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ORIGINAL PAPER

Combined biological treatments of olive mill wastewater using fungi and microalgae

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Abstract
Olive mill wastewaters (OMWs), which are mainly composed of phenolic compounds, present an important environmental issue. This effluent was treated using fungal laccases from *Tetraselmis* sp. and *Chlorella* sp. and two green microalgae. In the first step in the removal of phenolic compounds, enzymatic pretreatment of diluted OMWs (30% v/v) with the fungal laccases led to a 57.79 ± 2.21% decrease in phenol concentration and a 54.79 ± 3.44% decrease in the dark coloration of the diluted OMWs. This preatreated wastewater was suitable for microalgae culture under mixotrophy. The growth and biochemical composition of the two microalgae strains were determined and compared to those obtained from a control culture under autotrophy. Under mixotrophy, the growth of *Chlorella* sp. and *Tetraselmis* sp. reduced dramatically. Moroever, their photosynthetic pigment productivities (0.0071 ± 0.08 mg/10^4 cells/mL/day for *Chlorella* sp. and 0.011 ± 0.002 mg/10^4 cells/mL/day for *Tetraselmis* sp.) were decreased when compared to those attained under autotrophy. In terms of starch and lipid production, the highest final contents of starch and lipids were obtained under autotrophy: 13.38 ± 2.17% and 4.2 ± 0.35% for *Chlorella* sp. and 15.66 ± 0.92% and 5.22 ± 0.79% for *Tetraselmis* sp., respectively. The mixotrophic cultivation mode of *Chlorella* sp. reinforces protein synthesis. These results highlight the opportunity to achieve high-value biomass production by the mixotrophic cultivation of microalgae using pretreated OMWs as a low-cost raw material.

Keywords Laccase · *Chlorella* · *Tetraselmis* · Mixotrophy · Olive mill wastewater

Introduction

Olive oil production generates significant amounts of waste materials known as olive mill wastewaters (OMWs) and olive pomace. These by-products have harmful effects on land and water environments due to their pH values and high levels of toxic compounds (Dermeche et al. 2013; Hachicha et al. 2023; Khatib et al. 2009). The increasing demand for olive oil makes it an important sector in the food industries of Mediterranean countries (Malvis et al. 2019). The International Olive Council (IOC) has estimated the world's olive oil production and consumption for the 2021/2022 season at 3.1 and 3.215 million tonnes, respectively (https://www.internationaloliveoil.org/). The annual volume of OMWs generated by this production is estimated as 6×10^6 m^3, 98% of which is being discharged in Mediterranean countries (Foti et al. 2021). Notably, OMWs include organic matter such as sugars, polyphenols, lipids, proteins, mineral elements, photosynthetic pigments and polyalcohols. Furthermore, they are characterized by an acidic pH (3–5) and a high content of salts (EC ~ 5–10 mS cm^{-1}). The high levels of organic loads and the high levels of phenolic compounds (polluted matter) raise the chemical oxygen demand (COD) and the biological oxygen demand (BOD) and limit the biodegradability of the OMWs (Markou et al. 2012; Roig et al. 2006). Various studies aimed at promoting the degradation of these effluents using physical, chemical and biological technologies

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have been performed. Biological treatments, particularly those with white-rot fungus, sound promising (Cerrone et al. 2011). These fungi produce an extracellular oxidase enzymatic system [manganese peroxidases (MnPs), lignin peroxidases (LiPs) and laccases (Lacs)] that oxidizes and degrades a wide range of aromatic compounds and generates xenobiotics, including synthetic dyes, chlorinated phenols and polycyclic aromatic hydrocarbons (Jaouani et al. 2003; Quaratino et al. 2008). Laccases (benzenediol-oxygen oxidoreductase, EC 1.10.3.2) are blue-copper oxido-reductases that are abundant in nature. They are produced by plants, bacteria and insects (Mann et al. 2015; Strong and Claus 2011). The growth of microalgae using wastewaters as nutrients has been performed since the 1960s (Paddock 2019). Some of these photosynthetic microorganisms are characterized by easy culture and high growth and productivity rates. They have the potential to generate high-value products such as lipids, carbohydrates, chlorophylls, carotenoids and proteins (Hachicha et al. 2022; Rawat et al. 2011). As some of them have the ability to grow under mixotrophy and heterotrophy, microalgae are interesting biocatalysts for reducing the organic and inorganic contents (mainly N and P) of some wastewaters. Bioconversion processes that use these microorganisms could be a more ecofriendly alternative to traditional wastewater treatments (Giovanardi et al. 2013; Markou et al. 2012). OMWs have a black/dark color, limiting light penetration. They have a complex physicochemical composition, including high amounts of phenols, which may inhibit the growth and photosynthetic activity of algae (Guldhe et al. 2017). For these reasons, the use of pretreatments (selected based on the cost–benefit ratio calculated on a case-by-case basis) is necessary.

In contrast to the *Tetraselmis* genus, *Chlorella* species have been well studied for use in wastewater treatment (Abreu et al. 2012; Li et al. 2021; Pooja et al. 2022; Wirth et al. 2020) owing to their high capacity for and tolerance of soluble organic compounds.

In this study, we recommend a new process for OMW treatment based on enzymatic bioconversion (using laccases) followed by microalgae (*Chlorella* sp. and *Tetraselmis* sp.) culture. Physicochemical characteristics of real crude olive oil mill wastewater are analyzed. Additionally, the enzymatic pretreatment with laccases is determined and optimized. Then, two proportions of microalgal inoculum (20% and 50% v/v) are applied for autotrophic (BG11 medium) and mixotrophic cultivations using pretreated OMWs as substrate. The kinetic growth and biomass production of the two green microalgae are evaluated.

Materials and methods

Olive mill wastewater

OMWs were collected from an olive oil production unit located in the region of Sfax, Tunisia, during the 2019/2020 olive-oil campaign. Samples were taken from a vegetable water storage basin and transported in 5 L bottles for physicochemical assays and treatment. Then they were stored at – 20 °C. Prior to use, the OMWs were thawed at room temperature and centrifuged at 8000 rpm to remove insoluble particles.

Fungal and microalgal strains

The white rot basidiomycete *Trametes trogii* (CLBE55), selected for its laccase-producing potential, was isolated from acacia woods in the Bousalem region in the northwest of Tunisia. For short-term storage, the fungus was grown on malt extract agar at 30 °C for 5–7 days. Petri dishes were then stored at 4 °C. For laccase production, *T. trogii* was accrued on M7 medium containing (values in g/L): glucose, 10; peptone, 5; yeast extract, 1; ammonium tartrate, 2; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; and 1 mL of trace element solution. The trace element solution composition was (g/L): $B_4O_7Na_2.10H_2O$, 0.1; $CuSO_4.5H_2O$, 0.01; $FeSO_4.7H_2O$, 0.05; $MnSO_4.7H_2O$, 0.01; $ZnSO_4.7H_2O$, 0.07; $(NH_4)6Mo_7O_{24}.4H_2O$, 0.01. The pH of the solution was adjusted to 5.5.

