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Enhancement of Biomass and Lipid Productivities of *Scenedesmus* sp. Cultivated in the Wastewater of the Dairy Industry

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Abstract: Microalgae are photoautotrophic microorganisms capable of producing compounds with potential bioenergetic applications as an alternative energy source due to the imminent exhaustion of fossil fuels, their impact on the environment, and the constant population increase. The mass cultivation of these microorganisms requires high concentrations of nutrients, which is not profitable if analytical grade culture media are used. A viable alternative is the use of agro-industrial wastewater, due to the metabolic flexibility of these microorganisms and their ability to take advantage of the nutrients present in these substrates. For the reasons mentioned above, the effect of the cultivation in wastewater from cheese processing on the growth parameters and biomass composition of *Scenedesmus* sp. was evaluated, and its nutrient removal capacity determined. A high lipid concentration was obtained in the cultures with the dairy effluent (507.81 ± 19.09 mg g^{-1}) compared to the standard culture medium, while the growth parameters remained similar to the control medium. *Scenedesmus* sp. achieved high percentages of nutrient assimilation of the wastewater used (88.41% and 97.07% for nitrogen and phosphorus, respectively). With the results obtained, the feasibility of cultivating microalgae in agro-industrial wastewater as an alternative culture medium that induces the accumulation of compounds with potential bioenergetic applications was verified.

Keywords: *Scenedesmus* sp.; microalgae; wastewater; lipid production; nutrient removal

1. Introduction

Fossil fuels are hydrocarbons generated millions of years ago that appear in solid (coal), liquid (oil), or gaseous (natural gas) forms, which come from the decomposition of organic matter [1,2]. These molecules are responsible for 86% of the energy produced worldwide [3]. However, they are the primary source of greenhouse gas emissions [4], making their use unsustainable. Due to their environmental impact, imminent depletion (40–60 years), and the increase in world energy demand (↑50% by 2030), other sustainable energy sources are being sought, such as solar, tidal, and biofuels, among others, considered as clean energies [5–7].

The latter comprise any fuel derived from biomass or its residues such as plants, animals, or microorganisms. Biofuels emerge as a profitable alternative to the regular consumption of fossil fuels; the most developed are bioethanol, biodiesel, and biogas, whose consumption reduces carbon emissions by between 30% and 50%. Biomethanol, bioethers, synthetic biofuels, biohydrogen, and vegetable oils are used less frequently [8]. The use of biofuels for energy production has many advantages; however, only 10% of the energy is obtained from biomass and waste [9]. These bioenergetic compounds include liquid, solid, and gaseous fuels [8] classified according to the source of production in the first, second, third, and fourth generation.

First-generation biofuels are produced from food or edible vegetable oils (>90% of the biodiesel produced worldwide) such as rapeseed, soybeans, sunflower, and palm, which are readily available crops. However, their mass production is not profitable due to the high costs of biodiesel production and competition with other important food crops for land use [10]. Second-generation biofuels are produced from agricultural residues (residual vegetable oil, straw, rice husk), forestry (wood, wood chips), and industrial and non-food crops, which require less land and water and reduce the problems of deforestation. Despite this, they are not abundant enough to cover world energy demand [11]. Third-generation biofuels are produced from algae and microorganisms such as bacteria, yeasts, cyanobacteria, and microalgae, which have higher yields than other biofuels (10 times more) and help in the consumption of atmospheric CO_2, reducing environmental problems [12]. Biofuels produced by algae are profitable due to their high-efficiency rates (yield greater than 98,000 L ha^{-1} of biodiesel), they can supply a large part of the energy demand, they do not compromise food security, can be grown quickly (high growth rates), they are not toxic, and their cultivation does not need arable land [7,13].

Finally, fourth-generation biofuels result from the modification of photosynthetic microorganisms based on metabolic engineering, allowing easy extraction of microbial products, simplifying production stages, cost reduction, and performance improvement. Currently, the genetic manipulation of these microorganisms, such as microalgae, seeks a directed improvement of cellular activity that allows the generation of added value by-products, increasing the profitability of the process [14,15]. Microalgae have been prioritized as potential biofuel producers because they achieve yields between 15 and 300 times more than a traditional agricultural crop [7].

Microalgae can present with a eukaryotic cell organization (Chlorophyta, Bacillariophyta, Dinoflagellata) or prokaryote (Cyanophyta); however, cyanobacteria are not strictly within this classification, but they are considered for their high economic value. Microalgae can capture light energy and use it in organic molecules such as proteins, carbohydrates, or lipids through photosynthesis [16,17]. The cultivation of these microorganisms presents excellent advantages, such as high growth rates, high productivity, no requirement of agricultural land for their cultivation, short harvest cycles, ease of cultivation (few nutritional, environmental, and operational requirements), high lipid content, and high photosynthetic efficiency (4 times higher than plants). They are an inexhaustible-renewable resource, among others [18,19]. They also fulfill ecological functions such as global carbon sequestration (1.8 kg of CO_2 kg^{-1} of dry biomass), fixation of atmospheric nitrogen, and production of more than 50% of global oxygen [17–19].

These microorganisms constitute a potential source of biomass for the production of different types of biofuels. In addition, they contain between 20% and 50% lipids (under stress conditions) in terms of the dry weight of the biomass [20]. Microalgal biomass production is assumed to be between 15 and 25 t ha^{-1} $year^{-1}$, which would correspond to 4.5 and 7.5 t ha^{-1} $year^{-1}$ of lipid production (cellular lipid percentage of 30%) [21]. In this way, current studies seek to intensify the growth rate and production of lipids. Microalgae, under stress conditions, can accumulate lipids and carbohydrates in higher concentrations, decreasing protein synthesis [22]. Some of the stress conditions evaluated are limitation of nutrients such as N, P, or S (C becomes triglycerides or fatty acids), the addition of metal ions (Fe^{+3}, Mg^{+2}, Ca^{+2}), the addition of salts (EDTA), increase or decrease in light intensity, temperature, pH, and radiation [23,24].

