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RESEARCH PAPER

Mixotrophic growth regime as a strategy to develop microalgal bioprocess from nutrimental composition of tequila vinasses

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Abstract

The selection of a suitable growth regime can increase the physiological performance of microalgae and improve bioprocess based on these microorganisms from agro-industrial residues. Thus, this study assessed the biotechnology capacity—biomass production, biochemical composition, and nutrient uptake—from tequila vinasses (TVs) as the nutrient source of three indigenous microalgae—*Chlorella* sp., *Scenedesmus* sp., and *Chlamydomonas* sp.—cultured under heterotrophic and mixotrophic conditions. The results demonstrated that under the mixotrophic regime, the three microalgae evaluated reached the highest nitrogen uptake, biomass production, and cell compound accumulation. Under this condition, *Chlorella* sp. and *Scenedesmus* sp. showed the highest nutrient uptake and biomass production, 1.7 ± 0.3 and 1.9 ± 0.3 g L^{-1}, respectively; however, the biochemical composition, mainly carbohydrates and proteins, varied depending on the microalgal strain and its growth regime. Overall, our results demonstrated the biotechnological capacity of native microalgae from TVs, which may vary not only depending on the microalgal strain but also the culture strategy implemented and the characteristics of the residue used, highlighting—from a perspective of circular bio-economy—the feasibility of implementing microalgal bioprocess to reuse and valorize the nutrimental composition of TVs through biomass and high-valuable metabolite production, depicting a sustainable strategy for tequila agro-industry in Mexico.

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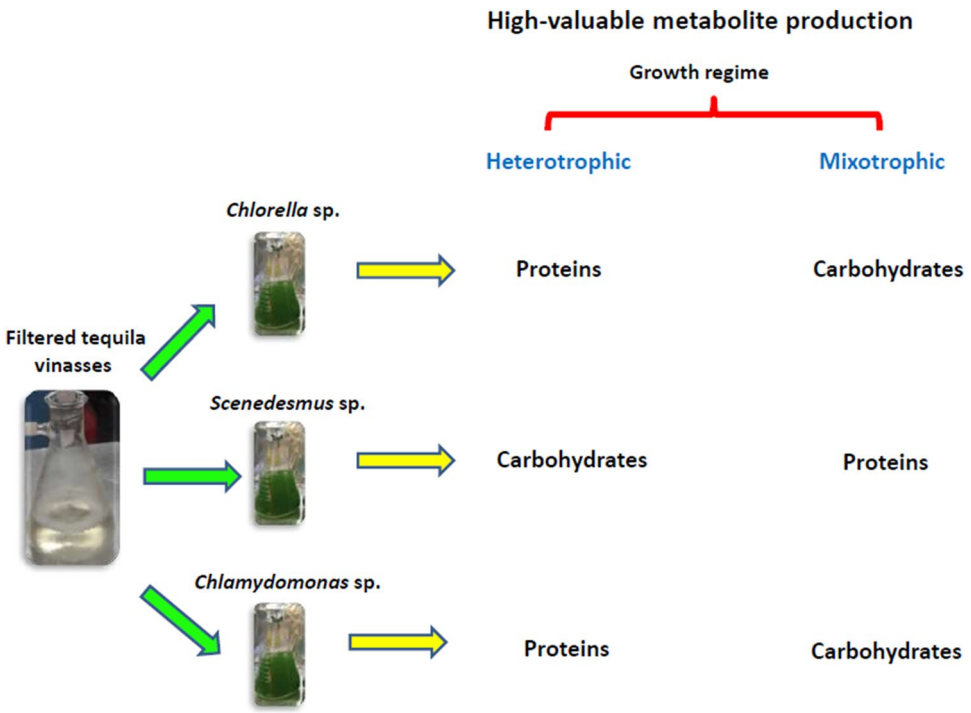
Graphic abstract



Keywords Agro-industrial residues · Tequila vinasses · *Chlamydomonas* · *Chlorella* · *Scenedesmus* · Mixotrophic · Heterotrophic · Nutrient uptake

Introduction

Microalgal biomass production with high-valuable metabolites, such as carbohydrates, proteins, lipids, and pigments, is a worldwide activity because of the diversity of biotechnological applications in several economic sectors, such as pharmaceutical, nutritional, environmental, and energetic [1]. Today, the main challenges of intensive microalgal production are water requirement and the high nutrient demand, such as carbon, nitrogen, and phosphorous [2]. In this regard, under the basic concept of circular bio-economy—reduce, reuse, and recycle—the use of agro-industrial residues or wastewater emerges as a valuable resource to reduce the cost of microalgal production, as well as an appropriate disposal and valorization of industrial effluents to comply with government regulations [3, 4].

Nowadays, the tequila agro-industry in Mexico has a considerable challenge to find and implement suitable disposal of TVs, a brown liquid generated in a proportion from 10 to 12 L per liter of tequila produced from blue agave (Agave tequilana Weber var. Azul), and with a composition rich of organic/inorganic carbon, macro and micronutrients [5, 6]. This effluent is used for biofuel production (biogas and hydrogen) through anaerobic digestion process, fertirrigation of agricultural lands, or simply discharged in municipal sewer [5, 6]. To date, vinasses generated from different feedstocks such as sugarcane, beet, grape, and corn have been used as a nutrient source for culturing yeast, bacteria, and microalgae [5]. Specifically, the microalgae of the genus *Chlorella*, *Scenedesmus*, *Chlamydomonas*, *Neochloris*, *Micractinium*, and the cyanobacterium *Spirulina* have been cultured in vinasses as an integral strategy of bioremediation and increasing biomass production to obtain fine products or biofuels [7–11]. However, they have shown different growth patterns, biomass production, and biochemical composition due to the different capacity of each microalgal strain to grow from the nutrimental composition of these effluents, as well as the culture strategies used [7–11].