The freshwater green microalga *Chlorella* sp. RCC288 was taken from the Roscoff culture collection (https://roscoff-culture-collection.org/). The marine unicellular green microalga *Tetraselmis* sp. was isolated from the Gulf of Gabes (Mediterranean Sea) along the coast of Sidi Mansour (Sfax, Tunisia) (Dammak et al. 2016).

Culture of fungi and microalgae

The fungal culture media were composed of sterilised (0.2 µm) OMWs diluted in sterile distilled water (10%, 20%, 30%, 40%, 50% or 100% v/v). They were supplemented with NH_4Cl (1 g/L) and and $CuSO_4$ (300 µM) before being inoculated with 2% of the *T. trogii* preculture (Zouari-Mechichi et al. 2006). Cultures were incubated at 30 °C and 150 rpm for 12 days.

Cultures of *Chlorella* sp. were grown in 1 L Erlenmeyer flasks containing 400 mL of BG-11 medium (Allen 1968) at 120 rpm and 25 (± 1) °C under a continuous light irradiance of 100 µmol photons m^{-2} s^{-1}. *Tetraselmis* sp. was grown in F/2 medium (Guillard 1975) at 120 rpm and 25 (± 1) °C under a continuous light irradiance of 84 µmol m^{-2} s^{-1}.

The cell concentrations of inocula were 3.5×10^6 and 1.7×10^6 cell/mL for *Chlorella* sp. and *Tetraselmis* sp., respectively.

Mixotrophic cultures were initiated in the OMWs using photoautotrophic inocula of 50 and 20% (v/w). The growth

parameters (temperature, stirring and light) were the same as those described for photoautotrophy.

Analytical methods

The dry weight of fungal biomass (DW) was determined by washing the mycelium with distilled water (filtration) and drying it overnight at 105 °C until a constant weight was reached.

Decolorization of OMWs was controlled by spectrophotometrically measuring absorbance at 395 nm (A_{395}) (Sayadi and Ellouz 1995).

Microalgae growth was determined on a Malassez slide under a light microscope (×40 magnification).

Chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoids (car) were quantified according to the procedure described by Ritchie (2006). The photosynthetic pigments were extracted using ethanol at 65 °C for 30 min, and A_{666}, A_{663} and A_{470} were measured to quantify the pigments, as shown in Eqs. 1–3:

$$[\text{Chlorophylla}](\text{mg/L}) = 15.65 \times \text{A}_{666} - 7.34 \times \text{A}_{653} \quad (1)$$

$$\left[\text{Chlorophyllb}\right]\left(\frac{\text{mg}}{\text{L}}\right) = 27.05 \times \text{A}_{653} - 11.21 \times \text{A}_{666} \quad (2)$$

$$[\text{Carotenoids}]\ (\text{mg/L}) = \left(1000 \times \text{A}_{470} - 2.86 \times \left[\text{chlorophylle a}\right] - 85.9 \times \left[\text{Chlorophylle b}\right]\right)/245 \quad (3)$$

Protein concentrations were evaluated according to the Bradford assay using bovine serum albumin as the standard (Bradford 1976).

An assay kit (Megazyme, AOAC Method 996.11, AACC Method 76-13.01) was used to quantify the total starch present in the microalgal biomass according to the supplier's recommendations.

Total lipids were extracted from the biomass by following the Folch chloroform-based lipid extraction protocol, after which they were gravimetrically quantified and expressed in % (w/v) (Bligh and Dyer 1959).

The pH and electrical conductivity (EC) were established according to the standard methods described by Sierra et al. (2001). Total solids (TS), volatile matter solids (VMS), mineral matter (MM), organic matter (OM), suspended solids (SS), and Kjeldahl nitrogen were determined in accordance with the standard methods (Daâssi et al. 2014a). Chemical oxygen demand (COD) was determined by following the standard methods for the examination of water and wastewater. Biological oxygen demand (BOD_5) was measured using the respirometric method (manometric) (Clesceri et al. 1996). The total phenolic content (TP) was discerned using the Folin–Ciocalteu method (Box 1983). The total sugar content was determined spectrophotometrically using the phenol sulfuric acid assay (DuBois et al. 1956).

The macro- and microelement and metallic trace element contents of the OMWs in a chemically digested sample were analyzed using an atomic absorption spectrophotometer (method EP 3051).

All the measurements were performed in duplicate.

Enzyme assays

Laccase (EC 1.10.3.2) activity was assayed using 10 mM 2,6-dimethoxyphenol (DMP) in 100 mM sodium acetate buffer, pH 5.0 (Molar extinction coefficient: $\varepsilon_{469\ \text{nm}} = 27{,}500\ \text{M}^{-1}\ \text{cm}^{-1}$). We define a unit of enzyme activity as the amount of enzyme that oxidizes 1 µmol of substrate per minute at 469 nm (Yaropolov et al. 1994).

Fourier Transform Infrared (FT-IR) analysis

Infrared spectra of the samples were recorded by attenuated total reflectance (ATR) (diamond) reflection at 21 °C (room temperature). Samples were deposited on an attenuated reflection cell equipped with a diamond crystal. Spectral software was employed to process the FT-IR spectra between 600 and 4000 cm^{-1} using 10 scans at a resolution of 4 cm^{-1}.

Statistical analysis

All analytical determinations were performed in duplicate or triplicate, and values are expressed as the mean ± standard deviation (SD). One-way ANOVA tests were used to compare results with significant differences ($p < 0.05$). Version 19 of the IBM SPSS statistics software (IBM Corp., Armonk, NY, USA) was used to perform all statistical analysis.

Results and discussion

Physicochemical characterization of OMWs

Table 1 illustrates the physicochemical characterization of the OMWs. The results highlighted a high organic matter content, as expressed in terms of turbidity = 1987 ± 5.65 NTU, TOC = 19.88 ± 2.21 mg/L, COD = 110 ± 2.12 g O_2/L, BOD = 9.7 ± 0.28 g O_2/L and TP = 7.07 ± 0.62 g/L. Likewise, we noticed a high level of salinity (11.09 ± 0.23 g/L).