The cultivation of these microorganisms on a large scale to obtain various metabolites of commercial interest generates a high demand for freshwater and nutrients, usually supplemented by chemically synthesized fertilizers due to the lower cost they present in relation to nutrients from formulated cultivation. However, industrial production with fertilizers is not profitable (>\$400 t^{-1}) if the costs of biomass production and processing are taken into account in the production of biofuels [25]. The use of agro-industrial wastewater emerges as a viable alternative in the cultivation of microalgae and cyanobacteria due to their outstanding capacity to adapt to various water bodies as sources of fresh, marine, brackish, and waste water, among others [26,27]. In this way, microalgae cultivation in

wastewater is a profitable, highly productive, and ecological process (decreased eutrophication that causes low oxygen levels and contamination in freshwater bodies) [28,29].

Due to the advantages mentioned above, the cultivation of *Scenedesmus* sp. in wastewater from dairy industries is proposed in this study, evaluating mainly the production of lipids and carbohydrates. The growth, productivity, and biochemical composition of the biomass and the removal of nutrients by *Scenedesmus* sp. will also be determined.

This investigation was carried out to induce an increase in the lipid and carbohydrate content in the biomass of the microalgae *Scenedesmus* sp. using wastewater as a cultivation substrate for the production of metabolites of bioenergetic interest, and at the same time cleaning it, avoiding the serious environmental problems caused by wastewater being directly discharged into the water bodies.

2. Materials and Methods

2.1. Microorganism and Wastewater Characteristics

The microalgae *Scenedesmus* sp. (Figure 1) evaluated in this research was obtained from the Bank of microalgae and cyanobacterial strains of the Laboratorio de Biotecnología Microbiana (BiotemLab) of the Universidad de Guayaquil (Universidad de Guayaquil, Guayaquil, Guayas, Ecuador), coded as RJ 3009 and conserved in solid BG11 standard medium [30] at a temperature of 20 ± 2 °C, light intensity of 100.0 µmol photon m^{-2} s^{-1}, and photoperiod 12 h light/12 h dark. Cultures of *Scenedesmus* sp. were acclimated to dairy industry wastewater by gradually adding increasing concentrations of cheese processing wastewater over several months, with an initial concentration of 10%.

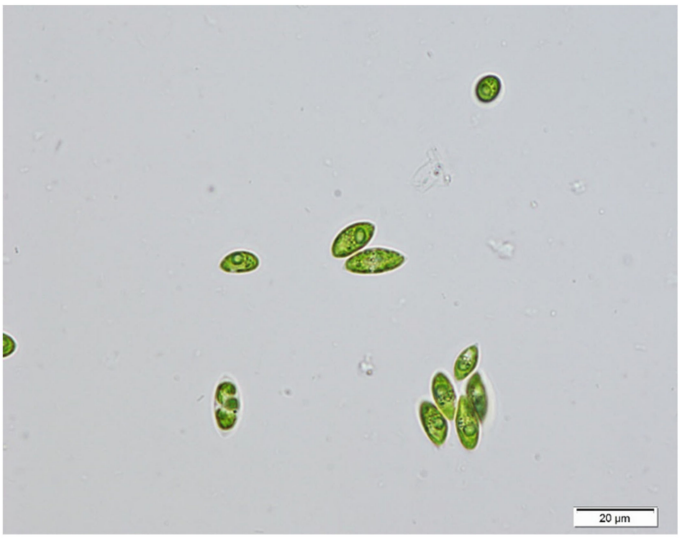


Figure 1. Light microscopic images (60x) of *Scenedesmus* sp.

The wastewater used as a culture medium was obtained from a dairy industry that produces cheeses and lactic derivatives. The dairy industry wastewater used in this research (DIWW) had the following characteristics: 2.22 mM total nitrogen (TN), 0.20 mM phosphate, 5.5 pH, 3500 mg O_2 L^{-1} chemical oxygen demand (COD), and heavy metal concentrations less than 0.09 mg L^{-1}, as shown in Table 1.

Table 1. Chemical characteristics of the dairy industry wastewater (DIWW).

Parameter	Value
TN	2.22 mM
PO_4^{3-}	0.20 mM
pH	5.5
COD	3500 mg O_2 L^{-1}
As	0.0060 mg L^{-1}
Cd	0.00047 mg L^{-1}
Hg	0.0890 mg L^{-1}
Pb	0.0001 mg L^{-1}

2.2. Culture Conditions

Scenedesmus sp. was cultivated in batch in triplicate in 150 mL photobioreactors (PBR) under a circadian illumination regime of 12 h light/12 h dark with a temperature of 30 °C (Figure 2), light intensity of 100.0 µmol photon m^{-2} s^{-1}, and a continuous supply of CO_2 pulses (1.5% *v*/*v*) to keep the pH stable (7.2 ± 0.2) and as a carbon source [31]. The cultures were inoculated with a concentration of 1.5×10^7 cell mL^{-1}.

For the control, the culture medium BG11 with the following composition was used: $NaNO_3$ 17.65 Mm, K_2HPO_4 0.23 mM, $MgSO_4{\cdot}7H_2O$ 0.30 mM, $CaCl_2{\cdot}H_2O$ 0.25 mM, citric acid 0.03 mM, ferric ammonium citrate 0.03 mM, EDTA 0.003 mM, Na_2CO_3 0.38 mM, H_3BO_3 0.046 mM, $MnCl_2{\cdot}4H_2O$ 0.009 mM, $ZnSO_4{\cdot}7H_2O$ 0.0008 mM, $Na_2MoO_4{\cdot}2H_2O$ 0.0016 mM, $CuSO_4{\cdot}5H_2O$ 0.0003 mM, and $Co(NO_3)_2{\cdot}6H_2O$ 0.0002 mM.

For the treatments, 100% dairy wastewater (DIWW) was used with a nutritional correction with the commercial fertilizer Bayfolan® (Bayer, Valencia, Spain) reaching a concentration of 20.80 mM of total nitrogen and 1.67 mM of PO_4^{3-}.

