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Nitrogen supplementation for the production of *Chlorella vulgaris* biomass in secondary effluent from dairy industry

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ABSTRACT

In this study, secondary effluent from dairy industry was employed for the cultivation of *Chlorella vulgaris* in a bench-scale tubular photobioreactor. We sought to evaluate the biomass production, the consumption of nitrogen in the medium, and the final biomass composition. Considering the need of nitrogen supplementation, we evaluated the nitrogen:phosphorus ratio: the same proportion found in Bold basal medium (N:P = 1.71:1) and Redfield ratio (N:P = 16:1), comparing with no nitrogen supplementation and Bold Basal Medium. The results showed that nitrogen supplementation improves biomass growth (up to 2824.93 $mg.L^{-1}$), also granting efficient nutrients consumption (up to 98 % of nitrogen removal). Nitrogen supplementation following the Bold medium ratio was the most suitable protocol, since it requires less nitrogen addition without impairment in biomass productivity (Px =259.90 $mg.L^{-1}.d^{-1}$), in comparison with cultures with supplementation following Redfield ratio (Px =296.73 $mg.L^{-1}.d^{-1}$) or control culture (Px =221.02 $mg.L^{-1}.d^{-1}$). In addition, the final biomass showed satisfactory amounts of proteins (up to 21.92 %) and lipids (up to 35.75 %), besides presenting a profile with high concentrations of saturated (C16:0) and monounsaturated fatty acids (C18:1).

1. Introduction

Dairy industries represent an important sector of the Brazilian economy, since this country is the 6th major milk producer in the world [1]. The state of Minas Gerais is the most important milk producer in Brazil [2]. Therefore, dairy industry wastewater treatment is of utmost importance in the State of Minas Gerais. Even after primary and secondary treatment, wastewater may contain significant amount of inorganic nutrients, such as nitrate and phosphate, which allow eutrophication of water bodies [3]. On the other hand, wastewater may be considered a potential sustainable growth medium for the algal biomass production. The use of microalgae in treatment and recycling of wastewater has attracted a great deal of interest because of excessive biomass generation at cheaper cost without extra input of nutrients [3,4].

Microalgae cultivation has shown to be an efficient option for wastewater treatment, mainly because of its fast growth in nitrogen and phosphate rich medium, besides bio-assimilating carbon dioxide. Moreover, the resulting biomass is of great commercial value, containing valuable bio-products, such as lipids and pigments [5]. One of these microalgae species is *Chlorella vulgaris*. *Chlorella* species have been largely applied for biotechnological purposes due to the relatively high growth rate and the possibility of growth under mixotrophic condition. There are several studies considering the application of its biomass in food supplements, animal feed, biofertilizers, and mainly for biofuel [6–8].

Several studies have already evaluated the integration of *Chlorella* species cultivation with wastewater treatment [9–11], including dairy farm wastewater [12,13]. Huo et al. [14] cultivated *Chlorella zofingiensis* in dairy industry wastewater, diluted with tap water (10 %), in bench-scale outdoor ponds, evaluating the pH regulation by the addition of CO_2 and acetic acid. They found that the use of CO_2 was the best strategy for pH control, resulting in better results in biomass production. Kothari et al. [4] cultivating *Chlorella pyrenoidosa* in dairy industry wastewater, evaluated the influence of different concentrations (25 % ~ 100 %) of dairy wastewater on the growth of *Chlorella pyrenoidosa,* as well as the efficiency in the removal of phosphorus and nitrogen. Considering pretreated wastewater, Hena et al. [15] employed

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secondary dairy farm wastewater for the cultivation of microalgae consortium, including *Chlorella saccharophila,* evaluating different levels of CO_2 for pH control and inorganic carbon supply.

More recently, Peng et al. [16] evaluated the influence of organic pollutants (carbohydrates, volatile fatty acids, and proteins) on the growth of *Chlorella vulgaris* in domestic wastewater. They observed that not only biomass productivity was enhanced by the mixotrophic growth but also the inorganic nutrients removal was improved. Additionally, Bellucci et al. [17] employed a microalgal community, including *Chlorella* spp., for tertiary treatment of municipal wastewater treatment, and proved that the photosynthetic microorganisms could assimilate the residual nutrients, coming from the secondary treatment, and also perform the disinfection of the wastewater, which could be an alternative to traditional physicochemical treatment (such as UV light) even for water reuse.

Despite the promising ability of microalgae to remove nutrients from wastewater and generate high quality biomass, variations in nutrients concentrations could hinder the implementation of tertiary agal treatment [18]. The secondary effluent employed in the present study contained almost 20 $mg.L^{-1}$ of residual phosphorus, but a very low concentration of residual nitrogen ($\leq$ 1 $mg.L^{-1}$). When applying secondary wastewater as medium for microalgae growth, it is important to ensure the availability of the main nutrients. Besides carbon, nitrogen and phosphorus are the main (quantitatively) nutrients required for cell growth. In fact, the so-called Redfield ratio (C:N:P = 106:16:1) is assumed to be important for the balanced growth of these photosynthetic microorganisms. Therefore, this study evaluated different protocols for nitrogen supplementation in the growth of *Chlorella vulgaris* by employing the secondary wastewater (after secondary treatment) from a local dairy industry as culture medium in a bench scale tubular photobioreactor.