Table 1 Physicochemical characterization of OMWs

Parameters	Values
pH	5.2 ± 0.28
EC (mS/cm)	13.055 ± 0.27
Salinity (g/L)	11.09 ± 0.23
TS (g/L)	67.92 ± 1.05
OM (g/L)	58.325 ± 1.096
MM (g/L)	9.61 ± 0.024
SS (g/L)	1.114 ± 0.42
VMS (g/L)	1.16 ± 0.021
COD (g O_2/L)	110.5 ± 2.12
BOD (g O_2/L)	9.7 ± 0.28
TOC (mg/L)	19.885 ± 2.21
Turbidity (NTU)	1987 ± 5.65
NTK (mg/L)	1071.5 ± 9.19
N–NH_4^+ (mg/L)	94.5 ± 4.94
TP (g/L)	7.075 ± 0.62
Proteins (g/L)	6.69 ± 5.65
Total sugars (g/L)	5.07 ± 0.29
Mg (mg/L)	72.41 ± 2.79
Na (mg/L)	182.5 ± 13.43
K (mg/L)	1334 ± 15.55
Ca (mg/L)	24.73 ± 1.73
Pb (mg/L)	< 0.1 (ND)
Zn (mg/L)	0.13 ± 0.007
Cu (mg/L)	< 0.02 (ND)
Cd (mg/L)	< 0.005 (ND)
Fe (mg/L)	1.179 ± 0.074
Total phosphorus (mg/L)	27.13 ± 2.20

The high values of these parameters contribute to the inhibition of microalgal growth due to phytotoxic and antimicrobial effects, as well as low biodegradability (Daâssi et al. 2014b; Dermeche et al. 2013; García and Hodaifa 2017). OMWs are characterized by an acid pH (5.2) as a result of the presence of some organic acids (notably phenolic and fatty acids) and a high salinity level due to the existence of K^+, Cl^-, Ca^{2+} and Mg^{2+} ions. Total inorganic nitrogen generated from olive fruit crushing and olive oil washing was low (Di Caprio et al. 2015). The mineral composition can play an important role in microalgae cell growth and metabolism via phosphorylation reactions. Therefore, OMWs contain appropriate nutrient contents: they are rich in carbon, nitrogen, phosphorus and the key elements required for algal biomass growth (Table 1).

Effect of OMW proportions on the growth of *Trametes trogii* and laccase production

The laccase production by *Trametes trogii* without effluent showed that maximal activity was obtained at 10 days of culture (9000 U/L). The final biomass reached 9.53 ± 0.98 g/L. Other white-rot fungal species in the same family as *Trametes trogii* have shown different rates of laccase activity and final biomasses. Xiao et al. (2017) cultivated *Pleurotus ostreatus* in Kirk's liquid medium at 28 °C with continuous stirring at 120 r/min for 11 days. The maximum laccase activity and mycelial dry weight were found to be, on average, 2000 U/L and 170 g/L, respectively.

To assess the OMW concentration that can be tolerated by *Trametes trogii*, OMW dilutions (10%, 20%, 30%, 40%, 50%, and 100% (v/v)) in water-based media were tested. The inoculum was 2%. Numerous studies have reported that dephenolization and decolorization rates are directly influenced by the size of the inoculum added to the initial culture. The addition of a low-density inoculum leads to maximum treatment and improves fungal biomass production. However, it may inhibit oxygen transfer and thus laccase activity (Daâssi et al. 2014a; Sayadi and Ellouz 1995). Many studies have revealed that OMWs are unbalanced due to their deficiency in nitrogen. However, the C/N ratio should be high to achieve optimal microbial growth (Ahmadi et al. 2006; Mwangi et al. 2012). The most appropriate nitrogen source to allow optimal growth of *Trametes trogii* strains and the greatest OMW biodegradation is ammonium salt (NH_4Cl) (Chakroun et al. 2009). For this reason, it was added to the medium at a concentration of 1 g/L.

Numerous results show that the type of nitrogen source as well as the inducer used influence not only the amount and type of enzymes produced but also the decolorization and degradation of the effluent (D'Souza Ticlo Diniz et al. 2005; Galhaup and Haltrich 2001). The effects of adding ammonium salts on OMW treatment by white rot fungi have been well studied (Ahmadi et al. 2006; Vassilev et al. 1997). This supplementation ameliorates mycelial growth and therefore improves polyphenol hydrolysis, causing decolorization (Lee and Han 2005; Sinigaglia et al. 2010). Some inducers, like copper, activate the transcription of laccase-encoding genes in almost all fungi, including *Trametes versicolor*, *T. pubescens*, *Pleurotus ostreatus*, *P. sajor-caju* and others, leading to higher enzymatic activity. Also, their addition at low concentrations (2–4 mM) to the culture medium stimulates laccase production (Ado et al. 2019; Hernández-Monjaraz et al. 2018). In this study, $CuSO_4$ was added as a laccase inducer to the medium at a concentration of 300 µM (Zouari-Mechichi et al. 2006). We recorded a maximum biomass production of 0.606 ± 0.024 g L^{-1} and a maximum laccase activity of 926.54 ± 10.28 U L^{-1} after 8 days of *Trametes*

trogii cultivation at 30 °C on 30% (v/v) OMWs/water-based media (Table 2).

Effect of laccase activity on OMW biodegradation

The decolorization of OMWs is logically inversely proportional to the increase in % OMWs in water (Table 2). The maximum rate of color removal, 71.31 ± 1.34%, was obtained with 10% dilution, and the color changed from near black to yellow-brown and became paler as growth proceeded. The same results were found for dephenolization (Table 2), where the maximum phenolic compound degradation rate was approximately 77–58%, which was achieved with concentrations in the range 10–30%. Similar results have been reported by Rahmanian et al. (2014), who showed that the high laccase activity of some *Pleurotus* spp. reduced the amount of phenol by about 70%. In addition, around 60% of the COD and color and 32% of the phenolic compounds were removed by *Phanerochaete chrysosporium* in the treatment of olive mill effluent (Díaz et al. 2021).

As shown in Table 2, the color removal changed from 39% to 11% as the OMW concentration was increased from 40% to 100%. In addition to their responsibility for the phytotoxic and antimicrobial properties of OMWs, high-molecular-weight phenolic compounds are also considered to be responsible for the dark brown color of these effluents, which means that the estimated dephenolization is correlated with the color removal (Daâssi et al. 2014a; McNamara et al. 2008).

Results suggest that the range of polyphenol concentrations used can be tolerated by the fungus cells with negligible substrate inhibition, especially at low phenol concentrations. Similarly, Aytar et al. (2011) and Daâssi et al. (2014a) demonstrated that substrate inhibition was negligible, especially at low concentrations of phenolic compounds. Moreover, substrate oxidation by laccases increased until the substrate concentration saturated the enzyme (Aytar et al. 2011; Daâssi et al. 2014a). However, Alaoui et al. (2008) and Fountoulakis et al. (2002) reported inhibitory effects of OMWs on fungi (*Pleurotus ostreatus* and *Pycnoporus coccineus*). Fungal remediation of OMWs using the white rot fungus *Aspergillus* sp. is well established. Besides reducing COD and removing simple phenolic compounds, these microorganisms are also effective at reducing OMW color, achieving removal rates of up to 88% for COD, 100% for phenolics and 81% for colorants (McNamara et al. 2008). Up to 60% reductions in COD and phenol concentrations were achieved using *A. niger*. Similar reductions in COD and phenolics were also observed with *A. terreus* (Hamdi et al. 1991; Martinez Nieto et al. 1993).

