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Evaluation of the Light/Dark Cycle and Concentration of Tannery Wastewater in the Production of Biomass and Metabolites of Industrial Interest from Microalgae and Cyanobacteria

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Abstract: The tanning industry transforms animal skins into leather and produces liquid effluents with a high organic and inorganic pollutant load. This work evaluated the effect of the tannery wastewater (TWW) concentration and the light/dark cycle on the production of biomass, carbohydrates, proteins, lipids, and pigments (carotenoids and phycobiliproteins) on two microalgae (*Chlorella* sp. and *Scenedesmus* sp.) and one cyanobacterium (*Hapalosiphon* sp.). A non-factorial central experimental design with a response surface was implemented using the STATISTICA 7.0 software. High removal percentages for nitrates (97%), phosphates (73.3%), and chemical oxygen demand (93.2%) were achieved with the three strains. The results also highlight that the use of a constant light regime (24:0) and the concentration of real TWW affect the biomass production, since the highest concentration of biomass recorded was 1.31 g L^{-1} of *Hapalosiphon* sp. with 100% undiluted wastewater.

Keywords: microalgae; tannery effluents; carbohydrates; lipids; proteins

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1. Introduction

The leather manufacturing process is a widely developed activity. Asia and Latin America are the highest producers globally, producing 5067 and 2359.12 tons per year, respectively, followed by Europe with 2359, Africa with 1616, North America with 1230 tons, and Oceania with 585 tons per year. To process these, an average of 10–25 m^3 of clean water is used in the different stages of the tannery process. This process generates a considerable volume of untreated wastewater, which is finally discharged into other bodies of water [1]. These effluents contain a high concentration of contaminating organic matter due to the presence of particles of blood, meat, hair, soluble proteins, and fertilizer. Additionally, different salts are used in the transformation process mainly sulfur and chromium, which are used as tanning agents thus generating a high concentration of heavy metal pollutants such as Fe Cd, Cr, Pb, Zn, and Cu, these metals are not biodegradable and their discharge in lakes and rivers can produce bioaccumulation in living organisms [2]. At present, the physicochemical methods employed in this process are expensive and generate secondary pollutants. Coagulation has been widely used due to its ease of operation; however, this process may generate secondary wastes. Likewise, compounds such as aluminum sulfate and ferric chloride affect the removal of suspended solids, COD, and chromium up to 46%, 37%, and 99%, respectively, at optimum coagulant concentrations and optimum

pH values (7.5) [3,4]. Other processes, such as electrocoagulation, can remove up to 82% of COD [5,6]. These technologies improve the quality of tannery wastewater and allow the removal of pollutant compounds; However, these treatment systems have disadvantages due to their high production of toxic sludge, high operating cost, complicated management and limited use in developing countries [7,8].

Microalgae and cyanobacterium are renowned for their high photosynthetic efficiency and ability to generate different metabolites of interest, such as lipids [9], proteins [10], pigments [11], and carbohydrates [12]. Three key elements are needed to produce this microorganism: (1) a carbon source (usually in the form of CO_2 or inorganic form); (2) a culture medium with a sufficient nutrient concentration; and (3) a source of energy—in this case, light, which is known as photosynthetically active radiation [13,14]. According to various Life Cycle Analyses (LCAs) of microalgal production, the energy required to obtain the nutrients necessary to produce microalgae biomass is very high [15,16]. Therefore, knowing the technical and economic feasibility of the use of different types of wastewater (domestic and industrial) as alternative sources of N and P (especially NO_3^- and PO_4^{-3}) is crucial in order to increase the sustainability of the process and improve the added value of the algal biomass [17,18]. Several species belonging to the genera *Chlorella*, *Scenedesmus*, *Arkistrodesmus*, *Arthrospira*, and *Hapalosiphon* sp. have been tested for their ability to grow in different types of wastewater [1,19,20]. In these systems, algae transform hazardous nutrients into biomass which can be later used to extract other metabolites, making it an interesting biotechnological tool for use in wastewater treatment [21,22] Another essential characteristic of these microorganisms is their capacity to bioaccumulate heavy metals, which may increase their potential as a sustainable alternative for the treatment of tannery wastewater [23–25].

It has been demonstrated that some strains of *Chlorella* and *Scenedesmus* sp. possess tolerance to heavy metals and other pollutants present in tannery effluents due to the activation of antioxidant enzymes (superoxide dismutase, catalase, and ascorbate peroxidase) that protect them from oxidative damage induced by the presence of metals [26]. However, to the best of the author's knowledge there are no references regarding the treatment of raw tannery effluent as a culture medium by algae or cyanobacterium. This could be due to the presence of high concentrations of toxic pollutants and the dark color of the raw effluent, which prevents light from entering the medium; the latter limits the growth of microalgae and suggests the application of dilutions of wastewater to allow better adaptation [27,28] and the determination of the appropriate photoperiod [29] to allow the adequate growth of the microalgal biomass and in order to obtain metabolites of interest.