In this context, the autotrophic growth regime—using sunlight and CO_2 from the atmosphere as a source of energy and inorganic carbon, respectively—is widely used to cultivate microalgae on industrial effluents [12]. Nonetheless, some microalgae can also grow under heterotrophic condition, in complete darkness, using organic compounds, such as acetic acid, glucose, glycerol, among others, as energy and carbon sources [13, 14], or under mixotrophic condition, that is, assimilating simultaneously inorganic and organic carbon under a light source [15, 16]. Today, several

studies have demonstrated that *Chlorella*, *Scenedesmus*, and *Chlamydomonas* strains can improve their physiological performance when grown under heterotrophic or mixotrophic conditions, achieving higher biomass production, high-value metabolite accumulation, and nutrient uptake than cultured under an autotrophic regime [13–17]. Under the heterotrophic regime, higher biomass yields are possible because the energy density of organic compounds (e.g. glucose, $\Delta H = 2801$ kJ mol^{-1}) is higher than that of CO_2 ($\Delta H = 395$ kJ mol^{-1}), whilst mixotrophic cultures are versatile since microalgae can obtain their energy needs from both organic and inorganic carbon [13, 18]. Nonetheless, each microalga genus is diverse and different strains may have different abilities to grow under heterotrophic or mixotrophic regime [9, 15]. Therefore, determining the appropriate regimen growth of each microalga should be evaluated to maximize its physiological performance and biomass production from efficient use of the composition of each residue as nutrient source.

From this standpoint, indigenous microalgae are preferred nowadays for biorefinery and bioremediation purposes because they are already adapted to the environmental conditions prevailing in a specific geographical location [4], *Chlorella* sp., *Scenedesmus* sp., and *Chlamydomonas* sp. microalgae were isolated from Cajititlán Lagoon-Jalisco, Mexico—a water body with high phytoplanktonic diversity [19]. The purpose of their isolation was to develop and propose a bioprocess to reuse and valorize the energy and nutrient content of TVs through biomass and high-valuable metabolite production with sustainable use in different economic sectors.

Considering the above, the aims of this study were to evaluate at laboratory scale the biotechnological capacity—cell density, biomass production, and biochemical composition—of three indigenous microalgae, *Chlorella* sp., *Scenedesmus* sp., and *Chlamydomonas* sp., using TVs as nutrient source and cultured under heterotrophic and mixotrophic conditions. Moreover, the nutrient uptake—chemical oxygen demand, nitrogen, and phosphorous—from TVs by each microalga growing in both growth regimens was also evaluated.

Materials and methods

Microorganisms and culture conditions

The green microalgae *Chlorella* sp., *Scenedesmus* sp., and *Chlamydomonas* sp. were isolated from Cajititlán Lagoon-Jalisco, Mexico (20° 25′ 26″ N 103° 19′ 23″ W) according to the methodology described by Smith et al. [20]. All microalgae were maintained in C30 + M medium [21] at 27 ± 2 °C, 200 µmol photon m^{-2} s^{-1}, and stirred at 120 rpm for 14 days.

Tequila vinasses

Tequila vinasses were obtained from Tequila Gran Padre distillery located in Arenal-Jalisco, Mexico; 50 L of vinasses was collected and stored at 4 °C until their utilization. In this study, TVs were used without sterilizing; but before each experimentation, TVs were filtered with filter paper grade 40 (Waltham, MA, USA) spreading 5 g of activated carbon (Sigma, St. Louis, MO, USA) on it to reduce the dark color of this residue (supplementary material Figure S1); subsequently, pH of filtered TVs was regulated at 6.00–7.00 with 1 M KOH (Sigma, St. Louis, MO, USA).

Experimental design

The experiments were set up by separately adding 25 mL (0.3 ± 0.1 g L^{-1}) of (1) *Chlorella* sp., (2) *Scenedesmus* sp., and (3) *Chlamydomonas* sp. in 275 mL of filtered TVs (Table 1) using 500-mL flasks with 300 mL of working volume. Under heterotrophic conditions, each microalga was maintained in complete darkness at 27.5 ± 2 °C, whilst under mixotrophic conditions, microalgae were maintained at 27.5 ± 2 °C with a light intensity of 200 µmol photon m^{-2} s^{-1}. Under each condition, microalgae culture was stirred by aeration for 15 days. Each experiment consisted of six treatments performed in triplicate and repeated thrice.

Table 1 Nutrient composition of filtered tequila vinasses (TVs)

Composition (mg L^{-1})	Filtered TVs
Chemical oxygen demand (COD)	18,469
Total nitrogen	115.9
NH_4^--nitrogen	7.9
NO_3^--nitrogen	5.9
NO_2^--nitrogen	< 0.1
Total phosphorous	286
Sulfates	346.1
Magnesium	248.9
Iron	10.3
Potassium	676
Calcium	414.8
Sodium	354
Zinc	1.7
Cupper	0.3
Glucose	2400
Acetate	3200
Lactate	2700
Propionate	800
Butyrate	1200
pH	3.8

Cell density and biomass production

Microalgal cell density (cell L^{-1}) was determined every 3 days by cell count with a Neubauer hemocytometer under light microscopy (Olympus BX40, Tokyo, JP) whilst biomass production (g L^{-1}) was quantified at the end of experimental time by cell dry weight. Briefly, samples were collected at the end of the experimental time and centrifuged at 10,000 rpm for 10 min; the microalgal pellet was washed twice with distilled water and dried at 80 °C for 12 h in Thermo Scientific Heratherm™ OGS100 Lab oven (Waltham, MA, USA). Biomass productivity (P; g L^{-1} day^{-1}) was calculated from Eq. 1, where X_f and X_i correspond to biomass production (g L^{-1}) at initial (t_i) and final time (t_f) [22].

$$P = \left(X_f - X_i\right)/\left(t_f - t_i\right). \tag{1}$$

Specific growth rate (μ_{max}; day^{-1}) was calculated from Eq. 2 [22], where N is the number of cells at initial (t_0) and final (t_1) sampling time of the exponential growth phase.

$$\mu = \left(\ln N_1 - \ln N_0\right)/\left(t_1 - t_0\right). \tag{2}$$

Microalgal biomass characterization

Biomass characterization of each microalga evaluated (carbohydrates, proteins, and lipids) was performed by Fourier transform infrared spectroscopy (FTIR). FTIR spectra from the dry biomass of each evaluated microalga growing in both growth regimes were collected using FTIR spectrometer CARY 630 (Agilent, Santa Clara, CA, USA) equipped with an attenuated total reflection (ATR) accessory; 20 scans per sample were carried out with a spectrum range from 4000 to 650 cm^{-1} at a spectral resolution of 4 cm^{-1}. FTIR spectra were recorded in transmittance units (U value) versus wavenumber (cm^{-1}), and data were assessed with Resolution-pro software (Agilent, Santa Clara, CA, USA). Subsequently, quantitative determination of carbohydrates and protein content was performed by the Phenol–sulfuric [23] and Lowry [24] methods, respectively. Protein and carbohydrate productivity (mg L^{-1} day^{-1}) were determined by Eq. 3 according to [25].