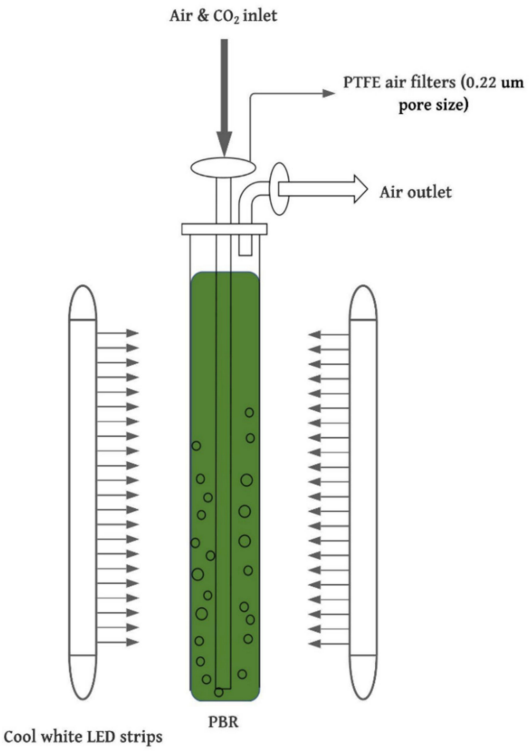


Figure 2. Schematic diagram of the 150 mL lab-scale photobioreactor (PBR), used for *Scenedesmus* sp. cultivation. The air and CO_2 inlet and air outlet were equipped with 0.22 µm PTFE (Polytetrafluoroethylene) filters (Sigma-Aldrich, Saint Louis, MO, USA).

2.3. Determination of Growth Parameters

Growth was evaluated by cell concentration (cell mL^{-1}) every 24 h: 10 uL of culture was introduced into a Neubauer chamber, and cells from all squares were quantified in the central area.

Cell concentration was calculated using the following equation [32]:

$$C = N \times 10^4 \times f \quad (1)$$

where C is the cell concentration (cell mL^{-1}), N the total number of cells counted in the area, and f the dilution factor.

The specific growth rate (μ:day^{-1}) was determined at the end of the assay based on the values obtained from the cell concentration using the equation proposed by Arredondo et al. [33]:

$$\mu = \ln (N_2 - N_1)/t_2 - t_1 \quad (2)$$

where N_1 and N_2 are the cell density values at times t_1 and t_2, respectively.

The dry biomass weight was determined using the protocol proposed by Zhu and Lee [34], which consists of the use of fiberglass filters of 47 mm diameter and a pore diameter of 0.7 μm (Whatman® GF/F). Previously, the filters were dried at 90 °C for 24 h in an oven, placed in a desiccator with SiO_2 to avoid humidity, and weighed on an analytical balance. 5 mL of *Scenedesmus* sp. culture was filtered with the addition of 0.5 mM ammonium formate (HCO_2NH_4) to remove the salts from the culture medium and the wastewater. The biomass filters were dried at 80 °C for 24 h and weighed on an analytical balance. The dry weight corresponds to the difference between the weight of the filter with the dry biomass and the weight of the dry filter. In addition, the dry weight value of the suspended solids in the wastewater was subtracted. The biomass productivity (g L^{-1} day^{-1}) was calculated using the following equation:

$$Pb = (X_2 - X_1)/t_2 - t_1 \quad (3)$$

where X_1 and X_2 were the biomass concentrations (g L^{-1}) on days t_1 (start of assay) and t_2 (end of the assay).

2.4. Determination of Biochemical Composition

The concentration of carbohydrates in the biomass was carried out by the Sulfuric Phenol method proposed by Dubois et al. [35]: 1 mL of centrifuged culture was used. The pellet was re-suspended in 1 mL of 1N NaOH. The pellet was shaken in the vortex, sonicated at 20 kHz and 4 °C for 20 min and centrifuged at 17,100× *g*, 4 °C for 15 min. From the obtained supernatant, 100 μL of each sample was transferred in triplicate, and 900 μL of distilled water, 25 μL of 80% phenol, and 2.5 mL of concentrated H_2SO_4 were added and shaken in the vortex. It was allowed to cool to room temperature for 30 min and was measured by spectrophotometry at 485 nm, using glucose as standard.

The protein content of the biomass was determined by the protocol of Lowry et al. [36] modified by Herbert et al. [37], where 2 mL of 1N NaOH are added to 6 mg of dry biomass, sonicated at 20 kHz and 4 °C for 15 min, followed by a water bath at 95–100 °C for 45 min. All samples were allowed to cool to room temperature and centrifuged at 17,100× *g* and 4 °C for 15 min. From the obtained supernatant, 100 μL of each sample was transferred in triplicate, 400 μL of distilled water, 300 μL of 1N NaOH, and 2 mL of saturated Cu-Tartrate solution were added, then it was mixed in the vortex and 10 min of reaction was expected. 400 μL of Folin Ciocalteu diluted *v*/*v* was added and left for 30 min at room temperature. Protein concentration was measured by spectrophotometry at 750 nm, using bovine serum albumin as standard.

The lipid concentration of the biomass was determined by the method of Bligh and Dyer [38]: 3 mL of methanol and 1.5 mL of chloroform *v*/*v* in a 2: 1 ratio were added to 5 mg of dry biomass. It was then sonicated at 4 °C at 20 kHz for 20 min, followed by a water bath at 50 °C for 30 min. The samples were allowed to cool to room temperature and centrifuged at 17,100× *g* and 4 °C for 10 min. 1.5 mL

of chloroform and 1.5 mL of distilled water were added to the supernatant and shaken in the vortex. It was centrifuged again at 17,100× *g* and 4 °C for 5 min. To the organic phase previously separated from the upper aqueous phase, 0.5 mL of acetone was added (remaining substances were removed), evaporated with a nitrogen flow, and re-suspended in 1 mL of chloroform. The lipid concentration was determined by spectrophotometry using the Marsh and Weinstein [39] simple carbonization method: 100 µL of each re-suspended sample was transferred in triplicate to glass tubes, 2.5 mL of concentrated H_2SO_4 was added, and it was brought to carbonization (200 °C, 15 min). Finally, the tubes were allowed to cool to room temperature, and 3 mL of distilled water was added, to determine the absorbance at 375 nm by spectrophotometry, using palmitic acid as standard.

2.5. Determination of the Lipid Profile

The fatty acid methyl esters (FAMEs) profile of Scenedesmus sp. grown in dairy wastewater was determined using the following methodologies: fat content by the extraction method with a mixture of ethers (Method 989.05) from AOAC [40], concentration and composition of saturated fatty acids (myristic, palmitic, stearic, arachidic), monounsaturated (oleic) and polyunsaturated (linoleic, linolenic, EPA, DHA) by AOAC Method 963.22 [41] by gas chromatography and the content of trans fats by AOAC Method 996.06 [42] which involves the use of gas chromatography after extraction with acid/alkaline hydrolysis and mixed ethers.