To the best of the author's knowledge, the literature on the utilization of tannery wastewater using microalgae is scarce. The use of microalgae consortia using tannery wastewater effluents after primary treatment has been reported [30]; similarly, the effect of light on the removal efficiency of *Scenedesmus* sp. has been reported for this type of wastewater [25]. To date, there have been no reports on the application of *Hapalosiphon* sp. in tannery wastewater; likewise, there have been no reports of the use of tannery wastewater for obtaining metabolites of interest from microalgae and *Hapalosiphon* sp. Therefore. this work is one of the first in this area. Currently, there is a gap in the literature regarding the influence of photoperiod and the concentration of tannery wastewater on biomass production and obtaining metabolites of interest (lipids, proteins, carbohydrates, phycocyanins, carotenoids, and phytohormones) from *Chlorella* sp., *Scenedesmus* sp., and *Hapalosiphon* sp. Therefore, this research evaluated the effect of the tannery wastewater (TA) concentration and the light/dark ratio on biomass production to find the optimal conditions for the extraction of lipids, carbohydrates, EPSs, carotenoids, proteins, and phycobiliproteins (PE, C-PC, and A-PC). We also evaluated the nutrient assimilation capacity of nitrogen and phosphorus and achieved a reduction in the pollutant load in terms of COD, BOD, and TOC.

2. Materials and Methods

2.1. Tannery Wastewater

The tannery wastewater was obtained from a business located in the city of Cúcuta (Norte de Santander, Colombia). The wastewater was evaluated regarding for its concentration of nitrates (NO_3), nitrites (NO_2), ammonia nitrogen (N-NH_3), phosphates (PO_4), chemical oxygen demand (COD), biochemical oxygen demand (BOD), fats and oils, color, and pH using standard methodology [31,32]. Heavy metals (Cr, Ni, Cd, Cu, Co, and Zn) were measured by atomic absorption spectrophotometry (ICE 3500, Thermo Scientific, Waltham, MA, USA) [31].

2.2. Microorganisms

Three strains from the INNOValgae collection (UFPS, Cúcuta, Colombia) were used. Two microalgae (*Chlorella* sp. and *Scenedesmus* sp.) and a cyanobacterium (*Hapalosiphon* sp.) were used. The inoculums were kept in Bold Basal medium for microalgae and in BG-11 medium for cyanobacterium. The culture conditions were: 12:12 photoperiod with a constant radiation of 110 µmol m^{-2} s^{-1}, 0.6 vvm air mixing, and 25 ± 2 °C.

2.3. Experimental Design

Tannery wastewater contains recalcitrant compounds that can inhibit the growth and production of metabolites of interest; likewise, the assimilation of carbon, nitrogen, and phosphorus compounds can be affected by the concentration in the effluent. To determine the effect of the concentration of tannery wastewater (TA) and the effect of the light/dark cycle, a central non-factorial experimental design was used with the STATISTICA 7.0 software, as shown in Table 1. Biomass was the main response variable, since the higher the biomass concentration was, the greater the capacity to assimilate the pollutants present in tannery wastewater was. Other response variables used were COD, TOC, nitrates, and phosphates.

Table 1. Experimental design for biomass production.

Experiment	Block	% TWW	Light/Dark Cycle
1	1	20	8
2	1	20	18
3	1	50	8
4	1	50	18
5 (C)	1	35	13
6	2	13.8	13
7	2	56.2	13
8	2	35	5.92
9	2	35	20.07
10 (C)	2	35	13

2.4. Biomass Production and Nutrient Removal

Prior to the inoculation of the strains, the tannery wastewater was autoclaved and its pH adjusted to 7.0. The different strains were cultured in 500 mL sterile glass flasks with a working volume of 250 mL of sample (30 mL of algal inoculum—equivalent to 0.1 g L^{-1} for *Chlorella* sp., 0.09 g L^{-1} *Scenedesmus* sp., and 0.103 g L^{-1} for *Hapasplosiphon* sp.). The flasks were kept in constant aeration with a flow of 0.6 vvm. The wastewater concentrations and the light/dark cycles were adjusted according to the experimental design.

To determine the biomass concentration (ash free), 5 mL was taken, filtered on GFC glass fiber filters and dried in an oven at 60 °C for 24 h on a silica gel bed. After drying, the filters were kept in the desiccator until they reached a constant weight (±2 h), then weighed [33]. Measurements of nitrates, phosphates, and COD were completed via the standard methodology [31]. For PO_4, the vanadomolybdophosphoric acid colorimetric method was used (SM-4500-P C). For nitrates, the ultraviolet spectrophotometric detection

method was used (SM-4500-NO_3-B). For COD, the closed reflux colorimetric method was used (SM-5220 D). The samples analyzed were taken in triplicate.

Total organic carbon (TOC) was determined using a TOC analyzer (Thermo Fisher Scientific). The operating conditions were a sample volume of 0.5 mL, water chase volume 1.0 mL, injection line rinse on, injection line rinse volume 0.5 mL, acid volume 0.5 mL, ICS parge flow 200 mL min^{-1}, carrier gas delay time 0.40 min, ICS parge time 50 min, detector sweep flow 500 mL min^{-1}, furnace sweep time 1.0 min, and system flow 200 mL min^{-1}.

