in the present study (Figure 1). According to the obtained VSS data, the highest microalgae biomass concentration were determined at TOPW percentages from 20 to 40%, who reached a mean value of 0.308 ± 0.005 g VSS/L, i.e. an enhancement around 243 % compared to the cultivation without TOPW (Figure 1). The increment of the percentage of TOPW to 80% entailed a lower increment of the microalgae biomass concentration, reaching a final concentration of 0.243 ± 0.007 g VSS/L, i.e. 22% lower than the highest concentration value. The highest biomass concentration was similar than that reported by Contreras et al.,^[2] which reached a concentration of *Nannochloropsis gaditana* of 0.32 ± 0.03 g VSS/L at TOPW percentages from 10 to 40%. Nevertheless, these authors described a 43% reduction in productivity for higher TOPW percentages. The lower biomass productivity at 80% TOPW reported by Contreras et al.^[2] compared to the present study could be explained by the sequential adaptation of *Nannochloropsis gaditana* to TOPW. Li et al.^[8] described that an appropriate stress should be critical to improving the growth phenotype of *Chlorella sp.* adapted to the desired conditions. Other authors suggest that microalgae biomass lineages may be able to adapt rapidly to stress conditions by a fast changing in the population characteristics.^[10] Therefore, sequential adaptation has been a useful strategy to adapt *Nannochloropsis gaditana* for recovering nutrients from TOPW and to enhance microalgae growth.

Recovery potential of proteins, lipids, carbohydrates and phenols

The adaptation of *Nannochloropsis gaditana* to more stressful conditions modifies the composition of the microalgae, which could affect the recovery of desirables compounds. The concentration of proteins, total carbohydrates, lipids and total phenols

were quantified in the microalgae biomass at the end of each growth experiment. Figure 2 shows the contents of these principal compound groups (expressed as g/g VSS) against the different TOPW percentages (%). As can be seen, the distribution among the different groups of compounds remains slight constant at TOPW percentages between 0% and 40%. Concretely, the mean concentration values were 0.44 ± 0.05 g proteins/g VSS, 0.16 ± 0.01 g total carbohydrates/g VSS, and 0.17 ± 0.02 g lipids/g VSS. When the TOPW percentage increased to 80%, the concentration of proteins and lipids decreased around 25% and 30%, respectively, whereas total carbohydrates concentration increased up to 13%, with respect to the values described for 0-40% of TOPW. These principal compound group concentrations were similar than the percentages of distribution described by Matos et al. ^[21] for *Nannochloropsis gaditana*, which reported as typical distribution values 41.6%, 18.6%, and 8.1% of proteins, total carbohydrates, and lipids, respectively. In present study lipid content was twice higher compared to the reported by Matos et al. ^[21] This difference could be explained by the imposition of stress conditions in the present research study, which derived in the accumulation of lipids by the microalgae biomass. ^[22-23]

TOPW is an important source of phenols, i.e. 229 ± 50 mg/L (Table 1), which can make an important contribution to the economic feasibility of the growth process if these compounds could be retained by the microalgae biomass. Figure 3 shows the concentration of total phenols in the microalgae biomass at the different percentages of TOPW. Total phenols concentration were in a range between 8.9 and 14.0 mg/g VSS, with a mean value of 12.1 ± 1.9 mg/g VSS. The increase in the percentage of TOPW did not imply a decrease in the capability of capture phenols by microalgae biomass, i.e. 12.9 ± 1.1 mg/g VSS at 80% of TOPW.

Microalgae culture grown at 80% of TOPW

According to the obtained results at the different TOPW percentages and the low dilution requirements, 80% of TOPW could be considered as the most interesting percentage for the future implementation of this treatment option. 80% TOPW medium resulted in interesting biomass production yield and a promising capacity of nutrient and phenols removal. The concentration of microalgae biomass was monitored versus time throughout the growth experiments at 80% TOPW. Figure 4 shows the solids concentration values (g VSS_t/L) against the culture period (d). As can be seen, the highest increment on the biomass concentration takes place on 2-day period, with a biomass production rate of 0.120 ± 0.006 g VSS/L·d. After the 2-days period, the biomass production rate was slowed down, with a mean value of 0.020 ± 0.002 g VSS/L·d. 80% of TOPW allowed reaching a high biomass production by employing very short hydraulic retention times. Biomass composition was also monitored throughout the growth experiments and remained constant. The mean concentrations from 2-day period to 15-day period of proteins, lipids, total carbohydrate and total phenols were 0.555 ± 0.013 g VSS/L, 0.141 ± 0.031 g VSS/L, 0.199 ± 0.018 g VSS/L, and 0.019 ± 0.002 g VSS/L, respectively.