2.6. Nutrient Removal and Wastewater Characterization

The total nitrogen concentration was determined by an adaptation of the persulfate digestion method proposed by D'Elia and Steudler [43], and the PO_4^{3-} content by the vanadate-molybdate method of Tandon et al. [44]. These concentrations were measured by the Lovibond MD600/MaxiDirect photometer (Lovibond®, Dortmund, North Rhine-Westphalia, Germany) based on the established protocols.

The characterization of the wastewater used in the test was carried out by determining the pH, chemical oxygen demand (COD), and the concentration of TN, PO_4^{3-}, and heavy metals (As, Cd, Hg, Pb). The COD expressed in mg O_2 L^{-1} was determined by an adaptation of the dichromate-H_2SO_4 method of APHA [45] measured by the Lovibond MD600 photometer based on respective protocols (Method M132). Mercury concentration was determined by applying the Evans, Johnson, and Leah protocol [46] by hot plate digestion with concentrated HNO_3 and cold vapor atomic absorption spectrophotometry. Cadmium and lead were determined following AOAC Method 999.10 [47], using microwave digestion with HNO_3 and H_2O_2 under pressure followed by atomic absorption spectroscopy in a graphite furnace. The arsenic concentration was determined using the programmed three-stage digestion technique with HNO_3 and atomic absorption spectrophotometry with a graphite furnace [48].

The assimilation of nutrients and COD removal were calculated using the percentage difference between the concentrations of nitrogen, PO_4^{3-}, and COD at the beginning and end of the assay (supernatant).

2.7. Morphological Changes

Cell size measurements (length, width) were made with Digital Image System Software (Digital Imaging Systems®, Buckinghamshire, England) while cell area and volume were determined by applying the following equations [49]:

$$A = \pi \, r1 \, r2 \quad (4)$$

$$V = \pi \, a^2 \, b/3 \quad (5)$$

where r1 and r2 correspond to half of the values of length and width of the cell (a: length b: width).

2.8. Statistic Analysis

The statistical and graphical analyses were conducted using Prism program version 8.3.0. (GraphPad Software, Inc®, San Diego, CA, USA). All the data obtained when evaluating the differences

between the control and the treatments were analyzed using one-way analysis of variance (ANOVA) and post hoc Tukey multiple comparisons test with a significance level of 95% ($p \leq 0.05$).

3. Results

The current demand for bioenergy generation that is clean, profitable, and productive has increased the study of microorganisms to produce compounds for energetic use such as bioethanol, biodiesel, biogas, among others. To achieve the research objectives, growth was determined by the daily cell density during the 11 days of culture and the dry weight of the biomass. At the end of the process, the biochemical composition of the biomass was evaluated to determine the increase in metabolites for bioenergetic applications.

3.1. Determination of Growth Parameters

The culture of *Scenedesmus* sp. for 11 days is shown in Figure 3, where the maximum cell density reached by the culture at 100% dairy industry wastewater is observed ($4.4 \times 10^7 \pm 1.4 \times 10^6$ cells mL^{-1}). These results did not register significant differences from the controls where a maximum cell concentration of $4.3 \times 10^7 \pm 3.4 \times 10^6$ cells mL^{-1} was obtained. These are expected results since the stress to which the microalgae were subjected to wastewater culture reduces cell growth. However, the results obtained when calculating the specific growth rate did show significant differences between controls and treatments, with 0.51 ± 0.06 day^{-1} and 0.54 ± 0.14 day^{-1}, respectively, as shown in Figure 4.

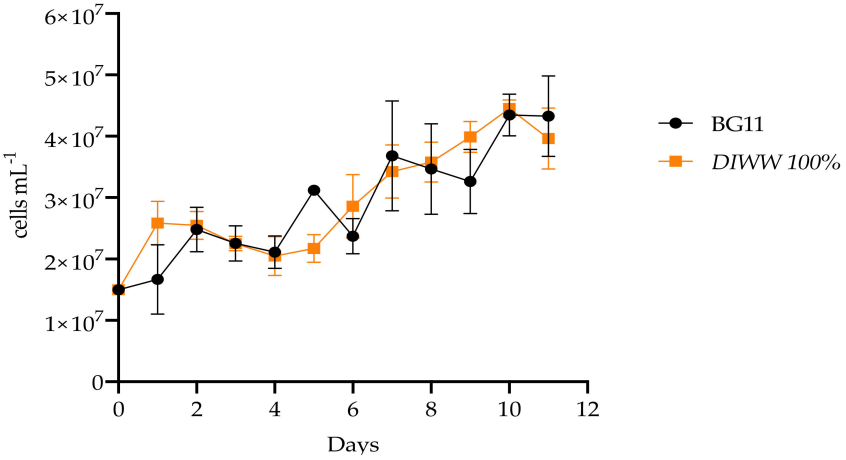


Figure 3. Cell density of the batch culture of *Scenedesmus* sp. in BG11 standard medium and dairy industry wastewater (n = 3).

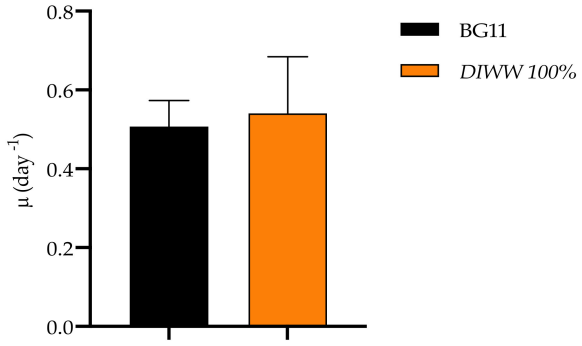


Figure 4. Specific growth rate of *Scenedesmus* sp. obtained in standard BG11 culture medium compared to the medium with the dairy industry wastewater (n = 3).

The biomass productivity determined based on the dry weight data does not show significant differences between controls and treatments with 1.84 ± 0.93 g L^{-1} day^{-1} and 1.75 ± 0.60 g L^{-1} day^{-1}, respectively (Table 2, Figure 5).