Several strains of bacteria (*Bacillus pumilus*, *Arthrobacter* sp., *Azotobacter vinelandii*, *Pseudomonas putida* and *Ralstonia* sp.) as well as other bacterial consortia have been tested in aerobic processes for OMW treatment (McNamara et al. 2008). Aerobic bacteria have been evaluated primarily as a method to remove cytotoxic compounds from these effluents. *B. pumilis* has been shown to effectively reduce the phenolic content of OMWs by 50% and to totally decompose protocatechuic and caffeic acids. It has less of an effect on tyrosol (Ramos-Cormenzana et al. 1996). Likewise, Ehaliotis et al. (1999) and Piperidou et al. (2000) report the outstanding ability of *A. vinelandii* to effectually decrease the phytotoxicity of OMW. This strain removed > 90% of the cytotoxic compounds from OMWs. On the other hand, OMW bioremediation using aerobic bacterial consortia was somewhat successful, as it decreased COD (by 50%) and the concentrations of phytotoxic compounds and it completely eliminated some simple phenolic compounds (Zouari and Ellouz 1996). In this regard, we refer the reader to a study by Mansour et al. (2012) in which they aimed to evaluate the in vivo toxicities of three wastewaters. They tested the abilities of textile wastewater, pharmaceutical wastewater and OMWs before and after treatment with *Pseudomonas putida* mt-2 to induce algogenic and convulsant effects in mice. They also suggested that the toxicity was entirely eliminated when the mice were treated with biologically rehabilitated

Table 2 Effects of OMW dilution on phenolic compound and color removal rates, fungal biomass production and laccase activity in exocellular medium after 12 days of *T. trogii* culture in conical flasks on OMWs/water-based media at different % OMWs:water ratios

% OMWs	Final biomass (g/L)	Dephenolization (%)	Decoloration (%)	Enzymatic activity (U/L)		
				$A_0 = 0$	$A_{max} = 8$	$A_f = 12$
10	0.095 ± 0.01	76.21 ± 1.62	71.31 ± 1.34	No activity	243.26 ± 2.09	31.16 ± 1.69
20	0.271 ± 0.025	66.34 ± 2.94	63.32 ± 2.82	No activity	462.54 ± 1.63	77.45 ± 2.05
30	0.606 ± 0.024	57.79 ± 2.21	54.79 ± 3.44	No activity	926.54 ± 10.28	323.27 ± 2.6
40	0.546 ± 0.028	38.81 ± 1.6	23.35 ± 2.29	No activity	750.9 ± 2.45	246.9 ± 2.9
50	0.4115 ± 0.014	34.07 ± 2.04	11.52 ± 1.18	No activity	680 ± 1.98	124.36 ± 1.54
100	0.377 ± 0.035	10.52 ± 0.6	7.17 ± 1.11	No activity	25.36 ± 1.7	No activity

Experimental conditions were as follows: 2% biomass, 30 °C, 150 rpm, 12 days incubation and 50 mL working volume; A_0, A_{max} and A_f represent the initial, maximal and final activity, respectively

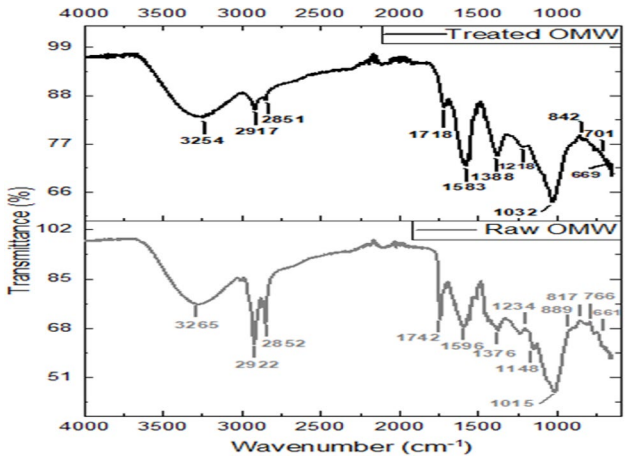

Fig. 1 FT-IR-ATR spectra of OMW samples

wastewater. This proves that *P. putida* is able to completely detoxify toxic industrial wastewater.

Spectroscopic analysis

The OMW contents before and after treatment with laccases were determined qualitatively by FT-IR analysis (Fig. 1).

The spectrum of untreated OMWs shows a broad and intense band at 3265 cm^{-1} attributed to C–H bonds and the elongation of O–H bonds in alcohol, water, phenol or carboxyl groups as well as the hydrogen vibrations of amide N–H functions. The bands in the region 2852–2922 cm^{-1} are assigned to the symmetrical and asymmetrical elongation of the aliphatic C–H stretching vibration in aliphatic structures (fatty acids, waxes and various aliphatics), which are mostly indistinguishable and embedded in a single shell. The band at around 1742 cm^{-1} is attributed to COOH groups, ketone groups and C=O stretching vibrations in esters. The region of 900–1570 cm^{-1} is mainly specific to N–H vibration deformations in secondary amides, C–H vibrations and deformations of OH functions, carboxyls, aromatic >C=C< bond elongation, the stretching of C=C bonds in aromatic groups, C=N bonds in amides, and the =C–O– functions of phenols, carbohydrates, aromatic ethers and polysaccharides (Jaouadi 2021; Zaier et al. 2017; Socrates 2004). Compared to the raw OMW spectrum, that of the treated OMWs shows a relative a decrease in O–H elongation at around 3254 cm^{-1} corresponding to the vibrations of alcohol, phenol and carboxylic groups. Also, a band centered between 1583 and 1718 cm^{-1} due to double bonds (C=C) in phenols and a band around the wavenumber 1388 cm^{-1} due to the vibration of OH groups in aromatic compounds appear (Hassani 2020). The spectrum also shows decreases in the signal at 2917 cm^{-1} and in the peak at 2851 cm^{-1}. Additionally, enzymatic treatment of OMW leads to a decrease in transmittance in the region 669–1218 cm^{-1} and the disappearance of the two peaks at around 766 cm^{-1} and 661 cm^{-1}. This accounts for the enzymatic treatment's effectiveness in terms of reducing the pollutant organic load of OMWs, especially the phenolic compounds. Indeed, bacteria and fungi can produce the enzymes polyphenol oxidase and β-glucosidase, which play a major role in the degradation of polyphenols and in hydrolysis during the decomposition of organic matter.

Effect of the culture conditions on microalgae strains

Numerous studies have reported the use of white-rot fungus species in OMW treatments (Cabana et al. 2007; McNamara et al. 2008). Untreated OMWs are toxic to microalgae cultivation and need dilution and pretreatment to reduce their initial organic loads and phenolic contents, as described in the literature (Paraskeva and Diamadopoulos 2006). In order to choose the concentration of pretreated OMWs that represents the best compromise between the need for a carbon source for the mixotrophic culture of microalgae and the need to reduce the organic pollutant load, the two strains of microalgae were cultivated with 30%, 40% and 50% OMW/water (v/v). The 30% dilution of OMWs promoted better growth of the two microalgae compared with the other dilutions (Fig. 2). Accordingly, the former was selected for mixotrophic cultivation.