$$\text{Protein productivity} = \text{Biomass productivity} \times \frac{\text{Protein content}}{100}, \tag{3}$$

$$\text{Carbohydrate productivity} = \text{Biomass productivity} \times \frac{\text{Carbohydrate content}}{100}, \tag{4}$$

where biomass productivity is in mg L^{-1} day^{-1} and protein and carbohydrate content in percentage per dry biomass weight. Pigment content (μg mL^{-1}), such as Chlorophyll a (*Chl a*), b (*Chl b*), and carotenoids (Car) of each microalga were quantified by Eqs. 5, 6, and 7, respectively, according to [26].

$$\text{Ca} = 11.97A_{664} - 1.93A_{647}, \tag{5}$$

$$\text{Cb} = 20.36A_{647} - 5.50A_{664}, \tag{6}$$

$$\text{Car} = 7.6\left(A_{480} - 1.49A_{510}\right). \tag{7}$$

Determination of nutrient uptake

Chemical oxygen demand (COD), nitrogen, and phosphorous uptake were quantified by the Hach Method-TNTplus (Loveland, CO, USA) for total nitrogen (TNT828), phosphorous (TNT845), and COD (TNT822) according to the indicated procedures. Removal efficiency (%) of each nutrient by each microalga assessed was quantified by Eq. 7, according to [25].

$$\text{Removal efficiency} = \left(\frac{\text{Initial concentration} - \text{Final concentration}}{\text{Initial concentration}}\right) \times 100. \tag{8}$$

Statistical analysis

The data from each treatment from the three experiments were combined for analyses, first by one-way analysis of variance (ANOVA) and then by Fisher's least significant difference (LSD) post hoc analysis with significance set at $P < 0.05$ using Statistica 6.0 software (StatSoft, Tulsa, OK).

Results

Cell density and biomass production

Figure 1 shows that the three microalgae evaluated showed 3 days of adaptation to filtered TVs in both growth regimes, but after this period cell density increased until the end of incubation time (15 d) showing a growth speed higher under mixotrophic than heterotrophic regime. At this time, the highest cell density of *Chlorella* sp., *Scenedesmus* sp., and *Chlamydomonas* sp. cultured under heterotrophic regime was $3.1 \times 10^7 \pm 0.3$, $3.5 \times 10^7 \pm 0.3$, and $2.3 \times 10^7 \pm 0.3$ cell mL^{-1}, respectively. However, under mixotrophic regime, the cell density reached was $4.7 \times 10^7 \pm 0.4$ (*Chlorella* sp.), $5.2 \times 10^7 \pm 0.5$ (*Scenedesmus* sp.), and $4.1 \times 10^7 \pm 0.3$ cell mL^{-1} (*Chlamydomonas* sp.). Besides, the cell density of each microalga was significantly higher in most of the interval times when they were cultured under mixotrophic rather

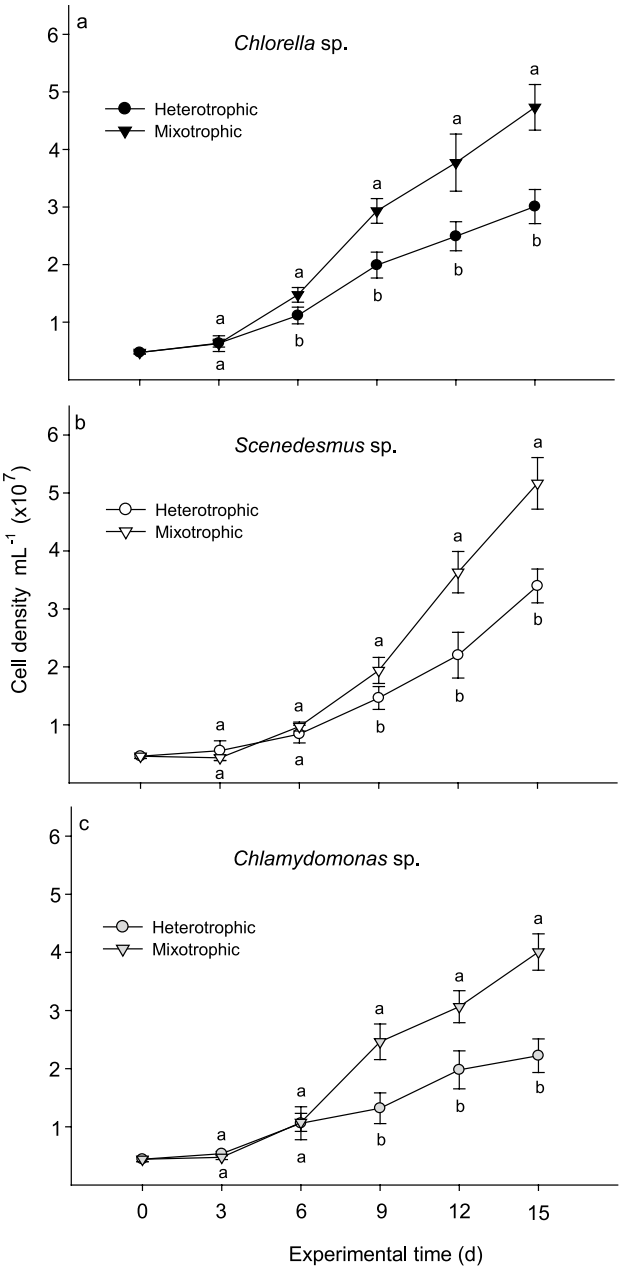


Fig. 1 Cell density by *Chlorella* sp. (**a**), *Scenedesmus* sp (**b**), and *Chlamydomonas* sp. (**c**) cultured in filtered tequila vinasses (TVs) under heterotrophic and mixotrophic growth regime. Points at each time interval denoted by different lowercase letters differed significantly when each microalga grew in different growth regimes. Statistical analyses were performed using analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis ($p < 0.05$). Bars represent standard error

than heterotrophic regime, as shown in Fig. 1a–c, lowercase analysis. Similarly at the end of experimental time (15 days), the biomass production of *Chlorella* sp., *Scenedesmus* sp., and *Chlamydomonas* sp. cultured under mixotrophy was significantly higher than in the heterotrophic regime, recording 1.7 ± 0.3, 1.9 ± 0.3, and 1.4 ± 0.2 g L^{-1}, respectively, as shown in Fig. 2a, lowercase analysis. Likewise, the growth rates and biomass productivities attained by each microalga cultured under mixotrophy were significantly higher than growing under heterotrophic regime, see Table 2.