2. Material and methods

2.1. Microalgae strain maintenance and inoculum preparation

This study employed *Clorella vulgaris* (CCMA-UFSCar 689) isolated in Jureia Itatins Ecological Park (Peruibe City, Sao Paulo State) [19]. This microorganism was maintained in Erlenmeyer flasks with Bold basal medium [20]. For inoculum preparation, 300 mL of the same medium was used in 500 mL Erlenmeyer Flasks. These flasks were agitated (100 RPM) under light intensity of 40 ± 5 μmol s photons.m^{-2}.s^{-1} and in room temperature (25 °C).

2.2. Culture of C. vulgaris in dairy industry secondary wastewater in Erlenmeyer flasks

Dairy industry wastewater, after secondary treatment, was obtained from a company located in the city of Santa Rita do Sapucaí (Minas Gerais State, Brazil). In the Laboratory, this wastewater was filtered (with filter paper), maintained under −20 °C, and autoclaved (121 °C for 20 min) before its use.

Cultivations were carried out in 500 mL Erlenmeyer flasks with 200 mL of culture medium, in accordance with Table 1, for evaluation of the best wastewater concentration (50 %, 75 %, 100 %) in comparison with control culture (Bold's basal medium). Distilled water was used for wastewater dilutions. With initial biomass concentration of approximately 50 $mg.L^{-1}$, cultures were kept on a rotary shaker at 100 RPM, at 25 °C and continuous light intensity of 40 ± 5 μmol photons m^{-2} s^{-1}. Since nitrogen concentration was much lower than in Bold Basal Medium, and mainly much lower than phosphate concentration, the secondary effluent was supplemented with $NaNO_3$ in sufficient amount for achieving the same N:P ratio found in Bold Basal Medium.

Table 1
Cultivations of *C. vulgaris* in Erlenmeyer flasks employing dairy industry secondary wastewater (mean values and standard deviation obtained by triplicates).

Run	Xm[a] ($mg.L^{-1}$)	Px[b] ($mg.L^{-1}.d^{-1}$)
Control[c]	665.2 ± 94.1 [A, B]	39.0 ± 5.9 [A, B]
50 %	498.0 ± 67.5 [B]	27.1 ± 4.7 [B]
75 %	814.1 ± 155.2 [A]	48.1 ± 0.45 [A]
100 %	547.6 ± 54.9 [B]	30.4 ± 3.6 [B]

[A,B]: Means that do not share a letter are significantly different according to the Tukey test ($p > 0.05$).
[a] Xm: maximum biomass concentration.
[b] Px: biomass productivity.
[c] Control: Bold Basal Medium.

2.3. Culture of C. vulgaris in dairy industry secondary wastewater in tubular photobioreactor

The best wastewater concentration (found in Erlenmeyer flasks) was applied for the cultivation in bench scale tubular photobioreactor (Fig. 1, adapted from Perez-Mora et al. [21]). This reactor was made of 20 transparent glass tubes (1.0 cm internal diameter and 50.0 cm long), with a slope of 2% for allowing liquid flow, and connected with silicone tubes with the same inner diameter. A degasser flask, at the top of the bioreactor, has an input for the entry of CO_2, which serves as carbon source and helps to maintain an optimal pH range (pH 7.0 ± 1.5). A timer was used for automated addition of CO_2 via the solenoid valve. Two fluorescent lamps of 18 W were used to provide light at an intensity of 40 ± 5 μmol photons m^{-2} s^{-1}. The total and illuminated volumes were 2 L and 1.26 L, respectively.

In tubular photobioreactor, the cultivations were performed diluting the wastewater with distilled water in the proportion 3:1, and testing different protocols for nitrogen ($NaNO_3$) supplementation: following the N:P ratio found in Bold Basal Medium (1.72:1); following Redfield proportion (16:1); and without supplementation (Table 2). These cultures were compared with control culture performed with Bold Basal Medium.

2.4. Analytical techniques

Biomass concentration was determined by turbidimetry (550 nm) [22], converting the values of absorbance with the aid of a calibration curve (Eq. 1)

$$X = 963,12^{*}abs + 40,086 \quad (1)$$

where X is biomass concentration ($mg.L^{-1}$, dry weight).

Culture medium (Bold Basal Medium) and secondary effluent from dairy industry were submitted to the following nutrients analysis before and at the end of each cultivation: nitrate, nitrite, ammonium, and phosphate. Samples were previously filtered with glass fiber membrane (0.45 μm).

Nitrogen concentration in the form of nitrate was determined in accordance with APHA [23] by spectrophotometric method. After acidification with HCl, samples were submitted to measurements of absorbance at 220 nm subtracting the absorbance at 275 nm. A calibration curve was made employing KNO_3.

Nitrite form nitrogen was quantified by spectrophotometric method in accordance with Mackereth [24] and Carmouze [25]. This method involves reactions with $C_6H_8O_2N_2S$ and $C_{12}H_{14}N_2.2HCl$ in acid solution. The absorbance was measured at 543 nm and a calibration curve was made with KNO_2.