The dependence of algal growth on the culture conditions is detailed in Fig. 3 for both *Chlorella* sp. and *Tetraselmis* sp. Autotrophic and mixotrophic cultures were tested with two microalgal inocula (20% and 50%). For both cultures, the highest cell growth was observed under autotrophy; more precisely, with the 50% inoculation. The autotrophic growth curves of *Chlorella* sp. and *Tetraselmis* sp. are linear. The short adaptation phase to the mixotrophic culture conditions

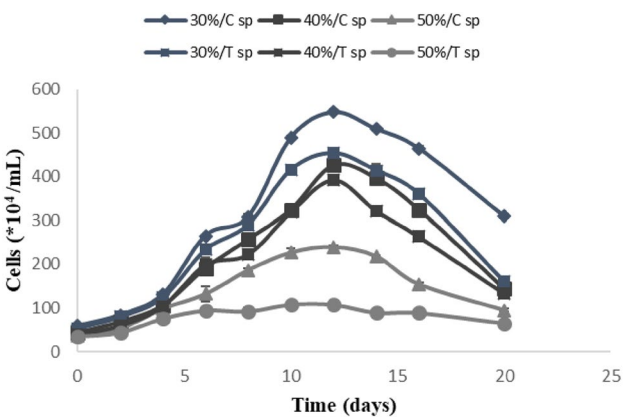

Fig. 2 Cultivation of *Chlorella* sp. (*C sp*) and *Tetraselmis* sp. (*T sp*) on diluted OMWs (30%, 40% and 50% dilutions)

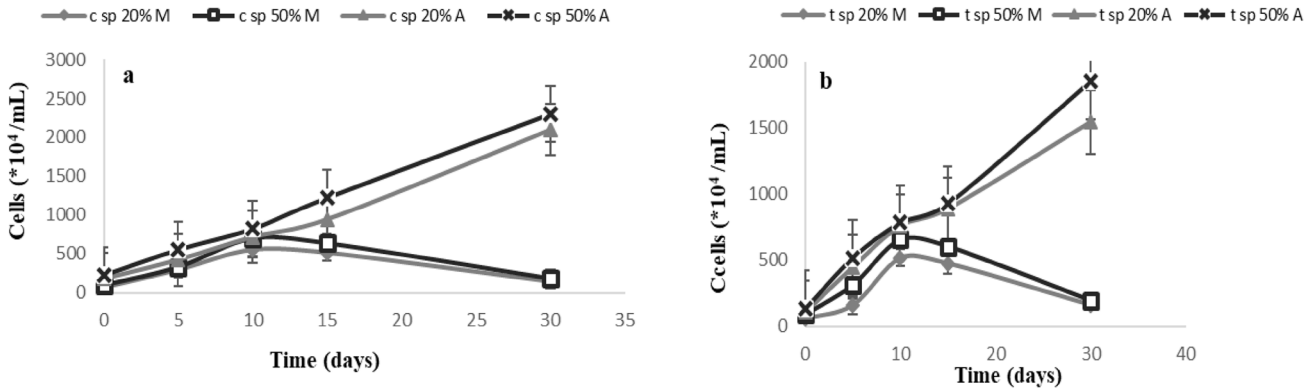

Fig. 3 Evolution of the cell concentration over time for *Chlorella* sp. (*c sp*) (**a**) and *Tetraselmis* sp. (*t sp*) (**b**) cultivated in autotrophic (*A*) and mixotrophic (*M*) conditions; *20%/50%*: volume of inoculum added

may be the result of the presence of organic carbon compounds derived from OMWs. The short lag phase is caused by the rapid transport and diffusion of organic carbon in the form of reducing sugars into the membrane (Piasecka et al. 2020). In mixotrophy, stationary phases of growth are reached on the 14th day of cultivation for both strains. *Chlorella* sp. cells grow faster than *Tetraselmis* sp. ones.

Most previous studies found that, regardless of the strain and source of carbon (synthetic or derived from waste) used, mixotrophy increases microalgal cell growth and biomass yields compared to autotrophy (Abreu et al. 2012; Arora and Philippidis 2021; León-Vaz et al. 2019; Piasecka et al. 2020). Other studies found no difference in the results obtained using the two cultivation modes (Burch et al. 2021).

The effects of the autotrophic and mixotrophic conditions on pigment content and productivity using the two inocula of both strains are summarized in Tables 3, 4, 5 and 6. The formation of the photosynthetic apparatus in *Chlorella* may be disrupted by the existence of organic matter, leading to a decrease in the production of photosynthetic pigments compared to those obtained in photoautotrophic growth.

It was found that the highest photosynthetic pigment contents and productivities are obtained under autotrophy with the 50% inoculum for *Chlorella* sp. (Tables 3 and 4) and *Tetraselmis* sp. (Tables 5 and 6).

The highest contents of chlorophylls and carotenoids (51.10 ± 0.1 mg/L and 2849.18 ± 0.55 mg/L, respectively) appeared on the 15th day in the photoautotrophic culture of *Tetraselmis* sp.

The chlorophyll and carotenoid contents of photoautotrophically cultivated *Chlorella* reached values of 45.28 ± 0.69 mg/L and 2674.6 ± 0.85 mg/L, respectively.

The application of the mixotrophic mode or the addition of a carbon source significantly reduced the photosynthetic pigment content. This phenomenon can be explained by negative feedback regulation by the end products of photosynthesis—a mechanism to relieve photoinhibition (Liu et al. 2009; Yan et al. 2012). Increased pigment biosynthesis by the *Chlorella* and *Tetraselmis* strains under autotrophy compared to mixotrophy has previously been reported by several authors (Abreu et al. 2012; Kong et al. 2011; Piasecka and Baier 2022). Bhatnagar et al.

Table 3 Effect of nutritional mode on the chlorophyll content and productivity of *Chlorella* sp.

T	[Chlorophylls] (mg/L)				Productivity (mg/10^4 cells/mL)			
	Chlorella sp.				*Chlorella* sp.			
	A		M		A		M	
	20%	50%	20%	50%	20%	50%	20%	50%
0	8.05 ± 0.12	10.70 ± 0.14	2.87 ± 0.01	3.63 ± 0.15	0.046 ± 0.001	0.047 ± 0.001	0.042 ± 0.0019	0.041 ± 0.053
5	10.67 ± 0.02	14.03 ± 0.30	4.56 ± 0.02	6.61 ± 0.26	0.022 ± 0.009	0.025 ± 0.003	0.015 ± 0.0023	0.0202 ± 0.0012
10	20.84 ± 0.22	23.16 ± 0.1	7.67 ± 0.2	9.97 ± 0.14	0.029 ± 0.0015	0.028 ± 0.0022	0.013 ± 0.00121	0.0142 ± 0.0002
15	44.42 ± 0.89	45.28 ± 0.69	5.19 ± 0.08	4.44 ± 0.08	0.47 ± 0.0033	0.037 ± 0.0039	0.010 ± 0.05	0.0071 ± 0.08
30	51.83 ± 0.18	74.58 ± 0.74	1.24 ± 0.04	2.96 ± 0.02	0.024 ± 0.0008	0.032 ± 0.0041	0.0085 ± 0.016	0.017 ± 0.0024

The experimental conditions were: pH 7, temperature = 25 °C, agitation speed = 120 rpm, and illumination intensity = 100 µmol photons m^{-2} s^{-1}
20%/50% volume of inoculum, *M* mixotrophy, *A* autotrophy

Table 4 Effect of nutritional mode on the carotenoid content and productivity of *Chlorella* sp.