Chemical oxygen demand, nitrogen, and phosphorous uptake

The removal efficiency of COD, nitrogen, and phosphorous uptake of filtered TVs was significantly higher in each microalga when they were cultured under mixotrophic than in the heterotrophic regime, as shown in Fig. 2b, lowercase analysis. Under this condition, *Chlorella* sp. recorded a removal efficiency of $74.0 \pm 4.9\%$ (COD), $97.8 \pm 1.3\%$ (nitrogen), and $35.6 \pm 7.4\%$ (phosphorous). Similarly, *Scenedesmus* sp. attained $78.8 \pm 4.0\%$ (COD), $99.4 \pm 0.4\%$ (nitrogen), and $40.2 \pm 8.20\%$ (phosphorous), respectively, whilst *Chlamydomonas* sp. showed a removal efficiency of $69.0 \pm 4.80\%$, $98.1 \pm 0.5\%$, and $38.8 \pm 2.60\%$ of COD, nitrogen, and phosphorous, respectively. Supplementary material Table S1 shows the nutrient uptake (mg L^{-1}) from filtered TVs by each microalga growing under heterotrophic and mixotrophic regimes.

Biochemical characterization

Figure 3 shows the qualitative biomass characterization of each microalga cultured under the different growth regimes and using filtered TVs as culture medium showed different patterns with respect to cell content. At the end of the experimental time (15 days), FTIR spectra showed that *Chlorella* sp. accumulated mainly proteins when it was cultured in heterotrophic regime since the elevated peaks shown at 1645 and 1530 cm^{-1} belonged to vibrations of C=O and N–H bonds of amide I and II, respectively, associated with proteins [27] that were higher than those cultured in mixotrophy. Whilst under mixotrophic conditions this microalga mainly accumulated carbohydrates due to the elevated peak at 1020 cm^{-1} attributed to vibration of C–O–C bond related to carbohydrates [28] that was higher under this growth regime than heterotrophic condition as shown in Fig. 3a. In contrast, the spectra of *Scenedesmus* sp. showed that this microalga mainly accumulated proteins when it was growing in mixotrophic regime, but interestingly, it mostly accumulated carbohydrates under both conditions, see Fig. 3b. Similarly, the protein content increased when *Chlamydomonas* was cultured under heterotrophic regime whilst under mixotrophic condition, the carbohydrate content was boosted, as shown in Fig. 3c. Nonetheless, under our experimental conditions, the peaks corresponding to lipids were low in the three microalgae evaluated.