2.5. Protein Extraction and Quantification

A filter with a known amount of biomass was submerged in a Falcon tube with 3 mL of 24% trichloroacetic acid (TCA) [34]. The sample was heated in a water bath at 95 °C for 15 min. A total of 9 mL of ultra-pure water was added and then centrifuged at 15,000× rpm for 20 min at 4 °C. The pellet was resuspended in 0.5 mL of Lowry D reagent and brought to 55 °C for 60 min in a water bath. Each tube was centrifuged twice at 4500 rpm for 15 min. A total of 175 µL of the supernatant was taken and 3325 µL of Lowry D was added. It was incubated 10 min at room temperature, 350 µL of Folin–Ciocalteu reagent was added, and finally it was left to rest for 30 min at room temperature before the absorbance at a wavelength of 600 nm was read [33]. To determine the concentration, a calibration curve was established in the range of 0 to 5000 ug L^{-1}; protein quantification was carried out according to Equation (1):

$$\text{Total protein (mg L}^{-1}\text{)} = (2038.5 * \text{OD 600}) + 59.706 \quad (1)$$

2.6. Carbohydrate Extraction and Quantification

A filter with a known amount of biomass was placed in a Falcon tube with 0.5 mL of 1 M H_2SO_4. This was homogenized in a vortex for 2 min, then 5 mL of 1 M H_2SO_4 was added and it was incubated in a water bath at 100 °C for 1 h. The sample was centrifuged at 4000 rpm at 5 °C for 10 min. A total of 2 mL of the supernatant was taken in a glass tube and 1 mL of 5% phenol was added. The mixture was stirred vigorously and 5 mL of concentrated H_2SO_4 solution was quickly added. The sample was shaken using vortex at medium speed for 1 min and allowed to rest at room temperature for 30 min before it was finally read at 485 nm [33]. To determine the concentration, a calibration curve was established in the range of 0 to 1.5 mg L^{-1}; the total carbohydrates were calculated using Equation (2):

$$\text{Total carbohydrates (mg mL}^{-1}\text{)} = (0.0116 * \text{OD 485}) + 0.0712 \quad (2)$$

2.7. Total Lipids Extraction and Quantification

A filter with a known amount of biomass was suspended in 100 µL of ultra-pure water and 2 mL of concentrated H_2SO_4, then heated at 100 °C for 10 min. A total of 5 mL of the freshly prepared Sulfo-Phospho-Vanillin (SPV) was added and incubated at 37 °C at 200 rpm for 15 min before it was finally read at a wavelength of 530 nm [35]. To determine the concentration, a calibration curve was established in the range of 0 to 1.5 mg L^{-1}; the analyses were performed in triplicate. The total lipids present in the sample were determined following Equation (3):

$$\text{Total lipids (µg)} = (\text{OD 530} - 0.0236)/0.0106 \quad (3)$$

2.8. Total Carotenoids Extraction and Quantification

Filters with a known amount of biomass were put in Falcon tubes. Then, 1 cm^3 of glass beads (0.5 mm) was added to each sample with 5 mL of ketone as a vehicle; then they were homogenized in a vortex at 100 rpm for 3 min and centrifuged at 4500 rpm for 10 min

at 4 °C. Finally, the supernatant was taken and measured at a wavelength of 450 nm [36]. The total carotenoid concentration was obtained using Equation (4):

$$\text{Total carotenoids (mg/mL)} = (\text{OD } 450 * \text{sample volume} * 10)/2500 \quad (4)$$

2.9. Phycobiliproteins Extraction and Quantification

The filters with a known amount of cyanobacterium biomass were put in Falcon tubes and suspended in 10 mL of 0.15 M phosphate buffer solution, pH 7.0, with 2 g of glass beads. Then, the samples were vortexed at maximum speed for 3 rounds of 2 min, letting the sample rest for 1 min. They were stored for 24 h, 4 °C, and then centrifuged at 3400 rpm for 15 min. Finally, the absorbance was measured at different wavelengths (620, 652, and 562 nm). The phycocyanin concentration was calculated using Equations (5)–(7), described by [37]:

$$\text{C-PC (g L}^{-1}) = \{[\text{OD } 620 - (0.474 * \text{OD } 652)]\}/5.34 \quad (5)$$

$$\text{A-PC (g L}^{-1}) = \{[\text{OD } 652 - (0.208 * \text{OD } 620)]\}/5.09 \quad (6)$$

$$\text{PE (g L}^{-1}) = \{[\text{OD } 562 - (2.41 * \text{C-PC}) - (0.849 * \text{APC})]\}/9.62 \quad (7)$$

3. Results and Discussion

3.1. Physicochemical Characterization of Tannery Effluents

Tannery wastewater is characterized by a dark brown color, a fetid odor due to the presence of volatile organic compounds, organic and inorganic carbon, phosphorus (P), nitrogenous compounds (N) [38–40], fats, and other compounds. These are highly polluting in terms of COD, BOD, and total dissolved solids (TDS). According to Table 2, the effluent has a high concentration of pollutant organic matter, BOD, COD, fats and oils, and total suspended solids, which do not comply with Colombian regulations for the discharge of wastewater.

Table 2. Physicochemical characterization.