Ammonium concentration was determined by the method with Berthelot reaction, with the use of phenol and sodium hypochlorite in alkaline medium. The absorbance of the resultant solution was measured at 630 nm [25,26]. NH_4Cl solution was used for calibration

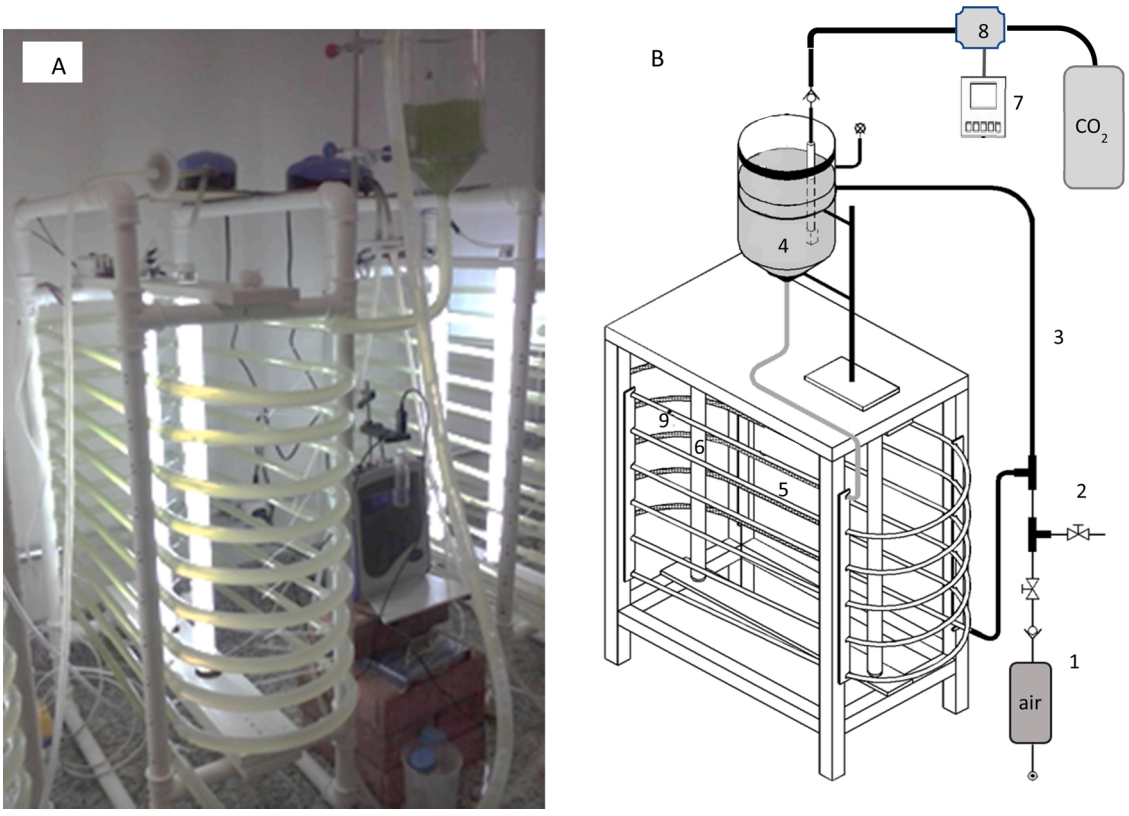

Fig. 1. A Bench-scale tubular photobioreactor in use at the Federal University of Itajubá (Brazil). 1B Tubular photobioreactor scheme: (1) aquarium air pump for cell circulation; (2) sampling system; (3) external silicon tube; (4) degasser flask; (5) glass tubes; (6) 18 W fluorescent lamps; (7) timer; (8) solenoid valve; (9) nylon sphere. (Adapted from Pérez-Mora et al [21], with kind permission from John Wiley and Sons).

Table 2
Cultivations of *C. vulgaris* in bench-scale tubular photobioreactor employing secondary effluent from dairy industry (mean values and standard deviation obtained by triplicates).

Run	Xm[a] ($mg.L^{-1}$)	Px[b] ($mg.L^{-1}.d^{-1}$)
Control (Bold Basal Medium)	1552.01 ± 199.5^{B}	221.02 ± 30.77^{A}
Effluent with no Nitrogen addition	813.58 ± 22.0^{C}	94.13 ± 3.14^{B}
Effluent with N:P from Bold	1966.95 ± 408.62^{B}	259.90 ± 56.56^{A}
Effluent with N:P from Redfield	2824.93 ± 90.64^{A}	296.73 ± 9.86^{A}

[A,B,C]: Means that do not share a letter are significantly different according to the Tukey test ($p > 0.05$).
[a] Xm: maximum biomass concentration.
[b] Px: biomass productivity.

curve.

Phosphate was also quantified by spectrophotometric method, which involves reaction with $(NH_4)_8$ $Mo_7O_{24}.4H_2O$, $K_2\mathbf{Sb}_2(C_4H_2O_6)_2$, and $C_6H_8O_8$ in acid medium. Absorbance was measured at 885 nm and KH_2PO_4 solution was employed for calibration curve.

Secondary effluent from dairy industry was also submitted to chemical oxygen demand (COD) analysis by the colorimetric method, with potassium dichromate as oxidative agent, in accordance with the Standard Methods for the Examination of Water and Wastewater [23]

At the end of each cultivation, biomass was recovered by centrifugation and dried at 60 °C overnight. Dry biomass was submitted to determination of total lipids and total proteins. Lipid fraction was submitted to analysis of fatty acids composition.

Total protein content was determined by the Kjeldahl method, employing 6.25 as conversion factor from total nitrogen content [27]. Total lipid content was gravimetrically determined after extraction with Chloroform:Methanol (2:1) using the Soxhlet methodology [28].

Lipid fractions were recovered with petroleum ether and the fatty acids were converted to fatty acid methyl esters (FAME) [29]. FAME fractions were analyzed by gas chromatography (model 7890, Agillent Technologies – USA) and the identification of fatty acids was performed by comparing retention time with that obtained in 37 Component FAME Mix (Supelco – USA) according to Pérez-Mora et al. [21].