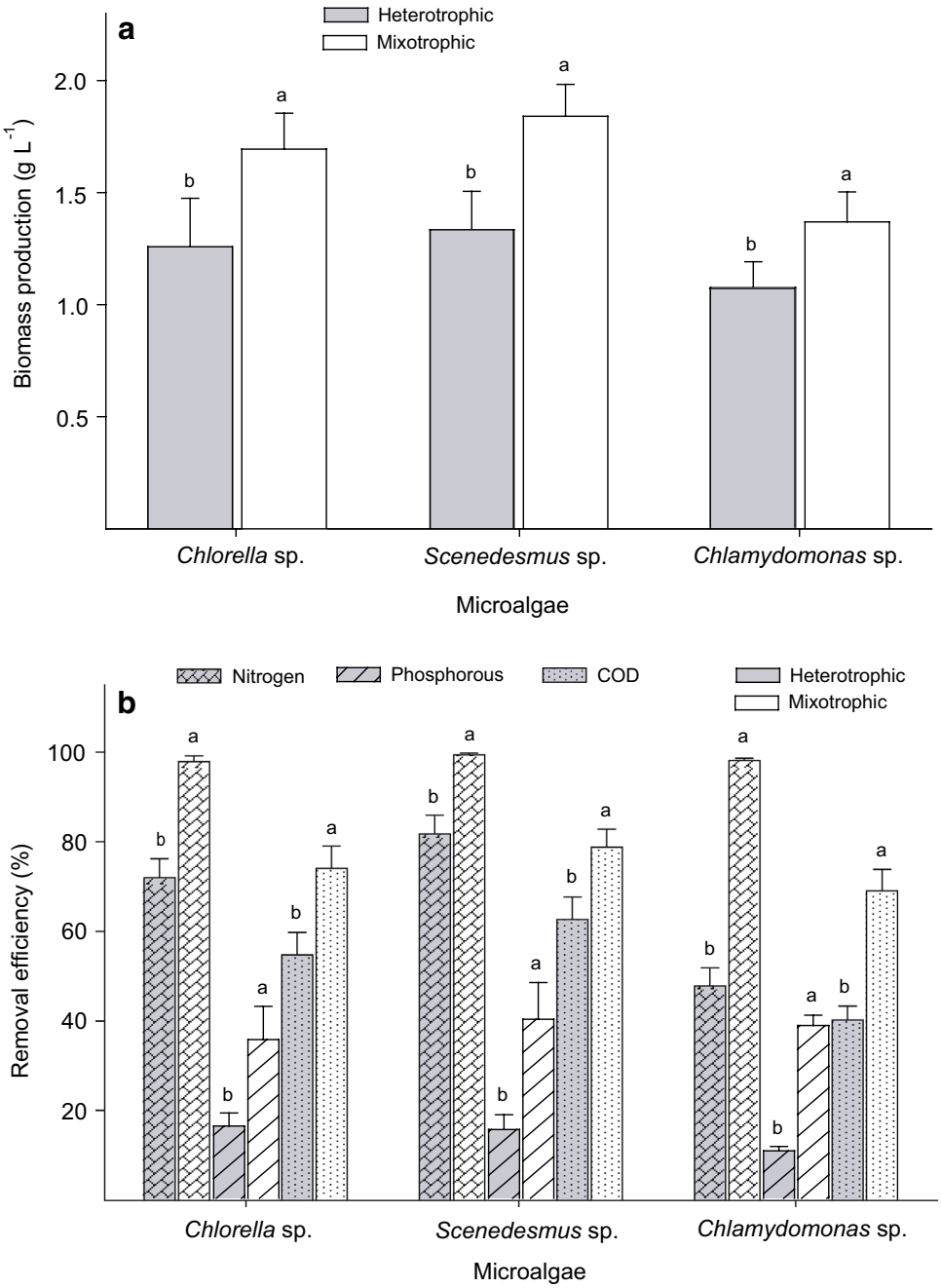


Fig. 2 Biomass production (**a**) and nutrient removal efficiency (**b**) by *Chlorella* sp., *Scenedesmus* sp., and *Chlamydomonas* sp. cultured in filtered tequila vinasses (TVs) under heterotrophic and mixotrophic growth regime. Columns denoted by different lowercase letters differed significantly when each microalga grew in different growth regimes. Statistical analyses were performed using analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis ($p < 0.05$). Bars represent standard error

On the other hand, the quantitative biomass characterization showed that under heterotrophic conditions the protein content (37.7 ± 4.3%) of *Chlorella* sp. was significantly higher than carbohydrates; however, when cultured under the mixotrophic regime, the carbohydrate content (37.4 ± 2.8%) showed significant differences with respect to proteins as shown in Fig. 4a, lowercase analysis. In contrast, the protein content (44.7 ± 4.8%) of *Scenedesmus* sp. was significantly higher than the carbohydrates growing under the mixotrophic regime whilst this latter compound significantly increased under heterotrophic conditions, 38.2 ± 3.9%, as shown in Fig. 4b, lowercase analysis. Similarly, *Chlamydomonas* sp. significantly increased protein content (41.4 ± 3.9%) under the heterotrophic regime although the carbohydrate content (40.6 ± 3.2%) was significantly higher than proteins under the mixotrophic regime, see Fig. 4c, lowercase analysis. Likewise, the highest carbohydrate and protein productivities recorded by each microalga were reached under the growth regime that induced the highest cell-compound accumulation, see Table 3.

Pigment content (*Chl a*, *Chl b*, and *Car*) produced by each microalga cultured under mixotrophy was also significantly

Table 2 Biomass productivity and growth rate of microalgae using filtered tequila vinasses (TVs) as culture medium under different growth regimes

Microalga	Growth regime	Growth rate (μ; d^{-1})	Biomass productivity (p; g $L^{-1}d^{-1}$)	pH
Chlorella sp.				
	Heterotrophic	0.3 ± 0.02b	0.07 ± 0.03b	5.9 ± 0.23b
	Mixotrophic	0.4 ± 0.01a	0.11 ± 0.02a	8.3 ± 0.11a
Scenedesmus sp.				
	Heterotrophic	0.3 ± 0.01b	0.08 ± 0.02b	5.7 ± 0.10b
	Mixotrophic	0.4 ± 0.01a	0.12 ± 0.02a	7.9 ± 0.08a
Chlamydomonas sp.				
	Heterotrophic	0.2 ± 0.01b	0.05 ± 0.02a	6.0 ± 0.13b
	Mixotrophic	0.4 ± 0.04b	0.08 ± 0.01a	8.2 ± 0.24a

Values denoted by different lowercase letters differ significantly when each microalga was cultured in a different growth regime. Statistical analyses were performed using analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis at $p < 0.05$; ± represents standard error

higher than growing in heterotrophy as shown in Fig. 5, lowercase analysis. Under mixotrophy, the *Chl a* content was 17.1 ± 0.2 (*Chlorella* sp.), 17.4 ± 0.4 (*Scenedesmus* sp.), and 13.7 ± 0.5 μg mL^{-1} (*Chlamydomonas* sp.), see Fig. 5a, whilst the *Chl b* accumulation attained was 10.9 ± 0.2, 9.8 ± 0.4, and 12.1 ± 0.4 μg mL^{-1}, respectively, showing also significant differences with respect to their heterotrophic growth as shown in Fig. 5a, b, lowercase analysis. Likewise, under this condition, the *Car* production recorded was 6.8 ± 0.2, 5.0 ± 0.6, and 6.1 ± 0.5 μg mL^{-1}, respectively, as shown in Fig. 5c, lowercase analysis. Likewise, the ratios *Chl a/Chl b* and *Chl/Car* of the three microalgae were significantly higher when they were cultured under mixotrophic than heterotrophic regimes, see Table 4.

Discussion

Several microalgae species can increase their biomass and metabolite production under heterotrophic and mixotrophic growth regimes from energy and nutrient content of wastewater [13, 15]. Thus, the aims of this study were to evaluate the biotechnological capacity to produce biomass and valuable metabolites of three indigenous microalgae, *Chlorella* sp., *Scenedesmus* sp., and *Chlamydomonas* sp., using filtered TVs as the nutrient source and cultured under heterotrophic and mixotrophic conditions.