Parameter	Units	Mean Value	Permissible Values RSL 631 (2015) of Colombia
Nitrates	mg L^{-1} NO_3	1641.00 ± 4.34	Analysis and report
Nitrites	mg L^{-1} NO_2	0.15 ± 0.0035	Analysis and report
Ammonia	mg L^{-1} N-NH_3	180.00 ± 2.4	Analysis and report
Total Nitrogen	mg L^{-1}	495.36 ± 5.28	Analysis and report
Turbidity	FAU	1120.00 ± 7.45	Analysis and report
Phosphate	mg L^{-1} P-PO_4	31.05 ± 0.67	Analysis and report
Total Phosphorus	mg L^{-1}	46.32 ± 1.06	Analysis and report
Color	m^{-1}	1300.00 ± 8.65	Analysis and report
COD	mg L^{-1} O_2	6720.00 ± 5.34	1200.00
BOD	mg L^{-1} O_2	4368.00 ± 2.34	600.00
pH	pH units	4.5 ± 0.1	6–9
Fats and oils	mg L^{-1}	387.42 ± 1.73	60.00
Total Suspended Solids (TSS)	mg L^{-1}	4960.56 ± 2.3	600.00
Settling Solids (SSOL)	mg L^{-1}	315.00 ± 1.51	2.00
Cr	mg L^{-1}	0.17 ± 0.002	1.5
Cd	mg L^{-1}	0.003 ± 0	0.05
Ni	mg L^{-1}	ND	N/A
Cu	mg L^{-1}	ND	N/A
Zn	mg L^{-1}	ND	N/A
Fe	mg L^{-1}	3.95	N/A

ND: not detectable; N/A: not available.

Goswami and Mazumder [41] reported the typical characterization of tannery wastewater, in which COD concentrations of 500–11,500 mg L^{-1} and total Kjeldahl nitrogen (TKN) of 200–550 mg L^{-1} were observed. Regarding pH, results of acidic pH between 3.4 ± 0.0351 and 5.96 ± 0.0351 [42–44] and basic pH between 8.0 ± 0.4 and 11.64 ± 0.53 have been reported [45–47]. In this work, the pH values were acidic, with values of 4.7 ± 0.12. Concerning TDS, typical values may be concentrations ranging between 2355 ± 85 mg L^{-1} and 10,000 ± 800 mg L^{-1} [48,49], which are very similar to the ones found in this work. For BOD, values in low ranges from 160 ± 15.8 mg L^{-1} to 1250 ± 38 mg L^{-1} have been reported [50], as well as values in high ranges that fluctuate between 1500 ± 41 mg L^{-1} and 6000 ± 30 mg L^{-1} [51]. As shown in Table 2, for this work similar results were found to those reported in the literature. Regarding the concentrations of nitrogen compounds, several authors have reported values between 129.69 ± 7.75 mg L^{-1} and 4000 ± 55.24 mg L^{-1} [52], which are similar to those obtained in this work (1641 mg L^{-1}). The high concentrations of ammonia nitrogen and nitrates are possibly due to the amount of protein residues and organic matter generated by the beamhouse and tanning operations [53]. In relation to heavy metals only, the presence of Cr (0.17 mg L^{-1}), Cd (0.003 mg L^{-1}), and Fe (3.95 mg L^{-1}) was detected; the remaining heavy metals were not detected. Concentrations of Cr have been reported in tannery wastewater in the range of 2–3500 ppm (1, 48.50); the high contents of heavy metals, mainly Cr and Cd, could be due to the excessive use of chemical compounds in the processes of the preservation of raw leather and the finishing of leather. Likewise, they could be due to the basic chromium sulfate used in the leather tanning process [54].

3.2. Biomass Production and Nutrient Removal

Figure 1 shows the biomass production, nitrates, phosphates, and COD and TOC removal of the treatments with the evaluated conditions that obtained the highest biomass production and pollutant removal, as well as the treatments with the lowest biomass production and pollutant removal for each strain. It can be seen in Figure 1 that the maximum biomass production values were obtained with the *Chlorella* sp. strain, 1.12 g L^{-1} (50% TWW and 18 h light), followed by 1.07 g L^{-1} (56.2% TWW and 13 h light) and 1.02 g L^{-1} (50% TWW and 8 h light). It is noteworthy that the higher the wastewater concentration and the greater exposure to light, the higher the values obtained were. For the *Scenedesmus* sp. strain, the highest biomass concentration was 0.59 g L^{-1} (56.2% TWW and 13 h light). The phosphorus and nitrogen metabolism in wastewater is directly related to the biomass production and metabolic activities [55]. Figure 1 shows nitrate removal with values ranging from 65% to 97% for the different microorganisms used. *Chlorella* sp. reached the highest removal rate (97%), while *Hapalosiphon* sp. obtained 94%. The percentage of elimination of the wastewater was directly proportional—that is, the higher the concentration was, the higher the percentage of elimination was. However, for *Scenedesmus* sp. it was inversely proportional; the lower the concentration was, the higher the percentage of elimination was. This behavior could be described by the ionic charge of the active sites on the surface of the microalgae due to changes in the pH and the concentration of wastewater, which can attract or repel metals and other contaminants in solution When the concentration of wastewater increases slightly, less pollutants are absorbed, due to competition for active sites in the cell wall for pollutant assimilation [26].

Regarding the phosphorus, the removal values (Figure 1) reached percentages ranging between 15 and 73% with the use of the three microorganisms. The cyanobacterium *Hapalosiphon* sp. achieved the highest levels of PO_4 consumption. It has been reported that phosphorus is an essential element for microalgae growth and performs many functions in cells. When it is in the form of phosphate (PO_4) the cell uses it to produce phospholipids, nucleic acids, and adenosine triphosphate (ATP); the latter os essential for all cellular processes [50], which explains the assimilation of this compound present in tannery wastewater by the strains studied. The COD removal percentages achieved in the three evaluated strains was higher than 85%, and although *Hapalosiphon* sp. presented the highest removal levels they did not differ much from *Scenedesmus* sp. and *Chlorella* sp. The COD level is

attributed to the growth rate and the photosynthetic activity of the microalgae strain [23]. According to the literature, effluents inoculated with microalgae had a greater reduction in COD [56], as can be seen in this work.