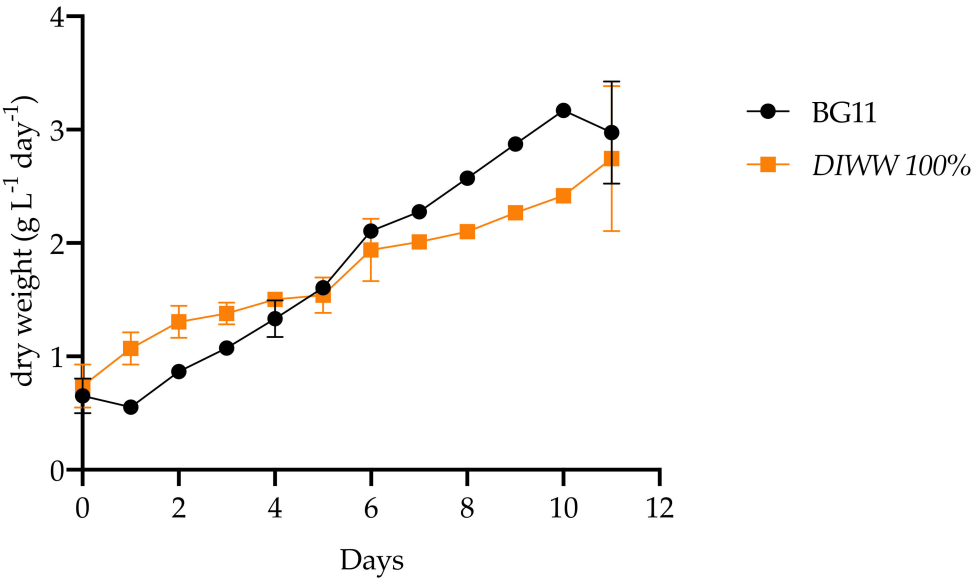


Figure 5. Biomass productivity obtained with *Scenedesmus* sp. in BG11 culture medium and wastewater from the dairy industry (n = 3).

Table 2. Kinetic parameters of *Scenedesmus* sp. grown in batch for 11 days of cultivation. X_m: maximum cell concentration obtained. μ: specific growth rate. Q_x: biomass productivity.

Culture Medium	X_m (Cell mL^{-1})	μ (day^{-1})	Q_x (g L^{-1} day^{-1})
BG11	$4.3 \times 10^7 \pm 3.4 \times 10^6$	0.51 ± 0.06	1.84 ± 0.93
DIWW 100%	$4.4 \times 10^7 \pm 1.4 \times 10^6$	0.54 ± 0.14	1.75 ± 0.60

3.2. Determination of the Composition of Biomass

Microalgae in standard growth conditions are characterized by lipid concentrations of 10–30% and carbohydrates of 5–30% of their composition [50], making them potential candidates to produce these compounds under stress conditions that induce their increase.

In this research, the cultivation of *Scenedesmus* sp. in dairy industry wastewater (DIWW) gave promising results compared to the standard BG11 culture medium in terms of compounds with potential use in bioenergy, reaching values of 276.96 ± 20.46 mg g^{-1} for proteins, 304.09 ± 31.65 mg g^{-1} for carbohydrates, and 350.81 ± 33.05 mg g^{-1} for lipids in the control; and 198.36 ± 12.73 mg g^{-1} for protein concentration, 268.51 ± 24.52 mg g^{-1} for carbohydrates, and 507.81 ± 19.09 mg g^{-1} for lipid production in the treatment. These values indicate that the biomass of *Scenedesmus* sp. cultivated in dairy industry wastewater reaches approximate percentages of 20%, 27%, and 51% of proteins, carbohydrates, and lipids, respectively.

These results show that the biomass composition of *Scenedesmus* sp. is modified when cultivated in dairy effluents, a substrate that acts as a physiological stress factor since it tends to increase lipid concentrations and decrease protein content as shown in Figure 6.

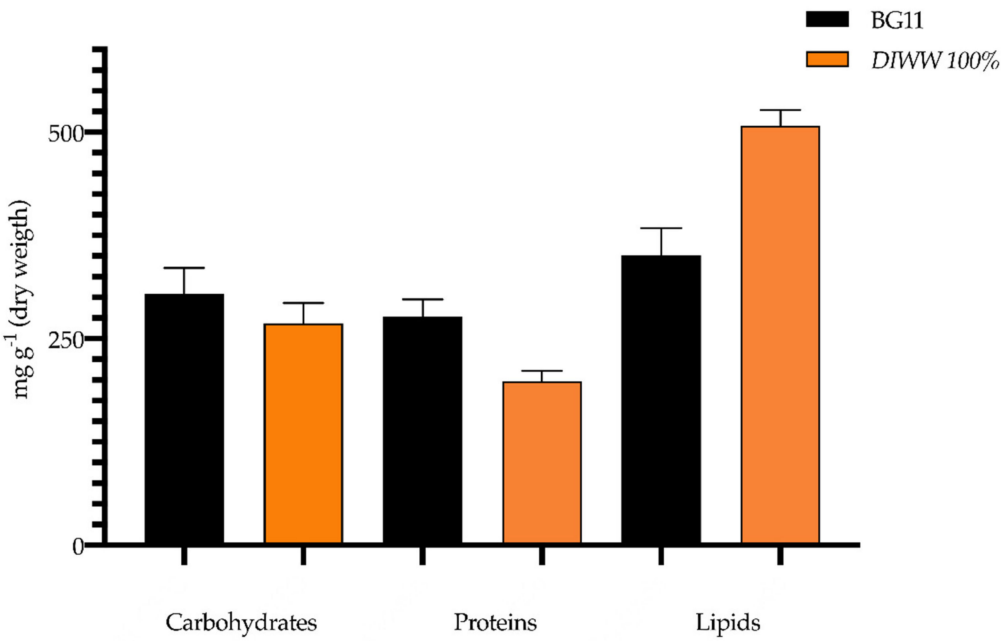


Figure 6. Biomass composition of *Scenedesmus* sp. grown in standard BG11 culture medium and dairy industry wastewater (n = 3).

3.3. Determination of the Lipid Profile

The analysis of fatty acid methyl esters (FAMEs) produced by *Scenedesmus* sp. (RJ 3009) grown in dairy effluents shows dominance of polyunsaturated fatty acids (44%), followed by saturated fatty acids (29%) and, in a lower concentration, fatty acids monounsaturated (21%). A higher proportion of linolenic, linoleic, and palmitic acid was obtained: 24%, 20%, and 19% of the total lipid profile, respectively (See Table 3).

Table 3. Fatty acid composition of *Scenedesmus* sp. grown in 100% dairy industry wastewater.