T	[Carotenoids] (mg/L)				Productivity (mg/10^4 cells/mL)			
	Chlorella sp.				*Chlorella* sp.			
	A		M		A		M	
	20%	50%	20%	50%	20%	50%	20%	50%
0	302.55 ± 2.1	521.92 ± 5.36	67.038 ± 0.85	89.98 ± 0.36	1.74 ± 0.084	2.31 ± 0.12	0.98 ± 0.015	1.016 ± 0.02
5	293.74 ± 2.03	600.48 ± 4.03	279.52 ± 1.05	330.27 ± 0.43	0.7 ± 0.021	1.084 ± 0.032	0.939 ± 0.028	1.011 ± 0.0156
10	1416.02 ± 0.82	1410.93 ± 0.36	515.46 ± 0.42	649.05 ± 2.02	1.98 ± 0.122	1.72 ± 0.038	0.932 ± 0.014	0.93 ± 0.036
15	2461 ± 0.9	2674.6 ± 0.85	233.95 ± 2.02	277.93 ± 0.74	2.61 ± 0.002	2.188 ± 0.06	0.45 ± 0.0159	0.43 ± 0.01
30	3538.6 ± 0.23	3192.04 ± 1.68	101.73 ± 1.1	105.95 ± 1.02	1.68 ± 0.012	1.390 ± 0.007	0.7 ± 0.008	0.6 ± 0.009

The experimental conditions were: pH 7, temperature = 25 °C, stirring = 120 rpm, and irradiance = 100 µmol photons $m^{-2}.s^{-1}$

20%/50% volume of inoculum, *M* mixotrophy, *A* autotrophy

Table 5 Effect of nutritional mode on the chlorophyll content and productivity of *Tetraselmis* sp.

T	[Chlorophylls] (mg/L)				Productivity (mg/10^4 cells/mL)			
	Tetraselmis sp.				*Tetraselmis* sp.			
	A		M		A		M	
	20%	50%	20%	50%	20%	50%	20%	50%
0	7.34 ± 1.4	10.88 ± 0.04	3.48 ± 0.51	3.78 ± 0.093	0.069 ± 0.017	0.083 ± 0.001	0.062 ± 0.012	0.043 ± 0.12
5	11.66 ± 1.5	16.54 ± 0.31	4.71 ± 0.43	6.70 ± 0.87	0.026 ± 0.002	0.03 ± 0.09	0.032 ± 0.016	0.021 ± 0.2
10	21.08 ± 0.68	25.78 ± 0.12	7.88 ± 0.7	9.27 ± 1.16	0.028 ± 0.0009	0.033 ± 0.03	0.015 ± 0.003	0.014 ± 0.036
15	43.6 ± 0.84	51.10 ± 0.1	5.52 ± 0.36	6.32 ± 1.96	0.049 ± 0.0007	0.05 ± 0.12	0.011 ± 0.002	0.01 ± 0.015
30	51.52 ± 0.87	76.04 ± 0.14	1.42 ± 0.32	3.38 ± 0.65	0.033 ± 0.0004	0.041 ± 0.02	0.0089 ± 0.0027	0.017 ± 0.01

The experimental conditions were: pH 7, temperature = 25 °C, stirring = 120 rpm, and irradiance = 100 µmol photons $m^{-2}.s^{-1}$

20%/50% volume of inoculum, *M* mixotrophy, *A* autotrophy

Table 6 Effect of nutritional mode on the carotenoid content and productivity of *Tetraselmis* sp.

T	[Carotenoids] (mg/L)				Productivity (mg/10^4 cells/mL)			
	Tetraselmis sp.				*Tetraselmis* sp.			
	A		M		A		M	
	20%	50%	20%	50%	20%	50%	20%	50%
0	328.3 ± 0.44	516.8 ± 0.52	82.21 ± 0.23	122.6 ± 0.52	1.8 ± 0.017	3.94 ± 0.27	1.47 ± 0.51	1.4 ± 0.61
5	283 ± 0.09	663 ± 0.09	267.7 ± 0.1	336.3 ± 0.11	0.67 ± 0.09	1.2 ± 0.12	1.7 ± 0.68	1.08 ± 0.14
10	1415.5 ± 0.75	1443.7 ± 0.12	508 ± 0.58	69 ± 0.09	1.9 ± 0.25	1.8 ± 0.14	0.98 ± 0.087	1.04 ± 0.07
15	2552.26 ± 0.36	2849.18 ± 0.55	236.9 ± 0.02	29 ± 0.23	2.71 ± 0.14	3 ± 0.28	0.5 ± 0.083	0.51 ± 0.83
30	3462.54 ± 0.21	3231.58 ± 0.84	99.4 ± 0.12	119.61 ± 0.2	1.64 ± 0.05	1.74 ± 0.09	0.61 ± 0.01	0.62 ± 0.15

The experimental conditions were: pH 7, temperature = 25 °C, agitation speed = 120 rpm, and irradiance = 100 µmol photons m^{-2} s^{-1}

20%/50% volume of inoculum, *M* mixotrophy, *A* autotrophy

(2011) cultivated *Chlamydomonas globosa*, *Chlorella minutissima* and *Scenedesmus bijuga* under mixotrophy and observed a decrease in the total chlorophyll content as well. The total chlorophyll/biomass ratio (µg/mg) is 58–85% lower under mixotrophy (industrial wastewater) than under photoautotrophy. Some authors suggest that the decrease in chlorophyll content may be due to reduced chlorophyll synthesis as organic carbon is taken directly from sugars. Also, it could be the result of the biodegradation of chlorophyll because of metabolic regulation.

Therefore, cells downregulate chlorophyll synthesis and conserve energy.

Regardless of the protein synthesized (Fig. 4a and b), the highest content was obtained for *Chlorella* sp. grown under mixotrophy (7.2 ± 0.079 µg/mL on the 15th day). The maximum protein productivity (0.032 µg/10^4 cells/mL/D) occurred when the cells were grow mixotrophically with a dilution of 20%. This result is similar to that reported by Abreu et al. (2012). Indeed, the cultivation of *Chlorella vulgaris* P12 using hydrolyzed cheese whey as the organic carbon source resulted in the highest protein content (63.5%) and protein productivity (474 mg/L/D). Piasecka and Baier (2022) revealed that the highest protein contents of *Chloroidium saccharophyllum* and *Chlorella vulgaris* were 6.85 mg/mL and 6.2 mg/mL, respectively, which were obtained under mixotrophy. The culture conditions did not definitely affect the protein content of *Chlorella sorokiniana*, as it did in our study with the *Tetraselmis* strain. In addition, mixotrophy did not affect the protein content of *Tetraselmis* sp. (Fig. 4c and d), and the highest protein content occurred in autotrophy mode with a dilution of 50% (6.05 ± 0.23 µg/mL). Pereira et al. (2019) mentioned that the *Spirulina platensis* biomass obtained from photoautotrophy presented a high total protein content (65%) compared to that from mixotrophy (with the addition of buffalo mozzarella cheese whey). The growth conditions or trophic mode may not affect the biochemical composition (Lari et al. 2019; Piasecka and Baier 2022).