Our results demonstrated that the three microalgae evaluated could grow in both growth regimes, but their higher cell density and biomass production were attained under the mixotrophic regime. These results were possible because of the physiological capacity of *Chlorella* sp., *Scenedesmus* sp., and *Chlamydomonas* sp. to assimilate and metabolize the nutrients and energetic compounds contained in filtered TVs under both conditions. In this study, under heterotrophic regime, each microalga assessed could assimilate almost completely the organic compounds contained in TVs to obtain energy and produce biomass (Supplementary material Table S2). In complete darkness, the carbon metabolism of microalgae depends first on their transport or diffusion systems of organic carbon through the plasmatic membrane, and subsequently assimilate it through the metabolic pathways of aerobic respiration for energy generation, biomass, and cell compound biosynthesis [13]. However, in this study, filtered TVs showed low concentration of organic compounds; thus, once these carbon sources exhausted, microalgae decreased their growth and nutrient assimilation. Nonetheless, higher biomass production obtained under the mixotrophic regime was due to the combination of light and organic carbon that reduced the dependence of microalgae on a single energy source and increased the carbon supply simultaneously through photosynthesis and respiration metabolism, inducing higher growth rates and biomass productivities than heterotrophic growth [15, 16]. These results explain the higher biomass productivities and COD removal efficiency by each microalga growing under this condition. By far, mixotrophy is considered the most efficient strategy to reduce organic matter and produce microalgal biomass from different industrial wastewater due to its physiological synergism [15–17]. For instance, *C. vulgaris* growing under mixotrophic regime reached higher biomass production (2.10 g L^{-1}) and growth rate (0.38 day^{-1}) from corn steep liquor than under the autotrophic regime [29]. Similarly, wastewater phycoremediation by *S. obliquus* also showed the highest biomass production (0.9 g L^{-1}) and growth rate (0.4 day^{-1}) of this microalga under mixotrophy [30]. The above indicates that the rich nutrient and energy content of TVs from *Agave tequilana* Weber var. Azul can be valorized as an alternative for developing microalgae bioprocesses. Recently, Tasic et al. [31] developed a bioremediation and ethanol fermentation bioprocess from anaerobically digested vinasses using *Chlamydomonas reinhardtii* CC-1093 which reached biomass productivity of 1129.2 mg L^{-1} day^{-1}. In another study, the biomass production of *C. vulgaris* increased proportionally

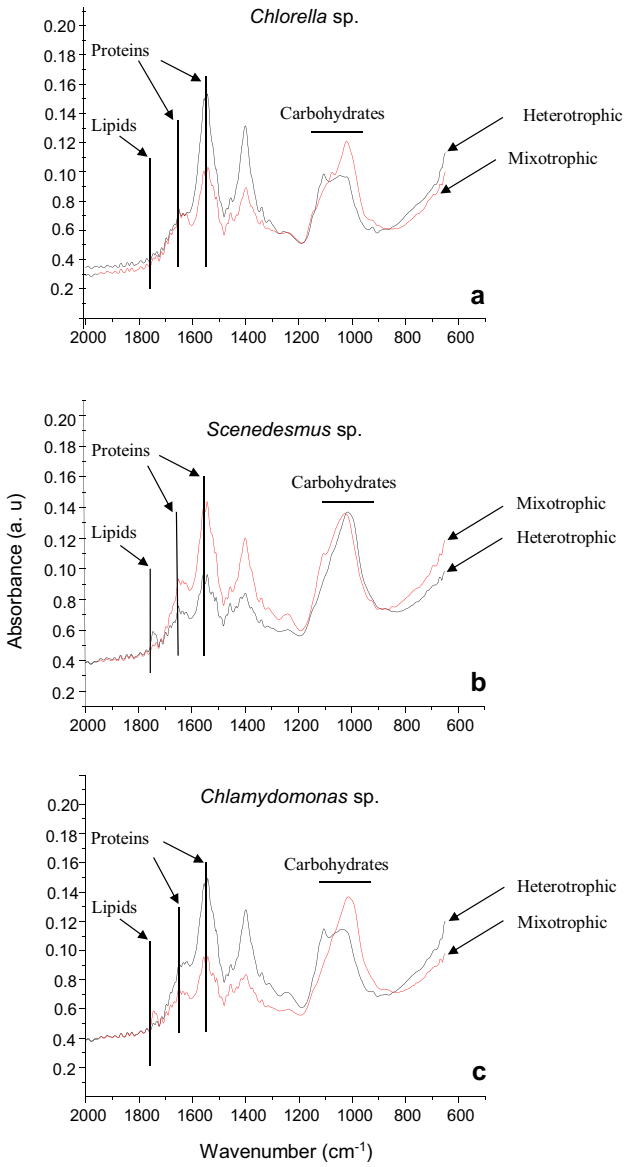


Fig. 3 Qualitative biomass characterization at the end of experimental time (15 d) of *Chlorella* sp. (**a**), *Scenedesmus* sp. (**b**), and *Chlamydomonas* sp. (**c**) cultured in filtered tequila vinasses (TVs) under heterotrophic and mixotrophic growth regime

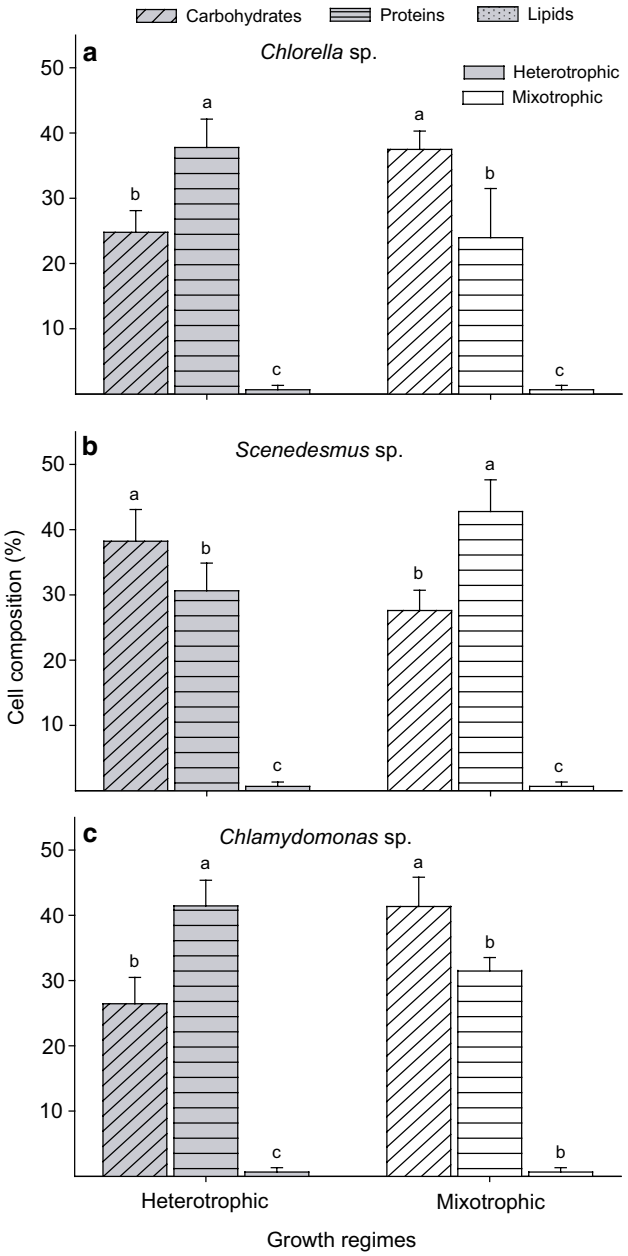


Fig. 4 Cell composition of *Chlorella* sp. (**a**), *Scenedesmus* sp. (**b**), and *Chlamydomonas* sp. (**c**) cultured in filtered tequila vinasses (TVs) under heterotrophic and mixotrophic growth regime. Columns denoted by different lowercase letters differed significantly in cell compound accumulation when each microalga grew in each growth regime. Statistical analyses were performed using analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis ($p < 0.05$). Bars represent standard error