2.5. Results evaluation

Maximum biomass concentration (Xm) was used for calculating Biomass Productivity (Px) in accordance with Eq. 2.

$$Px = \frac{Xm - Xi}{t} \quad (2)$$

Where Xi is the initial biomass concentration and t is the cultivation time. These results were compared by Analysis of variance (ANOVA) with a significance level of 0.05 and Tukey test, employing Minitab 17.

3. Results and discussion

3.1. Characterization of secondary effluent from dairy industry

For this study, we employed secondary effluent from dairy industry, i.e. the wastewater that had already been submitted to the primary and secondary (biological) treatment, mainly for the removal of suspended solids and organic load, respectively.

This secondary effluent was analyzed and the results were:

- Total inorganic nitrogen concentration (sum of nitrogen in the form of nitrate, nitrite, and ammonium): 0.90 $mg.L^{-1}$.
- Phosphorus concentration (in the form of phosphate): 19.75 $mg.L^{-1}$.
- Chemical oxygen demand (COD): 524 $mgO_2.L^{-1}$.

Considering that the nitrogen concentration was very low, this nutrient was supplemented in the secondary effluent for achieving the same N:P ratio found in Bold Basal Medium (1.72:1; molar ratio). Therefore, regarding the phosphorus concentration of 19.75 mg.L^{-1}, $NaNO_3$ was added for achieving 15.20 mg.L^{-1} of nitrogen. For the cultivations in tubular photobioreactor, Redfield N:P ratio (16:1, molar ratio) was also evaluated.

3.2. Growth of C. vulgaris in Erlenmeyer flasks employing dairy industry secondary wastewater

Table 1 shows the results of cultivations in Erlenmeyer flasks with different concentrations of wastewater and with Bold basal medium. Different wastewater concentrations had statistically significant influence on maximum cell concentration (Xm) and cell productivity (Px). In fact, p value obtained by ANOVA was 0.006 and 0.005, respectively. Considering Tukey test (Table 1), the best results of Xm and Px, in comparison with control culture, was obtained in cultivations with 75 % of wastewater. Kothari et al. [3] could efficiently cultivate the green microalgae *Chlamydomonas polypyrenoideum* employing dairy industry wastewater, and found that 75 % wastewater provided the best condition for cell growth, in comparison with 100 % wastewater or medium containing lower proportion of wastewater. For this reason, 75 % was the wastewater concentration applied for the cultivation in tubular photobioreactor, comparing the results of this run with a control culture, employing Bold Basal Medium.

3.3. Growth of C. vulgaris in bench-scale tubular photobioreactor employing dairy industry secondary wastewater

In bench-scale tubular photobioreactor, *Chlorella vulgaris* was cultivated employing 75 % of secondary effluent from dairy industry (in distilled water). Considering that the concentration of nitrogen was very low (0.90 mg.L^{-1}), two protocols for nitrogen addition were evaluated: following N:P ratio found in Bold Basal Medium or following Redfield proportion. These two cultivations were compared with secondary effluent (75 %) without nitrogen supplementation and Bold Basal Medium.

As it can be seen at Table 2 and Fig. 2, it is possible to infer that the growth of *Chlorella vulgaris* in our wastewater was negatively influenced by the absence of nitrogen addition. In this condition, even the maximum biomass concentration (Xm =813.58 mg.L^{-1}) or the biomass productivity (Px =94.13 mg.L^{-1}.d^{-1}) were lower than that obtained in other conditions. In fact, different cultivation conditions had statistically significant influence on Xm and Px ($P < 0001$ in ANOVA for both dependent variables), which justify the importance of supplementing our wastewater with nitrogen.

Considering only maximum biomass concentration, with the supplementation of N in accordance with N:P ratio of Bold Basal Medium, there was no statistically significant difference of this value (Xm =1966.95 mg.L^{-1}.d^{-1}) if compared with Bold Basal Medium (Control culture, Xm =1552.1 mg.L^{-1}.d^{-1}). On the other hand, with the Redfield N:P ratio, it was possible to obtain higher value of maximum biomass concentration (Xm =2824.93 mg.L^{-1}.d^{-1}), which may be justified by the higher initial nitrogen concentration. In fact, nitrogen is considered to be the second most quantitatively important element in the microalgae biomass [30].

Although maximum biomass concentration was higher in effluent with nitrogen supplementation following Redfield N:P ratio, the time required for this result was higher (9 days) than that required for effluent with Bold N:P ratio (7 days) and control (Bold Basal Medium, 6~7 days). As a result of these differences in cultivation time, there was no statistically significant difference between the values of biomass productivity (Px) obtained in these 3 experimental conditions (Table 2). Therefore, although the higher nitrogen concentration sustained the cell growth for a longer time, there was no difference in cell growth rate. In this sense, it is reasonable to indicate the supplementation following Bold N:P ratio as the most profitable one for *C. vulgaris* biomass production, since it required lower amount of nitrogen, reduced time for obtaining the same amount of biomass and, consequently, less amount of carbon dioxide and energy input.

It is important to note that the effluent with nitrogen supplementation following Bold N:P ratio contains much lower initial concentrations of nitrogen and phosphorus (15.20 and 19.75 mg.L^{-1}, respectively) in comparison with Bold Basal Medium (41 and 53 mg.L^{-1}, respectively). Notwithstanding, in the first case, the presence of organic compounds in the effluent (COD = 524 mgO_2.L^{-1}) was probably responsible for a mixotrophic metabolism of *Chlorella vulgaris,* which allows reduction in the loss of biomass during dark respiration and provides higher biomass productivity [6,31]. The beneficial effect of mixotrophic metabolism was also confirmed by Matsudo et al. [32] cultivating the green algae *Scenedesmus obliquus* with supplementation of ethanol.