The final total lipid content was highest under autotrophy with a dilution of 50%, reaching 4.2% ± 0.351% and 5.223% ± 0.795% for *Chlorella* sp. and *Tetraselmis* sp., respectively, but lipids were totally absent in the mixotrophic mode. With a dilution of 20%, the final total lipid content was 3.042% ± 0.205% and 3.397% ± 0.19% for *Chlorella* sp. and *Tetraselmis* sp., respectively. These results are similar to those found by López et al. (2019), who showed a significant impact of the trophic mode on lipid biosynthesis. The maximum lipid production by *Galdieria* sp. strain USBA-GBX-832 occurred under autotrophy (15.34 ± 3.3%). Abreu et al. (2012) highlight the high lipid content for *C. vulgaris* (42%) in photoautotrophic mode, and they also mention that the highest lipid productivity (253 mg/L/D) took place when cells were grown under mixotrophy (dairy wastewaters). Piasecka and Baier (2022) indicate that the total lipid

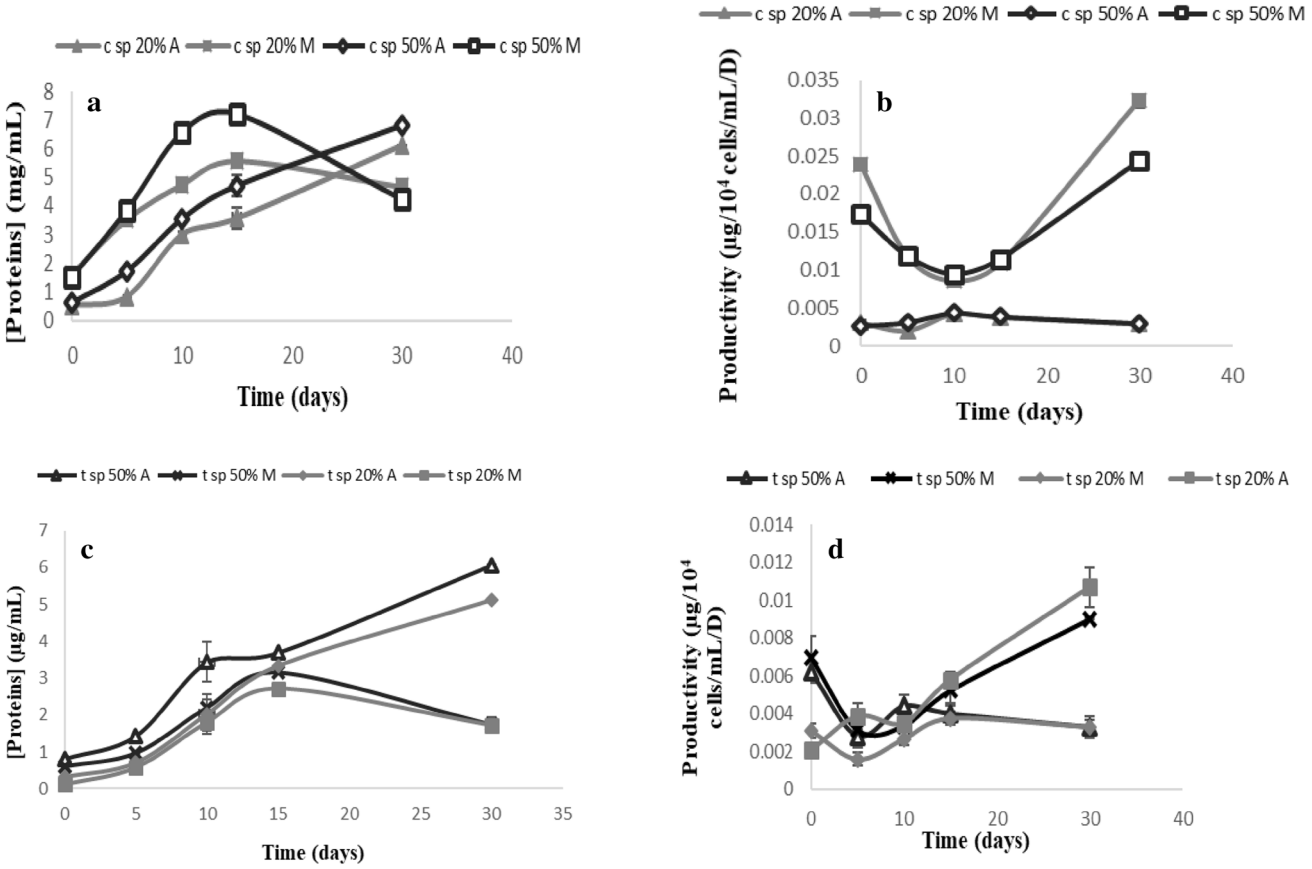

Fig. 4 Protein contents and productivities of *Chlorella* sp. (*c sp*) (**a** and **b**) and *Tetraselmis* sp. (*t sp*) (**c** and **d**) under different nutritional conditions; *20%/50%*: volume of inoculum added, *M* mixotrophy, *A* autotrophy

content of *Chlorella vulgaris* decreases significantly from 17.99% for photoautotrophy to 10.75% for mixotrophy with molasses.

Added to that, the different nutritional conditions (photoautotrophy and mixotrophy) have different effects on the starch content, as shown in Table 7 for *Chlorella* sp. and *Tetraselmis* sp. In all cultures, the highest starch contents occurred with the 50% inoculum (13% and 15% of the dry cell weight for *Chlorella* sp. and *Tetraselmis* sp., respectively). We also observe a substantial decrease in the total amount of starch under mixotrophy for the two strains. Similar to what was observed for lipids, both strains produce the maximum amount of total starch autotrophically. Abreu et al. (2012) reported similar results, and they demonstrated that *Chlorella vulgaris* yields a greater starch content (5.1%) in the photoautotrophic mode as compared to the mixotrophic mode (with a mixture of pure glucose and galactose and a hydrolyzed cheese whey solution). Dragone et al. (2011) also noted that starch accumulation was greater with autotrophically cultivated *Chlorella vulgaris* P12 under stressful growth conditions (nitrogen depletion).

Under optimal growth conditions, microalgae accumulate only limited amounts of lipids and carbohydrates (Behera et al. 2021). Microalgae produce lipids and carbohydrates as components of their internal defense mechanisms in response to stressful conditions. The protection of microalgae from the excess of reducing equivalents generated under stress (NADPH) leads to a massive enrichment in lipids (Piasecka and Baier 2022; Shi et al. 2020). Mixotrophy seems to be optimal and beneficial for protein production but does not favor the accumulation of lipids and starch. Many studies refer to the use of agro-industrial wastes and wastewaters in mixotrophic cultures of microalgae, with a focus on the production of lipids which can be transformed into biofuels by transesterification (Abreu et al. 2012; Arora and Philippidis 2021; Lau et al. 2014).

Degradation of phenolic compounds by *Chlorella* sp. and *Tetraselmis* sp.