Fatty Acid Methyl Esters (FAMEs)	% Total Fatty Acids
Saturated Fatty Acids	
Myristic (C14:0)	8.13
Palmitic (C16:0)	18.79
Stearic (C18:0)	0.32
Arachidic (C20:0)	1.58
Monounsaturated Fatty Acids	
Oleic (C18:1)	9.71
Polyunsaturated Fatty Acids	
Linoleic (C18:2)	20.05
Linolenic (C18:3)	23.79
Eicosapentaenoic (EPA) (C20:5)	0.00
Docosahexaenoic (DHA) (C18:3)	0.00
Total saturated fatty acids	28.95
Total monounsaturated fatty acids	20.82
Total polyunsaturated fatty acids	44.29
Trans fat	5.94

3.4. Morphological Changes

The culture in the dairy industry wastewater caused a substrate-induced modification in the size of the cells compared to the control. The same was observed concerning the area and cell volume (see Table 4).

Table 4. Cell morphological changes in *Scenedesmus* sp. in cultures with dairy wastewater compared to standard medium BG11.

Culture Medium	Length (μm)	Width (μm)	Area (μm^2)	Volume (μm^3)
BG11	17	6	80.1	1814.9
DIWW 100%	14	13	142.9	2666.9

These values show an increase of 1.78 and 1.46 times the cell area and volume in the treatments compared to the control, respectively.

3.5. Nutrient Removal

Microalgae have the ability to use nutrients from wastewater as culture substrates for growth, which gives an additional advantage to growing these microorganisms for wastewater treatment.

In the cultivation of *Scenedesmus* sp. with 100% dairy industry wastewater, removal percentages of 88.4% for total nitrogen, 97.1% for PO_4^{3-}, and 89.3% for COD were obtained (Table 5).

Table 5. Nutrient assimilation and chemical oxygen demand (COD) removal percentages for *Scenedesmus* sp. grown in 100% dairy industry wastewater.

Parameter	Initial Concentration	Final Concentration	Removal (%)
TN (mM)	20.80	2.41	88.41
PO_4^{3-} (mM)	1.67	0.049	97.07
COD (mg O_2 L^{-1})	3500	374.15	89.31

4. Discussion

Wastewater as an alternative culture medium induces the modification of the biochemical composition of the biomass of microalgae and cyanobacteria, which depends on the type of sewage used since they are generally toxic environments for microalgae and can cause some sensitivity in these microorganisms. However, it has been shown that microalgae can acclimatize to a series of stress conditions such as variations in salinity, light or temperature, the influence of heavy metals, nutrient deficiency, among others [51]. Microalgae are potential biological systems for treating various wastewater sources due to their metabolic flexibility [52]. Concerning heavy metals, it has been shown that microalgae have developed mechanisms to tolerate the toxicity of these elements, which constitutes an advantage in wastewater bioremediation processes. However, this will depend on the metabolism and resistance of the strain used [53]. The wastewater sample used in this investigation contains heavy metals concentrations within the permissible range except for mercury, which exceeds the limit by 44.5 times, as observed in Table 6.

Table 6. Recommended limits of heavy metals in wastewater [54,55].

Heavy Metal	This Research	WHO [1]	NEMA [2]	EPA [3]
As (mg L^{-1})	0.0060	0.010	NM*	0.05
Cd (mg L^{-1})	0.00047	0.003	0.01	0.01
Hg (mg L^{-1})	0.0890	0.001	0.005	0.00003
Pb (mg L^{-1})	0.0001	0.010	0.01	0.006

[1] World Health Organization, [2] National Environment Management Authority of Kenya, [3] Environmental Protection Agency of United States. NM*: not mentioned.

The results obtained in this research show that agro-industrial wastewater can be used as a culture substrate for the microalgae *Scenedesmus* sp., which is reflected in the increase in cell density similar to that obtained with the standard culture medium. This result is consistent with the research by Nagi et al. [56] and Koreivienė et al. [57], who used low initial cell concentrations and obtained an increase in cell density of up to 7.5 and 3600 times, respectively.

The growth of microalgae in dairy wastewater will depend on the metabolic capacity of the selected strains and the particular characteristics of the wastewater used as a culture substrate. Lu et al. [58] evaluated the growth of *Chlorella* sp. in wastewater from raw dairy products and observed after eight days that this strain is capable of adapting rapidly to the effluent used. This is evidenced in the thirteen-fold increase in the biomass concentration compared to the initial inoculum. This result agrees with what was obtained by Marazzi et al. [59], who demonstrated that dairy wastewater from whey processing, characterized by high contents of COD and organic nitrogen, allows the growth of *Scenedesmus acuminatus*, with percentages of removal of nutrients greater than 65%. Similar results obtained with other strains show that the native microalgae *Scenedesmus* sp. (RJ 3009) can adapt to dairy wastewater as an alternative growth medium, sustainable and profitable for culture at scale.

The growth rate of these microorganisms grown in agro-industrial wastewater generally correlates with the concentration of N and P in the wastewater [60]. The specific growth rate in our research was similar in cultures maintained with DIWW and cultures with standard medium (BG11). This result agrees with the work carried out by Jebali et al. [61] and Sweiss [62]. They demonstrated that strains that adapt to wastewater have better growth and show a high percentage of reduction in wastewater organic load. Hena et al. [63] used isolated strains from urban wastewater and evaluated their cultivation in municipal wastewater from a treatment plant, with *Scenedesmus* sp. LS40 obtained a specific growth rate of 0.468 day^{-1} and a lipid accumulation percentage of 29.4% in dry biomass, growth results similar to this investigation, but with lower lipid concentrations than our research. Ling et al. [64] isolated the microalgae *Scenedesmus obliquus* from wastewater from a dairy farm and later used it in municipal wastewater treatment, where they obtained a maximum specific growth rate of 0.48 day^{-1} with high removal percentages of ammonium and phosphate; however, the lipid content only reached 19.7% of the dry weight of the biomass. Wu et al. [65] evaluated the culture of *Scenedesmus* sp. in urban wastewater with different N:P ratios obtaining values for the specific growth rate in the range of 0.14–0.62 day^{-1}; the highest values for this parameter were with high N:P ratios.