Concerning pH, it is known that the optimum pH for *Chlorella vulgaris* is around 7 [33], and, besides the fact that dairy wastewater is generally alkaline [14] the phototrophic growth induce the increase in pH values, which hinder cell growth [34]. Although pH controller (coupled to pH meter) is recommended for this kind of process [35–37], in this study, a simple timer was used for automated CO_2 addition through a solenoid valve, aiming to reduce the cost of infrastructure. Our timer allowed up to 10 daily addition, which led to an increase of pH up to 8.5, without impairment in cell growth. Therefore, even the concentrated secondary wastewater from dairy industry (without dilution) could be used. Since the high pH in cultures without dilution was the most likely reason for the decrease in maximum biomass concentration, in comparison with diluted wastewater (3:1). In fact, these cultivations are the subject for the next study.

Moreover, Table 3 shows that *C. vulgaris* was very efficient in the removal of total nitrogen (96.6 ~ 98.7 %), with the exception of the run with effluent without nitrogen addition, since the initial nitrogen concentration was already very low (1.09 mg. L^{-1}).

Despite the promising results so far obtained, further studies should

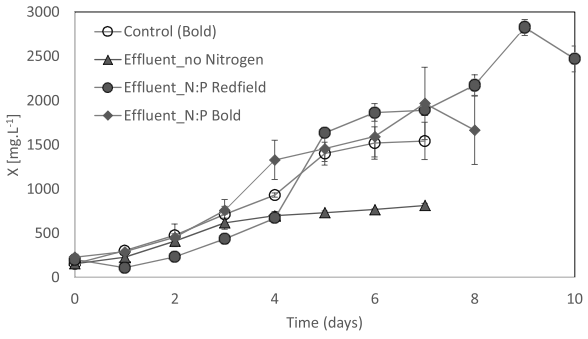

Fig. 2. Growth of *Chlorella vulgaris* in bench-scale tubular photobioreactor employing secondary effluent from dairy industry.

Table 3
Total Inorganic Nitrogen Concentration (sum of nitrogen in the forms of nitrate, nitrite and ammonium) and Inorganic Nitrogen Removal Efficiency.

Run	Initial [N] (mg.L^{-1})	Final [N]* (mg. L^{-1})	Removal efficiency* (%)
Control (Bold Basal Medium)	32.38	0.42 ± 0.04	98.70 ± 0.12
Effluent with no Nitrogen addition	1.09	0.70 ± 0.02	35.82 ± 1.43
Effluent with N:P from Bold	23.85	0.80 ± 0.11	96.65 ± 0.44
Effluent with N:P from Redfield	122.75	2.42 ± 0.22	98.00 ± 0.18

* Mean values and standard deviation from triplicates.

be performed, mainly outdoor, in order to evaluate the scalability of this process and verify if the natural light/dark cycle (with the use of sunlight) would provide the same results as that obtained with continuously illuminated cultures in this study.

3.4. Biomass biochemical composition

Total lipids and protein contents in microalgae biomass may vary depending on the cultivation condition (temperature, light intensity or nutrients quality and quantity, for instance) [37,38]. In fact, in the present study, different culture mediums had statistically significant influence on protein content and lipid content (ANOVA $p < 0.0001$ for both dependent variables).

In Table 4, it is possible to observe that protein content varied from 12.58 to 21.92 %. As expected, the highest initial concentration of nitrogen provided the highest content of total protein in *C. vulgaris* biomass, which is the case of the run with the nitrogen supplementation following Redfield N:P ratio. Considering that the residual nitrogen concentration was low (2.42 mg. L^{-1}) the addition of nitrogen by fed-batch protocol, for instance, could be suitable for obtaining biomass with higher protein content [39], when applications such as animal feed is the priority.

Also in Table 4, lipid content varied from 27.93 to 35.75 %. The use of secondary effluent supplemented with nitrogen following Bold N:P ratio seems the be the most suitable for obtaining oil rich biomass, since it allowed a satisfactory biomass productivity (as already mentioned above) and also provided the highest lipid content. The stress condition caused by nutrient limitation seems to be the most important lipid accumulating factor, if comparing with Control (Bold basal medium) culture and Effluent supplemented with nitrogen following Redfield N:P ratio. This increase in lipids in response to stress condition is in agreement with results obtained by Convert et al. [38] and Ávila-Leon et al. [37].

Table 5 shows the results of fatty acids content in *Chlorella vulgaris* biomass obtained in the tubular photobioreactor. It is possible to observe that there was no significative difference in fatty acids profile whether using Bold Basal Medium or dairy wastewater. As expected, and also observed by Converti et al. [38] in the same microalgae, Palmitic acid (C16:0) and oleic acid (C18:1n9) were the most abundant fatty acids, reaching up to 30.91 and 39.95 % (w/w).

Linoleic acid (C18:2n6), which is considered as essential for human body [40], was found in lower concentrations (up to 16.13 %), and the content of γ-linolenic acid (C18:3n6), which may also be important for fatty acid supplements [41], ranged from 8.64 to 24.07 %. The accumulation of these 2 fatty acids seemed to be favored by the higher initial amount of nitrogen found in the Effluent supplemented in accordance with Redfield N:P ratio.