To evaluate the effectiveness of the secondary dephenolization treatment, we determined the decrease in phenolic compounds after the cultivation of the two strains of microalgae. Results showed that the phenolic compounds decreased over the course of the culture (Fig. 5). The removal percentages increased with increasing OMW percentage (v/v) in the culture medium. The best removal rates were achieved with *Chlorella* sp. (Fig. 5a and b). Values increased from 57 to 72% when switching from 50% to 20% microalgae inoculum in the culture. Phenolic compound removal is therefore a function of their concentration and algae density. Many authors have demonstrated the ability of *Chlorella* and other microalgae to remove high levels of phenolic and other environmentally harmful compounds (Lindner and Pleissner 2022). The effect of *C. pyrenoidosa* (KX686118) on the phenol waste liquid from a coal gasification plant was investigated by Dayana Priyadharshini and Bakthavatsalam (2016). Some other authors have recorded removal percentages of higher than 90% after microalgae growth. Two studies by Pinto et al. (2002, 2003) showed the biodegradation of selected phenols from OMWs by green algae and cyanobacteria. This biodegradation led to the removal of more than 70% of the phenols within 5 days of incubation. However, phenolic compounds were not completely removed and seemed to be biotransformed to other aromatic ones.

Conclusions

This experiment tested two strains of microalgae (*Chlorella* sp. and *Tetraselmis* sp.) with specific environmental origins to validate their adaptability to OMWs. The aim was to reduce the phenolic compounds in these complex by-products and convert them into high-value biomass with great potential for applications in the cosmetic and food industries. However, OMWs have a complex composition, complicating their treatment, so a combined physicochemical and biological process for the efficient dephenolization

Table 7 Final starch contents of *Chlorella* sp. and *Tetraselmis* sp. grown under autotrophy and mixotrophy

	Chlorella sp.				*Tetraselmis* sp.			
	A		M		A		M	
	20%	50%	20%	50%	20%	50%	20%	50%
Starch content (%)	9.12 ± 0.73	13.38 ± 2.17	1.25 ± 0.4	2.12 ± 0.01	11.74 ± 0.64	15.66 ± 0.92	2.8 ± 0.01	3.01 ± 0.61

The common experimental conditions were: pH 7, temperature = 25 °C, agitation speed = 120 rpm, and illumination intensity = 100 µmol photons m^{-2} s^{-1}

20%/50%: volume of inoculum, *M* mixotrophy, *A* autotrophy

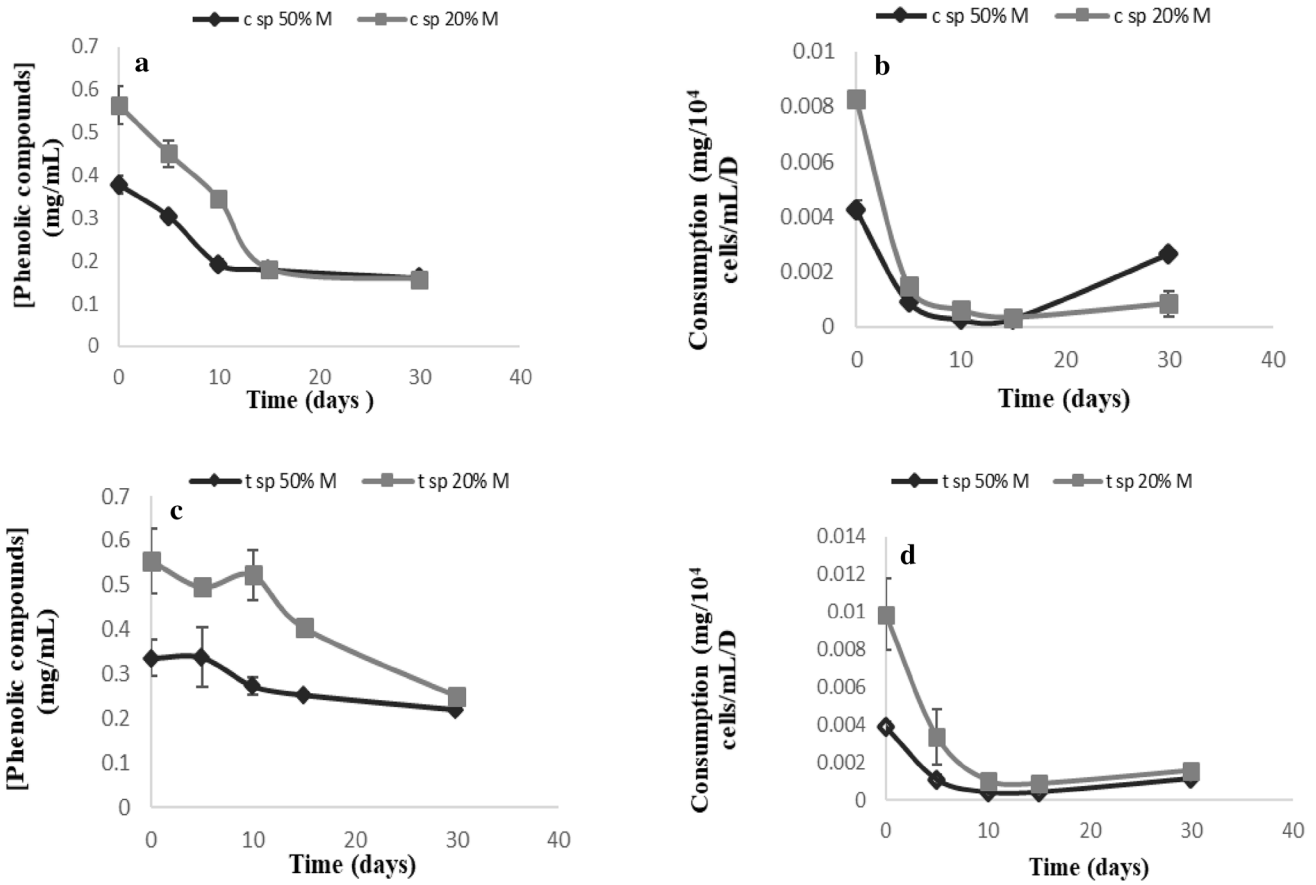

Fig. 5 Phenolic compound removal by *Chlorella* sp. (**a** and **b**) and *Tetraselmis* sp. (**c** and **d**). *c sp Chlorella* sp., *t sp Tetraselmis* sp., *20%/50%*: volume of inoculum added, *M* mixotrophy, *A* autotrophy

of the OMWs was proposed. Accordingly, and based on our overall findings, an environmentally friendly two-step approach to OMW biodegradation was proposed. The first step was an enzymatic treatment using laccases from *Trametes trogii* which aimed to eliminate most of the phenolic and colored compounds present in the effluent. The second step was phycoremediation using *Chlorella* sp. and *Tetraselmis* sp. This significantly reduced the phenol concentration in the pretreated OMWs. In the future, to effectively use the phenolic compounds, a consortium of microalgae should be employed. Regarding the biochemical transformation of phenolic compounds, further research is needed to clearly identify the biochemical pathways and metabolites.

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