Productivity is also influenced by the microalgal strain used and the characteristics of the environment where it grows (Table 7). Hena et al. [66] reported productivity of 0.20 g L^{-1} day^{-1} for *Chlorella saccharophila* and 0.21 g L^{-1} day^{-1} for *Scenedesmus* sp. in untreated wastewater from dairy farms, with lipid concentrations of 21.82 and 13.64%, respectively. Kuo et al. [67] cultivated *Chlorella* sp. in different concentrations of piggery wastewater (PWW) (0%, 25%, 50%, 75%, and 100%) which was previously sterilized and centrifuged. They obtained productivity of 0.68 g L^{-1} day^{-1} in the treatment with 100% PWW; however, the highest production of lipids was at 25% PWW, with 29.3%. In a more extensive study, Jeong and Jang [68] evaluated the lipid production of ten chlorophyte microalgae isolated from wastewater and an urban water treatment plant. The microalgae were cultivated in the Bold basal medium, and results showed a varied concentration of lipids. The lowest lipid content was 24.0% in *Micractinium pusillum* and the highest with 46.9% in *Chlorella sorokiniana*. We must emphasize that the lipid concentrations of *Scenedesmus* sp. grown in DIWW in our work are significantly higher (50.78%) than the studies mentioned above. This last characteristic, added to the high productivity obtained (1.75 g L^{-1} day^{-1}), makes these microalgae an interesting alternative biodiesel production source.

Table 7. Biomass productivity and lipid concentration of microalgae grown in wastewater.

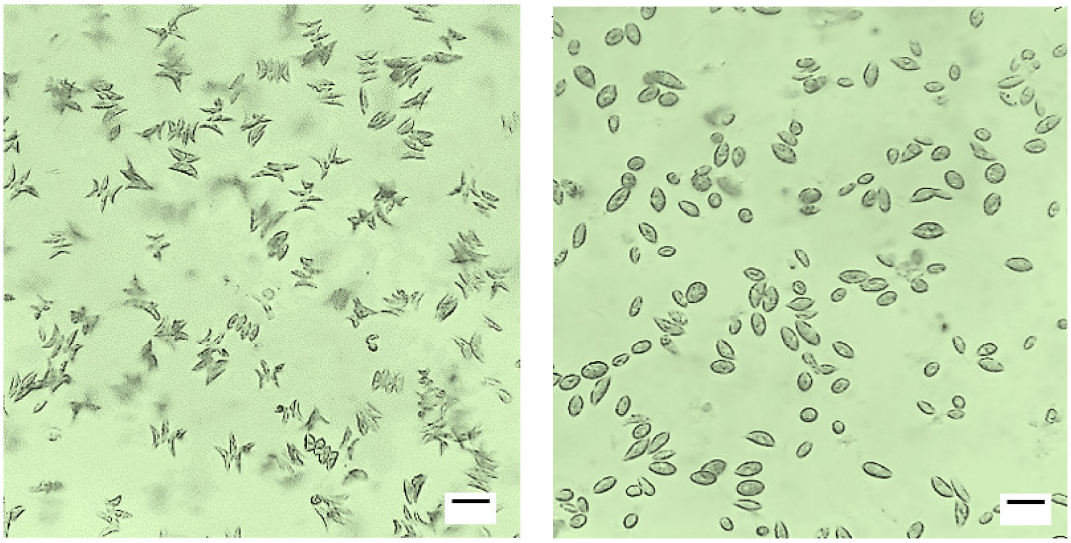
Microalgal Strain	Productivity (g L^{-1} day^{-1})	Lipid Concentrations (%)	Reference
Chlorella saccharophila	0.20	21.82	Hena et al. [66]
Scenedesmus sp.	0.21	13.64	
Chlorella sp.	0.68	29.3%.	Kuo et al. [67]
Chlorella sorokiniana	-	46.9%	Jeong and Jang [68]
Scenedesmus sp.	1.75	50.78%	This research

Scenedesmus has been widely studied concerning its production and composition of biomass in cultures with wastewater. Shen et al. [69] evaluated the municipal wastewater treatment (MWW) with *S. obliquus*, reaching a productivity of 0.578 g L^{-1} d^{-1}, and the composition of the dry biomass presented 19% of proteins, 64% of carbohydrates, and 17% of lipids. However, Ansari et al. [70] with the same species and wastewater (*S. obliquus*, MWW), obtained lower productivity, 0.085 g L^{-1} d^{-1}, and the composition of the dry biomass was 28.5% protein, 27.5% carbohydrates, and 26.5% lipids. The difference between both studies is that Shen et al. supplemented the culture with CO_2, and it is due to this that they had a high productivity, and percentage of carbohydrates (64%).

The results in terms of productivity and biomass composition of *Scenedesmus* spp. grown with standard media are varied. Thus, regarding *S. obliquus* grown in a one-liter PBR, with the standard Detmer culture medium, the cultures were supplemented with 2.5% CO_2, the maximum productivity reached was 0.573 g L^{-1} d^{-1}, and the composition of the biomass presented the following characteristics: 35.48%, 38.81%, and 8.94% for proteins, carbohydrates and lipids, respectively [71]. However, for *S. obtusiusculus* grown in the same system (PBR), with the standard culture medium BG11, with the supplement of CO_2 5.0%, the cultures reached productivity of 0.500 g L^{-1} d^{-1}, and the composition of the biomass presented the following profile: proteins 25%, carbohydrates 28%, and lipids 38% [72].

The content and composition of fatty acids in microalgae are influenced by culture conditions, directly affecting the concentration of FAMEs. In general, the composition of the fatty acids of microalgae grown in wastewater shows the dominance of unsaturated fatty acids (monounsaturated, polyunsaturated) and, in less concentration, saturated fatty acids. This is evidenced in investigations where 77% and 23% of unsaturated and saturated esters [73] have been obtained in a culture of *Chlorella vulgaris* in dairy wastewater effluents, 63% and 37% [66] with a microalgae-cyanobacteria consortium cultivated in wastewater from a dairy farm, 51% and 42% [74] with *Scenedesmus ecornis* using fertilizer plant wastewater as culture substrate, 51% and 44% [75] with *Scenedesmus* cultivated in 100% synthetic urban wastewater, and 65% and 29% obtained in this research (*Scenedesmus* sp.) in 100% DIWW. Good quality biodiesel is mainly composed of C14–C18 fatty acids, as obtained in this study where short-chain fatty acids reached high concentrations, represented by palmitic, linoleic, and linolenic acids. However, European Standard 14214 [76] determined that for quality biodiesel for vehicles, the maximum concentration of linolenic acid (C18:3) is 12%, a value that is two times lower than that obtained with *Scenedesmus* sp. (RJ 3009). Most of the microalgae oils do not comply with this standard due to polyunsaturated fatty acids in a higher proportion than the degree of unsaturation of this type of lipid compound. Despite this inconvenience, studies affirm that establishing different growing conditions, partial catalytic hydrogenation processes of the oil, mixtures with lipid substances from other raw materials and lipid extraction from wet biomass can reduce oxidative stability problems, inadequate cold flow control, and unsaturation levels, making the use of microalgal oil as a source of biodiesel viable [77,78].