Andrade and Andrade [8] reinforce the need of reducing costs of biomass production for making feasible the biotechnological application of microalgae such as *Chlorella* species. In this regard, the implementation of the process described herein could be made by dairy industries, next to the wastewater treatment plant. Thus, besides the extra income source, the company would also have the environmental benefits related to water quality and carbon dioxide assimilation.

Amongst several possibilities of microalgae biomass applications, biodiesel and animal feed could be highlighted for microalgae cultivated in dairy industry wastewater. Ferreira et al. [42] mention the need of finding alternative feed sources for livestock production, due to the difficulties faced during severe dry seasons in some areas. Therefore, defatted biomass (after removal of lipids for biodiesel) or even the whole microalgae biomass could be employed for this purpose.

4. Conclusion

This study showed the feasibility of employing secondary effluent from dairy industry for the production of *Chlorella vulgaris* biomass, which is also important as tertiary wastewater treatment. For this purpose, the analysis of nutrients is of the utmost importance and the N:P ratio is a good parameter for supplementation of nitrogen or phosphorus. For the production of biomass with high lipid content, the best protocol was the supplementation of nitrogen following the N:P ratio found in Bold Basal Medium. Although the amounts of nitrogen and phosphorus were lower than those in Bold medium, the availability of organic compounds in wastewater was probably important for obtaining satisfactory biomass productivity.

Table 4
Total proteins and total lipids contents in the biomass of *Chlorella vulgaris* cultivated in bench-scale tubular photobioreactor employing secondary effluent from dairy industry (mean values and standard deviations obtained by triplicates).

Run	Protein (%)	Lipid (%)
Control (Bold Basal Medium)	12.58 ± 0.82^{C}	30.16 ± 1.78^{B}
Effluent with no Nitrogen addition	17.05 ± 2.55^{B}	27.93 ± 1.01^{B}
Effluent with N:P from Bold	12.57 ± 0.91^{C}	35.75 ± 0.46^{A}
Effluent with N:P from Redfield	21.92 ± 0.15^{A}	28.68 ± 0.37^{B}

^{A,B,C}: Means that do not share a letter are significantly different according to the Tukey test ($p > 0.05$).

Table 5
Fatty acids content in *Chorella vulgaris* cultivated in tubular photobioreactor.

Fatty acid ocasionado (%)[a]	Control (Bold)	Effluent with no Nitrogen addition	Effluent with N:P from Bold	Effluent with N:P from Redifield
C11:0	0.33 ± 0.03	0.34 ± 0.02	0.34 ± 0.00	0.60 ± 0.27
C16:0	30.32 ± 0.48	30.91 ± 0.35	30.03 ± 0.07	24.97 ± 3.20
C16:1	1.89 ± 0.07	1.21 ± 0.02	0.93 ± 0.00	1.57 ± 0.38
N.I.[b]	1.96 ± 0.11	1.14 ± 0.03	1.19 ± 0.00	1.38 ± 0.31
C17:1	2.75 ± 0.15	2.80 ± 0.05	2.55 ± 0.21	8.24 ± 2.16
N.I.[b]	1.52 ± 0.20	2.27 ± 0.10	1.83 ± 0.00	4.20 ± 0.74
C18:0	3.25 ± 0.17	2.24 ± 0.08	2.97 ± 0.00	1.21 ± 0.17
C18:1n9C	39.85 ± 0.68	39.95 ± 0.55	39.61 ± 0.09	26.46 ± 0.33
C18:2n6c	9.49 ± 0.33	8.27 ± 0.12	9.68 ± 0.01	16.13 ± 4.26
C18:3n6	8.64 ± 0.30	11.49 ± 0.25	10.87 ± 0.02	24.07 ± 4.48

C11:0 undecanoic acid; C16:0 palmitic acid; C16:1 palmitoleic acid; C17:1 cis-10-heptadecenoic acid; C18:0 stearic acid; C18:1n9 oleic acid; C18:2n6 linoleic acid; C18:3n6 γ-linolenic acid.
[a] Percentage of fatty acids relative to the total content (weight/weight).
[b] Unidentified compound. Absent in the standard 37 FAME mix.

CRediT authorship contribution statement

Ana Elisa Rodrigues-Sousa: Investigation, Methodology, Writing - original draft. **Ivan V.O. Nunes:** Investigation, Methodology, Visualization. **Alex B. Muniz-Junior:** Investigation, Methodology. **João Carlos M. Carvalho:** Conceptualization, Resources, Funding acquisition, Visualization. **Lauris C. Mejia-da-Silva:** Methodology. **Marcelo C. Matsudo:** Conceptualization, Funding acquisition, Supervision, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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References

[1] FAO, Dairy Market Review, Food Agric. Organ. United Nations, 2018, pp. 1–8. http://www.fao.org/economic/est/est-commodities/dairy/milk-and-milk-products/en.

[2] EMBRAPA, Indicadores: Leite e Derivados, Empres. Bras. Pesqui. Agropecuária, 2019, pp. 1–16.

[3] R. Kothari, R. Prasad, V. Kumar, D.P. Singh, Production of biodiesel from microalgae Chlamydomonas polypyrenoideum grown on dairy industry wastewater, Bioresour. Technol. 144 (2013) 499–503, https://doi.org/10.1016/j.biortech.2013.06.116.

[4] R. Kothari, V.V. Pathak, V. Kumar, D.P. Singh, Experimental study for growth potential of unicellular alga Chlorella pyrenoidosa on dairy waste water: an integrated approach for treatment and biofuel production, Bioresour. Technol. 116 (2012) 466–470, https://doi.org/10.1016/j.biortech.2012.03.121.