to vinasse concentration reaching 255 mg L^{-1} with a load of 50% [32]. Candido and Lombardy et al. [33] demonstrated that under the mixotrophic regime *Chlorella* and *Desmodesmus* species were the most promising microalgae for diluted vinasse bioremediation reaching the highest growth rates, 1.5 and 1.2 day^{-1}, respectively.

According to Barclay and Apt [34], the most important factor in the successful development of bioprocess based on microalgae is selecting robust strains that grow rapidly from a residue and specific environmental conditions. In this regard, *Chlorella*, *Scenedesmus*, and *Chlamydomonas* genera are recognized to thrive in wastewater under different growth regimes [3, 4, 35]; they have the potential to endure the stressful conditions of these effluents because of their capacity to mediate anti-oxidative defense through the activities of various reactive oxygen species (ROS) scavenging enzymes, such as ascorbate peroxidase (APX; EC 1.11.1.11) [36]. In this study, the three microalgae were growing in

Table 3 Protein and carbohydrate productivity of microalgae using filtered tequila vinasses (TVs) as culture medium under different growth regimes

Microalga	Growth regime	Carbohydrate productivity (p_{Carb}; mg $L^{-1}day^{-1}$)	Protein productivity (p_{Prot}; mg $L^{-1}day^{-1}$)
Chlorella sp.			
	Heterotrophic	18.6 ± 4.3b	28.4 ± 5.6a
	Mixotrophic	41.8 ± 5.4a	26.7 ± 4.54a
Scenedesmus sp.			
	Heterotrophic	29.1 ± 6.4a	23.3 ± 5.6b
	Mixotrophic	32.6 ± 1.3a	50.6 ± 9.9a
Chlamydomonas sp.			
	Heterotrophic	14.5 ± 3.8b	22.8 ± 3.7a
	Mixotrophic	27.6 ± 2.5a	23.2 ± 5.2a

Values denoted by different lowercase letters differ significantly when each microalga was cultured in a different growth regime. Statistical analyses were performed using analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis at $p < 0.05$; ± represents standard error

100% filtered TVs without diluting since the choice of the microalgal strain also depends on the characteristics of the wastewater to be treated [4, 35]. Although raw TVs can contain phenolic compounds that inhibit microalgal growth, the filtration process with activated carbon—before experimentation—can remove or diminish the load of these compounds allowing the growth of these microorganisms from the nutrimental characteristics of this effluent, as reported previously by Candido and Lombardi [8] and Choix et al. [7]. Thus, the results in this study highlight the importance of not only selecting the appropriate microalga to grow from TVs but also determining its best culture strategy to maximize its physiological performance and using each agro-industrial residue efficiently.

As indicated above, the higher nitrogen and phosphorus removal efficiency from TVs by *Chlorella* sp., *Scenedesmus* sp., and *Chlamydomonas* sp. growing under mixotrophic rather than heterotrophic regime is due to the synergetic activity of photosynthesis and respiration metabolism maintained at constant growth and nutrient uptake [15, 16]. Under this condition, nitrogen was completely assimilated by the three microalgae at the end of the experimental period; this compound is essential for the synthess of amino acids and proteins vital to cellular machinery performing survival and ensuring tasks, such as light harvesting, photosynthesis, and energy generation in microalgae while phosphorous uptake (40%) is crucial to synthetize nucleotides, energy molecules as ATP, lipids, and polysaccharides [4, 35]; thus, the results indicated that the three microalgae evaluated were physiologically active from organic/inorganic carbon, as well as macro- and micro-nutrient content of filtered TVs under mixotrophy. Furthermore, this active metabolism of each microalga can be supported by the pH range (7.0–8.0) maintained during our experimental conditions; although pH is species specific, it usually ranges from 6.0 to 8.0 allowing nutrient availability and the activity of clue enzymes to assimilate them [12, 35]. In addition, the rich content of micronutrients (Fe, Zn, Ca, K) in filtered TVs performed an essential role on microalgal metabolism and the activity of key enzymes during carbon, nitrogen, and phosphorous assimilation [37]. The results in this study agree with numerous works reporting the high capacity of *Chlorella* sp. [29, 32], *Scenedesmus* sp. [17, 30], and *Chlamydomonas* sp. [31, 33] to bio-remediate different agro-industrial wastewater cultured in mixotrophy. Although, in this study, the highest biomass production obtained agreed with the highest nutrient removal efficiencies reached by each microalga evaluated, nutrient removal can be influenced by biotic or abiotic factors—microbial competition or temperature and alkaline pH (> 8.5), stimulating nitrogen volatilization or phosphorous precipitation—hindering precise determination of real nutrient assimilation by microalgae from wastewater [35]. Nonetheless, in this work microalgal growth was not surpassed by other microorganisms and no nutrient precipitation was observed during the experimental time (Supplementary material Figure S2). Thus, the nutrient uptake may be attributed to each microalga, confirming that the nutritional characteristics of TVs from blue agave are suitable to cultivate microalgae under mixotrophic conditions. Nevertheless, the effect of abiotic or biotic factors on nutrient uptake by microalga from filtered TVs will be assessed in depth in future studies.