The microalgae *Scenedesmus* sp. can grow in various types of sewage and accumulate lipids intracellularly, and this was verified in our research with the high values of productivity and lipid concentration obtained in the cultures maintained in the DIWW. However, the morphology of cells is altered by their tendency to accumulate lipids and carbohydrates in organelles as energy stores [79]. These morphological changes are evidenced in the increase in the surface-volume values, where the cells become spherical for better absorption of light and nutrients [80] (Figure 7).



(**a**) (**b**)

Figure 7. Light microscopes images (40x) of *Scenedesmus* sp. in different culture conditions (**a**) *Scenedesmus* sp. in BG11 standard culture medium. (**b**) *Scenedesmus* sp. in dairy industry wastewater [81]. Scale: 20 µm.

It is proven that microalgae can increase biomass production, while greater availability of nutrients exists in the environment. Tuantet et al. [82] used a mixture of male and female human urine characterized by high nitrogen and phosphorus contents as a culture substrate for *Chlorella sorokiniana*. The authors obtained productivity of 9.3 g L^{-1} day^{-1} and removal efficiencies of 60% for nitrogen and 100% for phosphorus. The productivity value achieved is due to the high concentrations of nitrogen and phosphorus in the urine used, promoting the growth of these microorganisms.

The content of organic compounds in agro-industrial wastewater, availability of nutrients, and cultivation conditions, influence the composition of the biomass of the microalgae cultivated in this type of substrate [83,84]. In this research, cultivation in the wastewater of the dairy industry influenced the metabolism of *Scenedesmus* sp., directing the flow of carbon fixed by photosynthesis to the synthesis of lipids in higher concentration (50.78% lipids) compared to the cultures maintained with the standard culture medium (35.08%). These results are similar to those obtained by Shen et al. [85], where they used the microalgae *Botryococcus* sp. isolated from a domestic wastewater treatment plant. This microalga was cultivated in synthetic wastewater, where it obtained a lipid production of 61.7% and nutrient removal of 64.5% of nitrogen and 89.8% of phosphorus. *Botryococcus* sp. obtained a high concentration of lipids explained by the heterotrophic culture conditions to which it was subjected (glucose 10 g L^{-1}, absence of light). However, the nutrient removal efficiency was lower compared to our research.

Shen et al. [86] used wastewater from a pig farm with the previous filtration of solid particles to cultivate a different microalgae strain in lipid production. The *Chlorella vulgaris* microalgae obtained a lipid concentration of 38.6% and high percentages of nutrient removal (96% nitrogen removal and 99.8% phosphorus removal). These results suggest that the microalgae used has a high capacity for removing nutrients from wastewater for the production of biofuels; however, the pre-treatment applied reduces the profitability of the process and increases production costs.

The efficiency of nutrient removal by microalgae is directly related to their ability to use inorganic nitrogen and phosphorus for their growth [87], which will depend on the strain used and the availability of nutrients in the wastewater. In the present investigation, nutrient removal percentages higher than 85% were obtained for nitrogen and phosphorus, making the use of *Scenedesmus* sp. viable for the treatment of this type of effluent. These results can be compared with those obtained by Gupta et al. [88], who used the *Scenedesmus obliquus* strain in different concentrations of municipal wastewater previously filtered and aerated for eight hours, obtaining removal percentages of 98% nitrogen and 97% phosphorus

at 15 days when cultivating the microalgae at 100% sewage; however, lipid production only reached 23% of the biomass composition. Although the strain used reached percentages of nutrient removal efficiency similar to those of the present investigation, a pre-treatment was carried out on the wastewater used, which reduces its profitability in possible industrial applications.

On the other hand, the chemical oxygen demand is an important parameter related to the amount of organic matter susceptible to oxidation processes, closely related to eutrophication and contamination in the environment. Ding et al. [89] using *Chlorella* culture with different percentages of milk residual water (5%, 10%, 20%) obtained removal rates of 84.2%, 87.9%, and 89.7% of COD, while Randi et al. [90] in the cultivation of *Chlorella vulgaris* and *Anabaena ambigua* in previously filtered dairy effluents obtained removal percentages of 96.36% and 95.9%, respectively. This is similar to that reported by Brar et al. [91], which shows a removal of 87.5% in cultures of *C. pyrenoidosa* in 75% of dairy wastewater; however, this percentage of COD reduction is achieved after 25 days of culture. The result agrees with that obtained with *Scenedesmus* sp., evidencing that microalgae can reduce COD in agro-industrial effluents. However, the present work shows advantages when using 100% of the residual, with high COD concentrations and no pretreatment process, as a cultivation substrate. It is worth mentioning that a high COD removal can be obtained by using microalgal consortia, increasing the efficiency of nutrient assimilation, as reported by Hena et al. [63]. They observed that the use of a consortium of microalgae and cyanobacteria represented by *Chlorella*, *Ankistrodesmus*, *Chlamydomonas*, and *Scenedesmus* reached 98.8% COD removal.

5. Conclusions

Agro-industrial wastewater is a sustainable alternative for the cultivation of microalgae for bioenergetic applications to use the nutrients present in these substrates for the accumulation of energy compounds and, at the same time, purify them, avoiding contamination of freshwater sources.

The native microalgae *Scenedesmus* can be grown in dairy industry wastewater with similar culture results to standard BG11 medium concerning productivity and growth. *Scenedesmus* sp. could increase its concentration of lipids in this culture system without presenting a significant decrease in cell density, diverting the carbon flux to synthesize these compounds.

The cultivation of *Scenedesmus* sp. in agro-industrial wastewater constitutes a profitable and sustainable biotechnological alternative for obtaining biomass with the high added value of commercial interest.

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