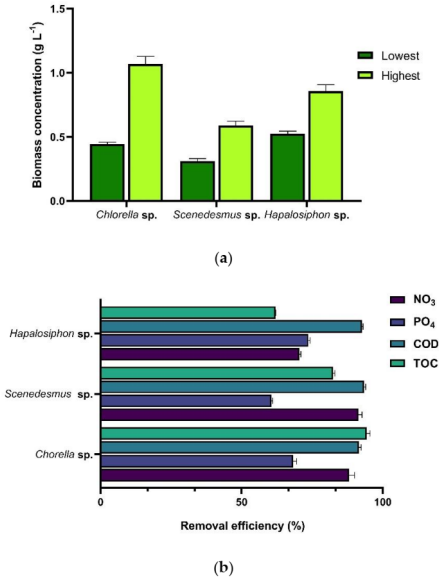


Figure 1. Biomass production (**a**) and removal of NO_3, PO_4, COD, and TOC (**b**) by *Chlorella* sp., *Scendesmus* sp., and *Hapalosiphon* sp.

Figure 2 shows the results of the experimental design for the biomass production of *Chlorella* sp., *Scendesmus* sp., and *Hapalosiphon* sp. regarding the TWW concentration effect and the light/dark cycle.

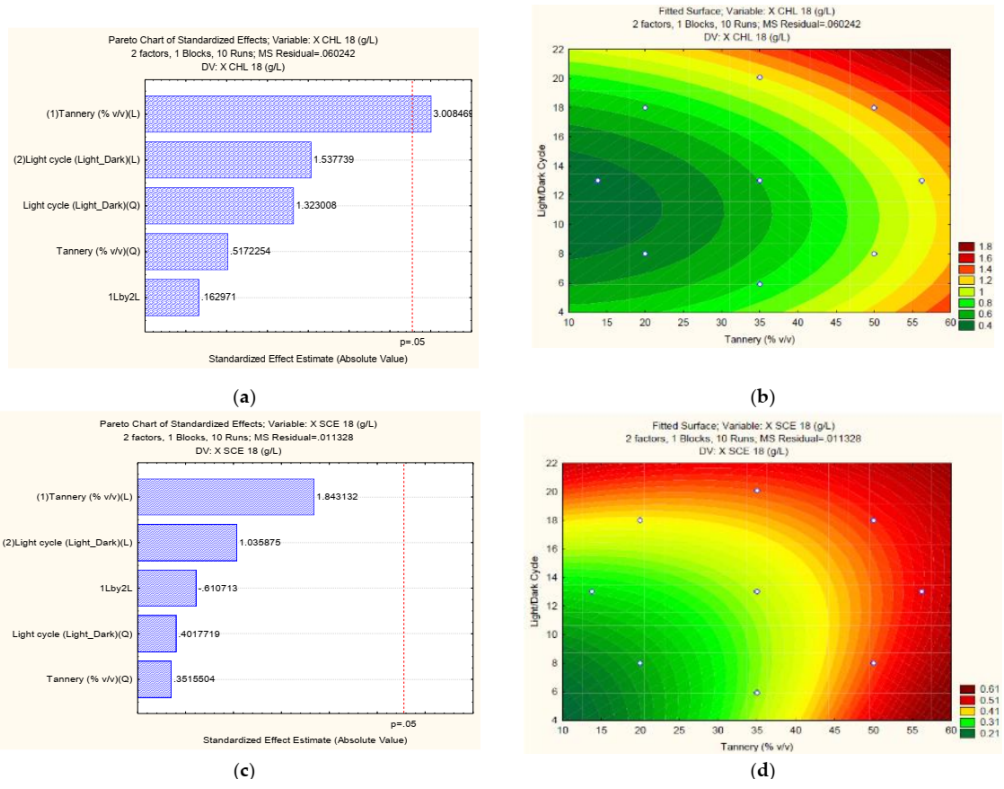
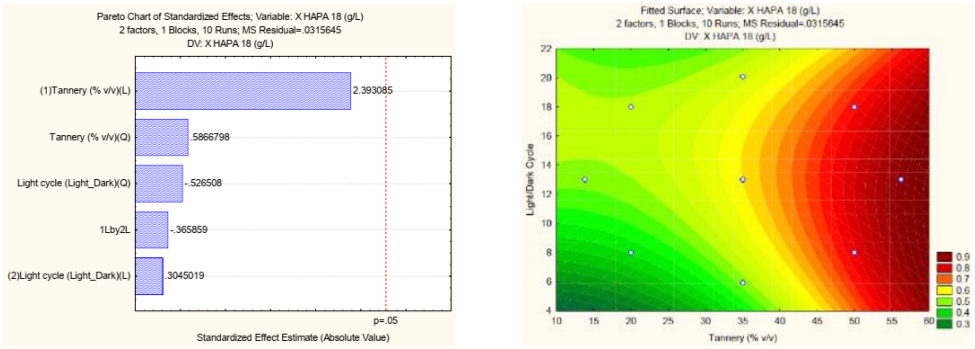


Figure 2. *Cont.*



(**e**) (**f**)

Figure 2. Pareto plot and response surface diagram in relation to biomass production and tannery wastewater. TWW concentration for *Chlorella* sp. (**a**,**b**) *Scenedesmus* sp. (**c**,**d**) *Hapalosiphon* sp. (**e**,**f**).