[5] R.B. Derner, S. Ohse, M. Villela, S.M. De Carvalho, R. Fett, Microalgae, products and applications, Microalgas, Prod. e Apl. 36 (2006) 1959–1967, https://doi.org/10.1590/S0103-84782006000600050.

[6] C. Safi, B. Zebib, O. Merah, P.Y. Pontalier, C. Vaca-Garcia, Morphology, composition, production, processing and applications of Chlorella vulgaris: a review, Renewable Sustainable Energy Rev. 35 (2014) 265–278, https://doi.org/10.1016/j.rser.2014.04.007.

[7] S. Chader, B. Mahmah, K. Chetehouna, E. Mignolet, Biodiesel production using Chlorella sorokiniana a green microalga, Rev. Des Energies Renouvelables. 14 (2011) 21–26. http://www.cder.dz/download/Art14-1_3.pdf.

[8] C.J. de Andrade, L.M. de Andrade, An overview on the application of genus Chlorella in biotechnological processes, J. Adv. Res. Biotechnol. 2 (2017) 1–9, https://doi.org/10.15226/2475-4714/2/1/00117.

[9] P.L. Gupta, S.M. Lee, H.J. Choi, Integration of microalgal cultivation system for wastewater remediation and sustainable biomass production, World J. Microbiol. Biotechnol. 32 (2016) 1–11, https://doi.org/10.1007/s11274-016-2090-8.

[10] L. Zhu, Z. Wang, J. Takala, E. Hiltunen, L. Qin, Z. Xu, X. Qin, Z. Yuan, Scale-up potential of cultivating chlorella zofingiensis in piggery wastewater for biodiesel production, Bioresour. Technol. 137 (2013) 318–325, https://doi.org/10.1016/j.biortech.2013.03.144.

[11] Y. Li, Y.-F. Chen, P. Chen, M. Min, W. Zhou, B. Martinez, J. Zhu, R. Ruan, Characterization of a microalga Chlorella sp. Well adapted to highly concentrated municipal wastewater for nutrient removal and biodiesel production, Bioresour. Technol. 102 (2011) 5138–5144, https://doi.org/10.1016/J.BIORTECH.2011.01.091.

[12] Y.K. Choi, H.M. Jang, E. Kan, Microalgal biomass and lipid production on dairy effluent using a novel microalga, Chlorella sp. Isolated from Dairy Wastewater, Biotechnol. Bioprocess Eng. 23 (2018) 333–340, https://doi.org/10.1007/s12257-018-0094-y.

[13] W. Lu, Z. Wang, X. Wang, Z. Yuan, Cultivation of Chlorella sp. Using raw diary wastewater for nutrient removal and biodiesel production: characteristics comparison of indoor bench-scale and outdoor pilot-scale cultures, Bioresour. Technol. 192 (2015) 382–388, https://doi.org/10.1016/j.biortech.2015.05.094.

[14] S. Huo, Z. Wang, S. Zhu, W. Zhou, R. Dong, Z. Yuan, Cultivation of Chlorella zofingiensis in bench-scale outdoor ponds by regulation of pH using dairy wastewater in winter, South China, Bioresour. Technol. 121 (2012) 76–82, https://doi.org/10.1016/j.biortech.2012.07.012.

[15] S. Hena, S. Fatimah, S. Tabassum, Cultivation of algae consortium in a dairy farm wastewater for biodiesel production, Water Resour. Ind. 10 (2015) 1–14, https://doi.org/10.1016/j.wri.2015.02.002.

[16] Y.Y. Peng, F. Gao, W.J.W. Hang, H.L. Yang, W.H. Jin, C. Li, Effects of organic matters in domestic wastewater on lipid/carbohydrate production and nutrient removal of Chlorella vulgaris cultivated under mixotrophic growth conditions, J. Chem. Technol. Biotechnol. 94 (2019) 3578–3584, https://doi.org/10.1002/jctb.6161.

[17] M. Bellucci, F. Marazzi, L.S. Naddeo, F. Piergiacomo, L. Beneduce, E. Ficara, V. Mezzanotte, Disinfection and nutrient removal in laboratory-scale photobioreactors for wastewater tertiary treatment, J. Chem. Technol. Biotechnol. 95 (2020) 959–966, https://doi.org/10.1002/jctb.6010.

[18] J. Chamberlin, K. Harrison, W. Zhang, Impact of nutrient availability on tertiary wastewater treatment by Chlorella vulgaris, Water Environ. Res. 90 (2018) 2008–2016, https://doi.org/10.2175/106143017x15131012188114.

[19] M.C. Matsudo, C.L. Sant'Anna, L.S. Pérez-mora, R.C. da Silva, J.C.M. De Carvalho, Isolation and evaluation of microalgae from Mangrove Area in South Coast of Sao Paulo (Brazil) for lipid production, Int. J. Curr. Microbiol. Appl. Sci. 9 (2020) 1293–1302.

[20] UTEX, The Culture Collection of Algae at the University of Texas at Austin, (n.d.). http://www.sbs.utexas.edu/utex/ (accessed September 3, 2011).

[21] L.S. Pérez-Mora, M.C. Matsudo, E.A. Cezare-Gomes, J.C.M. Carvalho, An investigation into producing Botryococcus braunii in a tubular photobioreactor, J. Chem. Technol. Biotechnol. 91 (2016) 3053–3060, https://doi.org/10.1002/jctb.4934.

[22] W. Becker, Microalgae in human and animal nutrition, in: A. Richmond (Ed.), Handb. Microalgal Cult. Biotechnol, Appl. Phycol., Blackwell Science, London, 2004, pp. 312–351.