Microalgae as wastewater treatment for TOPW

The retention of nutrients from TOPW by *Nannochloropsis gaditana* might be used to obtain a cleaner final effluent as already done with wastewaters.^[7,24] The main nutrients and valuable compounds presented in TOPW, i.e. TN, phosphate, TOC and total phenols, were determined at the beginning and at the end of each growth experiment in order to obtain removal efficiencies. Phosphate concentration was provided in the same range for both substrates, TOPW and synthetic medium, whereas TN was mainly

provided by the synthetic medium, which presented a concentration of TN around 4 times higher than that TOPW. TOC and total phenols were only provided by TOPW (Table 1). Figures 5A and 5B show the removal efficiencies of TN, phosphate, TOC and total phenols at the different percentages of TOPW. The highest removal efficiencies for TN, phosphate and TOC were reached at 80% of TOPW. TN and phosphate removal efficiencies reached values of 50.9% and 54.3%, respectively, which were around twice higher than the values obtained for the other percentages of TOPW. TOC removal efficiency at 80% TOPW increased 25.4% compared to the second highest value, which was obtained at 20% TOPW. Total phenols removal efficiency was similar to percentages of TOPW between 20% and 80%, with a mean value of $65.9 \pm 7.6\%$. The low concentration of total phenols in growth medium composed of 10% of TOPW allowed the degradation of these compounds up to undetectable concentrations. The removal efficiencies for nitrogen and phosphate are lower than the values found for some species of *Chlorella* and *Scenedesmus*, which could reach efficiencies higher than 80% by growing on municipal wastewater. ^[25] TOPW as an agro-industrial process water presented lower removal efficiencies most probably due to the stressful conditions presented in TOPW medium compared to urban wastewater. ^[24]

Figure 6 shows the concentrations of TOC, TN and total phenols during the experimental time at an 80% TOPW. According to Figure 6, the removal efficiency of TOC, TN, and total phenols reached values of $71.9 \pm 3.2\%$, $54.1 \pm 4.9\%$ and $70.0 \pm 3.7\%$, respectively, after a just 4-day period. Sequential adaptation can ensure reaching a sustainable microalgae culture as a treatment method for TOPW at such short retention times. According to the characteristics of the final effluent, this effluent could be employed for irrigation purposes according to the Andalusian regional regulation, i.e. pH = 6-9; Suspended solids < 600 ppm; Biochemical Oxygen Demand (BOD₅) < 2000

ppm; Chemical Oxygen Demand (COD) < 2500 ppm; limitation for spreading on the terrain: 30 m³/Ha every 7 days.^[26] In areas with more restrict legislation it could be necessary to carry out a tertiary treatment to accomplish with the local legislation.

Conclusions

Nannochloropsis gaditana was effectively growth up using TOPW to a proportion of 80% TOPW, although the maximum microalgae biomass productions were achieved for percentages of 20-40%. The composition of microalgae biomass remained constant at the different growth experiments. Proteins were the majority compound in the grown microalgae biomass (0.44 ± 0.05 g/g VSS). Phenols were retained in microalgae biomass up to a mean concentration of 12.1 ± 1.9 mg total phenols/g VSS. The highest efficiency of TOC, TN and total phenols removal was found at 80% of TOPW, reaching removal efficiencies of 69.1%, 50.9%, 54.3% and 71.8% for TOC, TN, phosphate and total phenols, respectively. The highest microalgae biomass production rate, at a percentage of TOPW of 80%, takes place in the first two days, when also takes place most nutrients removal. Sequential adaptation of *Nannochloropsis gaditana* allowed minimal dilution without marked adverse consequences.

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FIGURE CAPTIONS

Figure 1. Variation from the initial point of the cultivation period to the final of turbidity (g VSS_t/L) and VSS (g VSS/L) for the different percentages of TOPW (%).

Figure 2. Content of proteins, lipids and total carbohydrates (g/gVSS) at the end of growing period for the different percentages of TOPW (%).

Figure 3. Content of total phenols (g phenols/g VSS) retained in the microalgae biomass for the different percentages of TOPW (%).

Figure 4. Variation of solids concentration (g VSS_t/L) versus time throughout the culture period for experiments at 80% of TOPW.

Figure 5. (A) Removal efficiencies for TN and phosphate for the different percentages of TOPW (%), and (B) removal efficiencies for TOC and Phenols for the different percentages of TOPW (%).

Figure 6. Variation of the concentration of total phenols (mg/L), TOC (mg/L), and TN (mg/L) versus time throughout the culture period for experiments at 80% of TOPW.

Figure 1

Figure 2

Figure 3

Figure 4

A)

B)

Figure 5A and 5B

Figure 6

Table 1. Physicochemical characterization of table olives processing water and synthetic medium

		TOPW			Synthetic medium		
pH		5.55	±	0.04	7.6	±	0.3
Conductivity	mS/cm	17.5	±	0.1	50.1	±	0.3
TS	g/L	11.6	±	0.1	25.7	±	0.2
VS	g/L	1.9	±	0.2	N.D		
VSS	mg/L	104	±	1	N.D		
TOC	mg/L	843	±	1	50	±	1
TN	mg/L	31	±	1	123	±	1
Phosphate	mg/L	19.23	±	0.06	24.18	±	0.22
Phenols	mg/L	229	±	50	N.D		

N.D: non-determined; TS: Total Solids; VS: Volatile Solids; VSS: Volatile Suspended

Solids; TOC: Total Organic Carbon; TN: Total soluble Nitrogen