The response surface diagrams and the Pareto analysis of the experimental design (with a confidence >99.95) showed that, in the case of *Chlorella* sp., the dilution of the residual water affects the final biomass concentration, demonstrating that the higher the concentration of residual water (50%) is, the higher the biomass concentrations that can be reached are. For this same strain, it is evident that although the Pareto analysis shows that the light/dark cycle has no significant difference in each treatment, the surface diagram shows that the greater the exposure to light (20/4) is, the higher the biomass concentrations obtained are. In relation to *Scenedesmus* sp., it was found according to the Pareto analysis, that there are no significant differences for the concentration of residual water and light/dark cycle in the final biomass concentration, although according to the response surface diagram it is found that the higher the light/dark cycle (20/4) is, the higher the biomass concentrations that can be obtained are. Finally, for the case of *Hapalosiphon* sp. strain, the statistical analysis reported similar values to the *Scenedesmus* strain. The Pareto analysis shows that there are no significant differences for the variables evaluated, the surface diagram shows that the higher the concentration of residual water (50%) (with a light/dark cycle between 14/10 and 18/8), the higher the biomass concentrations obtained.

De Cassia et al. [27] evaluated the influence of light intensity and the TWW concentration on biomass production and nutrient removal by *Scenedesmus* sp. Their results show that with a higher TWW concentration (88.4% TWW) and light intensity (182.5 µmol photons m^{-2} s^{-1}), a higher biomass production was reached (0.90 g L^{-1}). Thus, a direct proportional relationship for biomass production was observed. Bellén et al. [50] reported the use of *Scenedesmus* sp. in three different wastewater dilutions from complete leather manufacturing (20%, 50%, and 100%). They found that the microalgae growth was directly proportional to the effluent concentration, with the elimination of chromium (>98%), nitrates (>90%), phosphates (>99%), and BOD (>88%). In this work, similar results to those reported in the literature were found for *Scenedesmus* and *Chlorella* sp.; however, it is important to indicate that there are no reports on the application of *Hapalosiphon* sp. in the removal of nutrients from tannery wastewater by evaluating the wastewater concentration and the light/dark cycle. The content of other compounds present in the wastewater also influences the removal efficiency of different pollutants present in tannery wastewater, as they compete for interaction with the functional groups of the microalgae. Likewise, it was found that the interactions of pH, COD concentration, strain used, and temperature also play an important role in biomass production using these waters [23,56]. Positive results have been reported [40] for *Scenedesmus* sp. using a central composite design, demonstrating that wastewater concentration and light intensity influence the amount of biomass produced and the removal of nitrogen and phosphorus. In this study, concentrations of wastewater of between 20% and 100% were used, and removals of 65% to 97% for nitrogen in the form of nitrate, 76.58% for phosphorus, 85% to 93% for COD, and 60.53% to 95.06% for TOC were found.

3.3. Culture Optimization Using Tannery Wastewater

For biomass optimization, the results of the response surface design were used using the equations provided by Statistica 7.0 software. The equations were solved by substituting the values of photoperiod and dilution of the wastewater evaluated, obtaining the optimum photoperiod for each species, and evaluating three concentrations of tannery wastewater (50%, 75% and 100%). The light/dark cycle for the strains were 24/0 for *Chlorella* sp. and *Scenedesmus* sp. and 18/6 for *Hapalosiphon* sp. Table 3 shows the biomass production, growth rate (μ), and nutrient removal for the three TWW concentrations evaluated and the control; the control is a BOLD and BG11 medium where the organisms evaluated grow normally. The operating conditions were 1 vvm aeration, 500 mL photobioreactors, with an operational volume of 300 mL, pH 7 ± 0.2, and temperature of 28 °C ± 2 °C.

Table 3. Optimal culture for biomass production and nutrient removal.

Strains	Response	% TWW			
		50	**75**	**100**	**CONTROL**
***Chlorella* sp.**	Biomass (g L^{-1})	0.9 ± 0.1	1.06 ± 0.02	1.08 ± 0.0	0.41 ± 0.03
	NO_3 (%)	73.6 ± 0.84	78.0 ± 1.73	82.3 ± 1.11	98.6 ± 0.16
	PO_4 (%)	53.1 ± 1.17	59.9 ± 2.65	64.8 ± 4. 27	98.2 ± 0.02
	μ (d^{-1})	0.0876 ± 0.0001	0.0225 ± 0.0006	0.0197 ± 0.005	0.09 ± 0.0003
	Cr (mg L^{-1})	0.07	0.02	0	0
	Cd (mg L^{-1})	0	0	0	0
	Fe (mg L^{-1})	1.1	0.6	0.01	0
***Scenedesmus* sp.**	Biomass (g L^{-1})	0.71 ± 0.15	0.89 ± 0.10	1.03 ± 0.16	0.30 ± 0.12
	NO_3 (%)	84.4 ± 0.24	85.1 ± 1.20	88.1 ± 0.27	98.9 ± 0.33
	PO_4 (%)	54.2 ± 2.92	55.5 ± 2.75	61.9 ± 4.45	98.6 ± 0.016
	μ (d^{-1})	0.0187 ± 0.00034	0.0236 ± 0.0012	0.0156 ± 0	0.1 ± 0.001
	Cr (mg L^{-1})	0.9	0.06	0.01	0
	Cd (mg L^{-1})	0	0	0	0
	Fe (mg L^{-1})	0.7	0.1	0	0
***Hapalosiphon* sp.**	Biomass (g L^{-1})	0.65 ± 0.76	1.18 ± 0.18	1.31 ± 0.23	0.31 ± 0.016
	NO_3 (%)	70.1 ± 19.15	87.9 ± 1.77	90.0 ± 0.87	91.1 ± 0.19
	PO_4 (%)	49.5 ± 1.17	52.2 ± 2.09	54.1 ± 5.60	92.4 ± 0.01
	μ (d^{-1})	0.0436 ± 0.0003	0.0352 ± 0.0005	0.035 ± 0.0001	0.039 ± 0.0003
	Cr (mg L^{-1})	0.8	0.1	0.03	0
	Cd (mg L^{-1})	0	0	0	0
	Fe (mg L^{-1})	0.3	0.05	0	0