Interestingly, the biochemical biomass composition of the three microalgae was composed mainly of carbohydrates and proteins although their proportion varied depending on the microalga and cultivation regime. The above was mainly attributed to the different metabolic pathways used to assimilate and metabolize carbon under each growth regime [13], as well as the nutrimental characteristics of each residue [3, 4]. Although biochemical composition varies among microalga species [3, 34] as well as culture conditions [38], the excess carbon and energy of filtered TVs could induce rapid carbohydrate production. This result can be explained because its biosynthesis is energetically lower than lipids

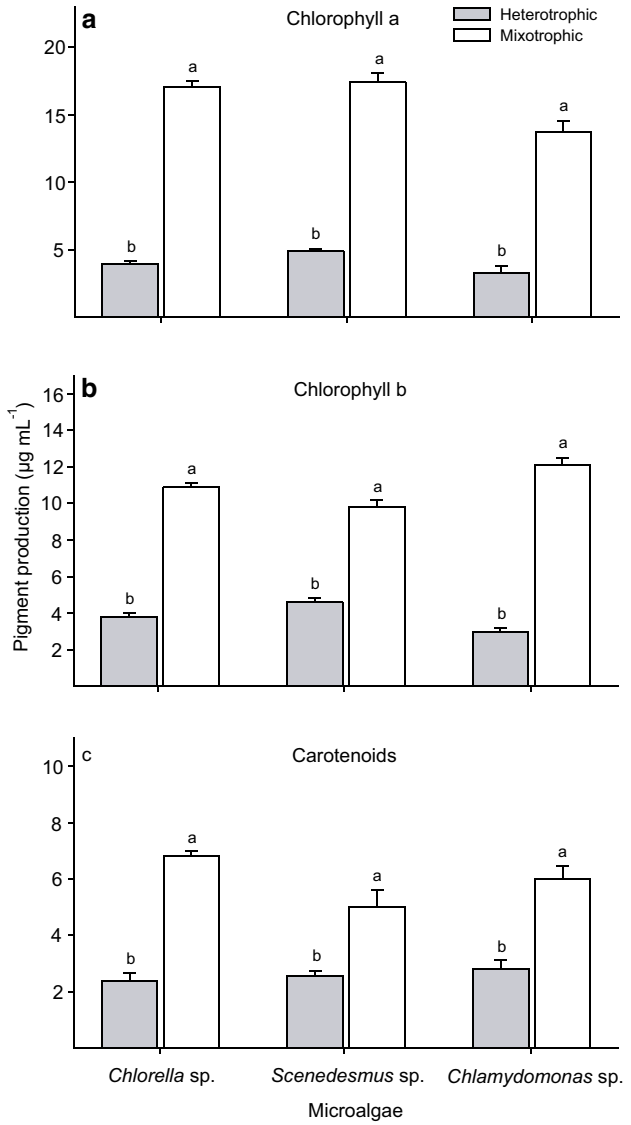


Fig. 5 Production of *Chlorophyll a* (**a**), *Chlorophyll b* (**b**), and carotenoids (**c**) of *Chlorella* sp., *Scenedesmus* sp., and *Chlamydomonas* sp. cultured in filtered tequila vinasses (TVs) under heterotrophic and mixotrophic growth regime. Columns denoted by different lowercase letters differed significantly in pigment production when each microalga grew in different growth regime. Statistical analyses were performed using analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis ($p < 0.05$). Bars represent standard error

[39] whilst nitrogen uptake and protein production indicate a high microalgal culture activity [40]. Earlier, Smith et al. [41] demonstrated that *Tetraselmis suecica* and *Cyclotella cryptica* changed their carbon allocation depending on the microalgal strain, carbon source, and heterotrophic or mixotrophic regime. In another study, Nzayisenga et al. [42] demonstrated that *Chlorella* sp. cultured in wastewater and supplemented with glycerol as carbon source accumulated mainly lipids (39.5%) under the heterotrophic regime, but

Table 4 Pigment ratio of microalgae from filtered tequila vinasses (TVs) under different growth regimes

Microalga	Growth regime	Pigment ratio	
		Chl a/Chl b	*Car/Chl a*
Chlorella sp.			
	Heterotrophic	1.0 ± 0.2b	0.3 ± 0.2b
	Mixotrophic	1.6 ± 0.2a	0.7 ± 0.1a
Scenedesmus sp.			
	Heterotrophic	1.1 ± 0.1b	0.3 ± 0.1b
	Mixotrophic	1.8 ± 0.1a	0.8 ± 0.1a
Chlamydomonas sp.			
	Heterotrophic	1.1 ± 0.2a	0.2 ± 0.2b
	Mixotrophic	1.4 ± 0.1a	0.8 ± 0.4a

Values denoted by different lowercase letters differ significantly when each microalga was cultured in different growth regimes. Statistical analyses were performed using analysis of variance (ANOVA) and least significant difference (LSD) post hoc analysis at $p < 0.05$; ± represents standard error

under autotrophic and mixotrophic growth, it produced mainly carbohydrates, 53.1 and 50.3%, respectively. On the other hand, higher *Chl a*, *b*, and *Car* contents under mixotrophy indicated that the photosynthetic activity of the three microalga under this condition induced pigment production due to light absorption whilst under heterotrophy it was repressed, explaining the higher pigment ratio of the three microalgae under mixotrophy. Earlier, Kong et al. [43] and Li et al. [40] also obtained the highest pigment ratio in *C. vulgaris* 31 and *Asterarcys* sp. SCS-188, respectively, cultured under mixotrophic rather than heterotrophic regime. Therefore, the results in this study confirm the importance of determining the suitable culture strategy for each microalga cultured in agro-industrial residues to induce the accumulation of high-valuable metabolites, making each bioprocess desired efficient.

Conclusions

Overall, the obtained results demonstrated the biotechnological capacity of native microalgae—mainly *Chlorella* sp. and *Scenedesmus* sp.—cultured under mixotrophy to produce biomass and valuable metabolites such as pigments, proteins, and carbohydrates from the nutritional characteristics of filtered TVs, but did not show the ability to produce lipids. Moreover, this study highlighted—from a perspective of circular bio-economy—the feasibility of implementing microalgal bioprocess to reuse and valorize the nutrimental composition of TVs through biomass and high-valuable metabolite production to obtain pigments, or animal feed supplements, depicting a sustainable strategy

for tequila agro-industry in Mexico. Finally, in a near future, a techno-economical study should be performed to cultivate microalgae from TVs at an industrial scale, develop a vinasse filtration process to a larger scale, and evaluate microalgal–bacterial consortium to improve the performance of these microalgae.

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Data availability The datasets generated during and/or analyses during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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