[23] APHA, Standard Methods for the Examination of Water and Wastewater, 21st ed., American Public Health Association, Washington, 2005.

[24] F.J. Mackereth, J. Heron, J.F. Talling, Water Analysis: Some Revised Methods for Limnologists, Freshwater Biological Association, Ambleside, 1978.

[25] J.P. Carmouze, O Metabolismo Dos Ecossistemas aquáticos : Fundamentos teóricos, métodos De Estudo E análises químicas, Edgard Blucher, 1994.

[26] F. Koroleff, Determination of nutrients, in: K. Grasshoff (Ed.), Methods Seawater Anal., Verlag Chemie, Weinheim, 1976, pp. 117–181.

[27] AOAC, Official Methods of Analysis of the Association of Official Analytical Chemists, 14th ed., American Public Health Association, Arlington, 1984.

[28] L.H. Pelizer, C.E. Sassano, J.C.M. Carvalho, S. Sato, L.A. Gioielli, I.O. Moraes, Padronização do método de secagem da biomassa de Spirulina platensis, Farmácia e Química. 32 (1999) 37–40.

[29] L. Hartman, R.C. Lago, Rapid preparation of fatty acid methyl esters from lipids, Lab. Pr. 22 (1973) 475–477.

[30] J. Grobbelaar, Algal nutrition: mineral nutrition, in: A. Richmond (Ed.), Handb. Microalgal Cult. Biotechnol. Appl. Phycol., Blackwell Publishing Ltd., 2004, pp. 97–115.

[31] K.-L. Yeh, J.-S. Chang, Effects of cultivation conditions and media composition on cell growth and lipid productivity of indigenous microalga Chlorella vulgaris ESP-31, Bioresour. Technol. 105 (2012) 120–127, https://doi.org/10.1016/j.biortech.2011.11.103.

[32] M.C. Matsudo, T.F. Sousa, L.S. Pérez-Mora, R.P. Bezerra, S. Sato, J.C.M. Carvalho, Ethanol as complementary carbon source in Scenedesmus obliquus cultivation, J. Chem. Technol. Biotechnol. 92 (2017), https://doi.org/10.1002/jctb.5059.

[33] C. Wang, H. Li, Q. Wang, P. Wei, Effect of pH on growth and lipid content of Chlorella vulgaris cultured in biogas slurry, Chin. J. Biotechnol. 26 (2010) 1074–1079.

[34] J.C.M. Carvalho, M.C. Matsudo, R.P. Bezerra, L.S. Ferreira-Camargo, S. Sato, Microalgae bioreactors, in: R. BAJPAI, A. PROKOP, M. ZAPPI (Eds.), Algal Biorefineries, Vol. 1, Cultiv. Cells Prod., SPRINGER, Dordrecht, 2014, pp. 83–126, https://doi.org/10.1007/978-94-007-7494-0_4.

[35] M.C. Matsudo, R.P. Bezerra, A. Converti, S. Sato, J.C.M. Carvalho, CO2 from alcoholic fermentation for continuous cultivation of Arthrospira (Spirulina) platensis in tubular photobioreactor using urea as nitrogen source, Biotechnol. Prog. 27 (2011) 650–656, https://doi.org/10.1002/btpr.581.

[36] M.C. Matsudo, R.P. Bezerra, S. Sato, A. Converti, J.C.M. Carvalho, Photosynthetic efficiency and CO2 assimilation rate of Arthrospira (Spirulina) platensis continuously cultivated in tubular photobioreactor, Biotechnol. J. 7 (2012) 1412–1417.

[37] I.A. Avila-león, M.C. Matsudo, L.S. Ferreira-camargo, J.N. Rodrigues-ract, J.C.M. Carvalho, Evaluation of Neochloris oleoabundans as sustainable source of oil-rich biomass, Brazilian J. Chem. Eng. (2020), https://doi.org/10.1007/s43153-020-00011-3.

[38] A. Converti, A.A. Casazza, E.Y. Ortiz, P. Perego, M. Del Borghi, M. Del Borghi, Effect of temperature and nitrogen concentration on the growth and lipid content of Nannochloropsis oculata and Chlorella vulgaris for biodiesel production, Chem. Eng. Process. Process Intensif. 48 (2009) 1146–1151, https://doi.org/10.1016/j.cep.2009.03.006.

[39] J.C.M. Carvalho, R.P. Bezerra, M.C. Matsudo, S. Sato, Cultivation of Arthrospira (Spirulina) platensis by Fed-Batch Process, in: J. Lee (Ed.), Adv. Biofuels Bioprod., Springer New York, New York, NY, 2013, pp. 781–805, https://doi.org/10.1007/978-1-4614-3348-4_33.

[40] J.E. Teitelbaum, W.A. Walker, Review: The role of omega 3 fatty acids in intestinal inflammation, J. Nutr. Biochem. 12 (2001) 21–32, https://doi.org/10.1016/S0955-2863(00)00141-8.

[41] H. Tallima, R. El-Ridi, Arachidonic acid: physiological roles and potential health benefits – a review, J. Adv. Res. 11 (2018) 33–41, https://doi.org/10.1016/j.jare.2017.11.004.

[42] M.D.A. Ferreira, F.M. Da Silva, S.V. Bispo, M. De Azevedo, Strategies for the supplementation of dairy cows in the Brazilian semi-arid, Rev. Bras. Zootec. 38 (2009) 322–329, https://doi.org/10.1590/S1516-35982009001300032.