The results in Table 3 show that the cultures with the highest TWW concentration (100% TWW) have the highest values of biomass produced. *Chlorella* sp. produced 1.08 g L^{-1}, while *Scenedesmus* sp. produced 1.03 g L^{-1}. In comparison, their control reached 0.41 ad 0.30 g L^{-1}, respectively. Finally, *Hapalosiphon* sp. produced 1.31 g L^{-1}, while its control reached 0.31g L^{-1}. For each of the concentrations evaluated, it was found that the biomass production was higher compared to the controls. The results in relation to the growth rate show that the strain of *Hapalosiphon* sp. reached the highest growth rate, which was even higher than that of the control. It was possible to demonstrate how the cyanobacterium managed to grow rapidly regardless of the high coloration of the medium. In relation to the strains of *Scenedesmus* sp. and *Chlorella* sp. the growth rates were very similar and lower than those of the control. The different toxic compounds in high concentrations and the dark color of the effluent, the product of the different compounds used during the tanning process, can inhibit or minimize growth. Likewise, this prevents the entry of light into the medium and limits the growth of microalgae and cyanobacterium [1,57], which explains the growth rates achieved by the strains of *Chlorella* sp. and *Scenedesmus* sp.

For nutrient consumption, the highest removals were achieved in cultures with a concentration of 100% TWW for the three strains evaluated. Regarding nitrate and phosphate, the three strains reached NO_3 removal percentages of 82.3%, 88.1%, and 90%, respectively,

and PO_4 removal percentages of 64.8%, 61.9%, and 54.1%, respectively. In comparison with the controls, it was observed that the nitrogen consumption was higher, while the phosphate consumption was lower than that of the control. This may be due to the physicochemical characteristics of the wastewater, which may decrease the assimilation capacity of these compounds [51]. However, it was found that it does not significantly affect the growth of the strains evaluated in this type of effluent. Studies with *Chlorella* vulgaris showed significant removals of COD, NO_3, and PO_4 of 94.74%, 100%, and 91.73%, respectively, between days 6 and 21 of culture [39]. In strains of *C. vulgaris* and *Pseudochlorella* pringsheimii, significant reductions in the concentration of pollutants were observed, higher than 65% for NH_3-N, 100% for PO_4, and 63% for COD. It should be emphasized that these results were achieved with a dilution of up to 30% of the wastewater [58]. Regarding *Hapalosiphon* sp., to the best of the authors' knowledge there are no reports in the literature on the cultivation of this organism in tannery waters, this being one of the first works.

3.4. Metabolites Production

Figure 3 shows the metabolite production percentage by *Scenedesmus* sp., *Chlorella* sp., and *Hapalosiphon* sp. in undiluted TWW. For the metabolite production and extraction, the three strains were cultivated with 100% TWW. It was found that *Chlorella* sp. produced the highest percentages of protein (35.38%), carbohydrates (42.94%), and carotenoids (0.35%). On the other hand, *Hapalosiphon* sp. produced the highest lipid percentages (23.33%) and an interesting concentration of phycobiliproteins such as A-PC (4.6%), C-PC (3.81%), and PE (2.51%). The protein content in microalgae varies from 20% to 70% depending on the species and the culture parameters and exhibits an amino acid profile that is suitable for human nutrition. In addition, these substances are currently being extensively researched in relation to their techno functional potential to stabilize fluid interfaces as well as their potential to satisfy the global demand for proteins. Furthermore, they can be functional ingredients such as bioactives or bio-based dyes, thus providing added value to the microalgae biorefinery [53]. Fertilizers based on microalgae and cyanobacterium have been studied to incorporate sustainability both in the recovery of soils and in the reduction in the amount of chemical fertilizers used [55]. In this work, it was found that the evaluated strains can grow using the TWW as a culture medium and generate metabolites with potential use as biofertilizers. The carbohydrate percentages obtained from each strain were greater than 25%, allowing the potential use of the biomass as a carbon source for microbial cultures to obtain biofuels [56]. Saranya and Shanthakumar [28] obtained a biomass concentration of 3.51 g L^{-1} using tannery wastewater (30% concentration) for *C. vulgaris*, but the highest amount of lipids produced (9.3%) was obtained from a 20% concentration. In this work, the microalgae *Chlorella* sp. with TWW without dilution—that is, at 100%—obtained a lipid percentage of 21.04%, thus evidencing a higher production compared to the work previously mentioned.

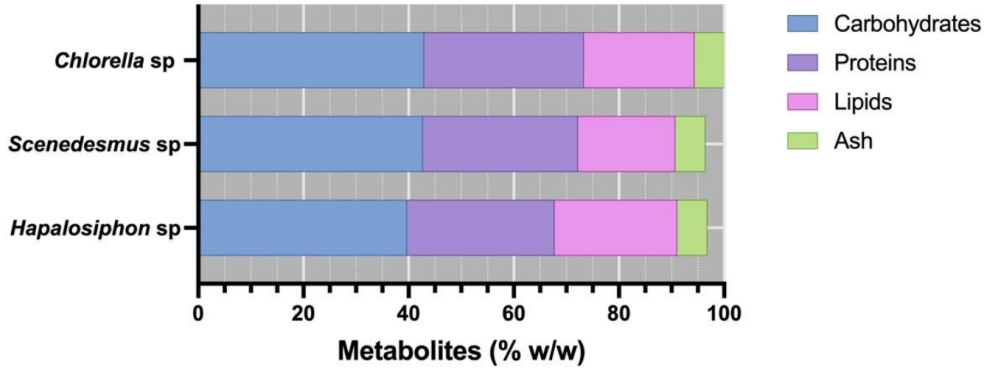


Figure 3. Metabolites produced by *Scenedesmus* sp., *Chlorella* sp., and *Hapalosiphon* sp.

The metabolite production rates between microalgae and cyanobacterium differ mainly due to the metabolic characteristics of these organisms. The central carbon metabolism of

microalgae and the biochemical composition differ between microalgae such as *Chlorella* or *Scenedesmus* and cyanobacterium such as *Hapalosiphon*, since microalgae are eukaryotic organisms and cyanobacterium are prokaryotic [57]. The physicochemical characteristics of wastewater affect the growth of these organisms, and the presence of emerging contaminants can affect the growth and the accumulation. It has been reported that the thermodynamics of adsorption are determined by the physicochemical characteristics of the surrounding conditions, such as pH, temperature, and redox potential [58]. If there are a wide range of contaminants present in the wastewater, this can lead to the saturation of the binding sites with contaminants, generating the inhibition or slight accumulation of metabolites [59]. Likewise, aspects such as irradiation affect metabolite production. During irradiation periods, photosynthesis produces carbohydrates that are consumed in respiration during dark periods. In general, irradiation periods are crucial for carbohydrate localization and content; the effects are different between microalgae and cyanobacterium strains. Therefore, carbohydrate accumulation can occur with particular irradiation regimes for each strain or species of microalgae and cyanobacterium [60]. The results obtained demonstrate the capacity of the organisms evaluated to reduce most of the highly environmentally polluting compounds present in the tannery wastewater used in this work, allowing the use of these effluents for the cultivation of microalgae and cyanobacterium and obtaining metabolites with potential use as biofertilizers. The low concentration of heavy metals in the wastewater and the productivities obtained allow us to continue advancing our studies for the optimization and use of the biomass generated. The use of microalgal biomass generated from the bioremediation process of tannery effluents to obtain lipids with potential use as biofuels has been reported, [61]. The success of the commercial application of these biofuels is associated with the biorefining of other metabolites such as pigments or renewable polymers that can also be used [62]. Some authors have described the behavior of microalgae in the biosorption of heavy metals; the ionic charge of the active sites of the membranes of these organisms is an important aspect of this process and depends to a great extent on the pH. At low values, the carboxyl, hydroxyl, and sulfonate groups remain free and can therefore attract metallic cations chelating the heavy metals [63]. This aspect could be a sustainable ecological strategy that could improve the productivity of the process by recovering chromium for reuse in the tanning process, promoting sustainable economic development by obtaining a metal-free biomass that would allow diversifying its use in substances other than biofuels. [64].

4. Conclusions

This work demonstrates that *Chlorella*, *Scenedesmus*, and *Hapalosiphon* sp. can efficiently grow in wastewater from tanning processes. When the wastewater concentrations percentages were higher and the light/dark cycles more intense, high removal percentages of inorganic nutrients such as NO_3 and PO_4 were obtained. The removal values were higher than 90% for the three strains studied. Due to the higher wastewater concentration, there were more nutrients available in the medium and the microorganisms could thrive and grow, thus obtaining a higher biomass production. It can be seen in the experiments with a TWW concentration percentage of 100% that the microorganisms were able to metabolize the nutrients and produce value-added metabolites with a high percentage of content within the biomass. The *Chlorella* sp. presented the highest metabolite production values. Thus, it is concluded that the residual water from tanneries can be used as a source to produce metabolites of commercial interest.

For *Chlorella* and *Scenedesmus* sp., it can be concluded that the light/dark cycle in which they grow and assimilate the nutrients present in the tannery wastewater is 24/0 h. On the other hand, this work shows that the optimal light time for *Hapalosiphon* sp. is between 16 and 20 h. Regarding the wastewater concentration and microorganism growth, it is not necessary to decrease its concentration since the three strains adapted and managed to grow in undiluted water. This represents an environmental advantage by avoiding the use of water to reduce the pollutant load.

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