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Sanitary Wastewater Supplemented with Glycerol to Obtain Lipid-Rich Microalgal Biomass

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A R T I C L E I N F O

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ABSTRACT

Introduction: Mixotrophic microalgae systems have great potential for bioenergy production and wastewater treatment. Anaerobic-treated wastewater supplemented with carbon can improve biomass yield and quality, as it presents low carbon content. Alternative carbon sources in microalgae cultivation, such as glycerol, are essential for minimizing the economic and environmental impacts caused by biomass production, and improving the profile of fatty acids. This study aimed to increase biomass production and the lipid content with glycerol as the carbon source for microalgae cultivation from sanitary wastewater.

Materials and Methods: The microalgae behavior in the wastewater was pilot tested using glycerol supplementation at 7.5, 10.5, and 12.5 g L^{-1}.

Results: In all the experiments with sanitary wastewater, the microalgae production presented *Chlorella* sp. as the predominant species. The best biomass (3.78 ± 1.12 g L^{-1}) and lipid (35.67 ± 0.80%) yields were found at 12.5 and 10.5 g L^{-1} of glycerol, respectively.

Conclusion: The microalgae produced more lipids with glycerol supplementation. An attractive profile for biodiesel was found regarding the fatty acids in the biomass.

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Introduction

The lipids in microalgae make it a suitable raw material for biodiesel production and pharmaceutical and food industries. Compounds such as proteins and carbohydrates are also classified as valuable substances for commercial applications [1]. Microalgae can grow in different ways (autotrophic, mixotrophic, or heterotrophic) and should be consider for lipid production.

Some microalgae species can survive under phototrophic or heterotrophic or both conditions. Furthermore, the CO_2 released by the microalgae via respiration can be captured and reused in a phototrophic mode (mixotrophic growth). Compared with phototrophic and heterotrophic cultures, mixotrophic culture can still be an interesting way to produce microalgae lipids [2]. Heterotrophic and mixotrophic microalgae grow much faster than phototrophic microalgae and generate a more significant amount of cellular lipid [3]. Table 1 shows different metabolic conditions (mixo or heterotrophic) for some microalgae species

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with their respective lipid content.

According to Tan, et al. the organic load of wastewater can positively affect lipid production by microalgae mixotrophically [4]. Thuy Lan Chi, Mathimani study showed that nutrient scarcity in the stationary phase increased lipid and reduced protein content of the biomass [5]. The fatty acid composition of microalgal triacylglycerols can also change, depending on the cultivation conditions. In a study conducted on *Chlorella*, Anto, Pugazhendhi and Mathimani observed a change in the ratio between saturated and unsaturated fatty acids and lipid content when the medium was nitrogen-deprived [6].

In nature, the adaptation of microalgae to climate change is usually the result of changing lipid patterns and the synthesis of various other compounds. It is possible to induce or increase lipid production using stress factors, such as nitrogen limitation/depletion in the medium. The lipid content of microalgae typically comprises 1 to 70% of the dry weight, and can reach up to 90% under certain conditions. This lipid content exceeds the value found in most land plants. Therefore, nutritional conditions, processing, and cultivation can affect lipid profiles. Careful selection should be done based on several factors, including growth rate, lipid quality and quantity, the strength of adaptation to environmental changes, determination of preferred nutrients, and nutrient assimilation rates. In addition, the unfavorable environmental stress condition can also stimulate the accumulation of lipids in microalgae [1].

During optimal growth conditions, *Chlorella vulgaris* can yield 5-58% lipids in dry biomass weight, especially during unfavorable growth conditions [7], consisting of neutral lipids, glycolipids, hydrocarbons, phospholipids, and small amounts of free fatty acids [8]. Thus, many microalgae species can be induced to accumulate substantial amounts of lipids. Moreover, microalgae can be cultivated for lipids production on land unsuitable for terrestrial oilseed crops in a cost-effective manner and for shorter cultivation times [9].

In order to reduce microalgal production costs, it is essential to find cheap organic substrates that meet the nutritional needs of lipid production by microalgae [10]. Thus, substituting a traditional carbon source, such as glucose, for less expensive sources positively affects the economics of bioprocesses. In large-scale biodiesel production, the market offers glycerol as a surplus co-product, which makes it crucial to discover new applications for this substance. Glycerol is a water-soluble molecule, suitable for microorganisms as a carbon source [1]. Some studies have already reported glycerol as a substrate for microalgae, as shown in Table 1.

Another way to reduce production costs is using sanitary or industrial wastewater as a cultivation medium. Microalgal wastewater treatment is one of the most promising technologies for advanced treatment and nutrient recovery from wastewater. However, it is sometimes necessary to adjust effluent parameters, such as total nitrogen and phosphorus, organic pollutants, and color, after the anaerobic process.

The effectiveness of using microalgae as an adjunct to tertiary wastewater treatment has been proven by many researchers for its high efficiency in nutrient removal in advanced municipal, agricultural, and industrial wastewater treatment [9]. Algae/microalgae grown in wastewater provide significant benefits over traditional cultivation and treatment processes, including (1) treating sewage at a reduced cost, as many pollutants are assimilated by the cells [11]; (2) achieving a substantial level of nutrient removal from wastewater, and meeting increasingly stringent discharge and reuse standards [12]; (3) producing biomass as a renewable source of proteins, lipids, carbohydrates, etc.; and (4) adding value to the process by converting biomass to biogas, liquid biofuels, fertilizers, animal feed, and biocomposites [14-16]. Anaerobic-treated wastewater supplemented with carbon can improve biomass yield and quality, as it presents low carbon content and enough N and P for microalgae growth. Therefore, the objectives were to (1) evaluate the microalgae growth in sanitary wastewater with glycerol supplementation to lower the costs associated with the cultivation medium, and (2) improve microalgae's lipid content.

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Table 1: Lipid content and fatty acid profile of different microalgae species supplemented with glycerol under different metabolic modes

Microorganism	Metabolic mode	Lipid content (%)	Main fatty acids	Reference
Botryococcus terribilis	Mixotrophic	9.5 – 25	C16:0, C16:1, C17:0, C18:0, C18:1, C18:2	17
Botryococcus braunii	Mixotrophic	9.3 - 16.41	C14:0, C16:0, C16:1, C17:0, C18:0, C18:1, C18:2, C18:3, C20:0, C20:1, C22:0, C22:1, C24:0, C24:1	18
Chlorella protothecoides	Heterotrophic*	47.0 – 50.6	-	19
Chlorella pyrenoidosa	Mixotrophic	30.76	C12:0, C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, C18:3, C20:0, C22:0	10
Chlorella sp.	Mixotrophic	20,94	C16:0, C18:0, C16:1, C18:1 e C18:3	20
Chlorella sorokiniana	Mixotrophic	25 – 35	-	21
		15.07	C14:0, C16:0, C16:1, C18:1, C18:2, C20:1	22
Chlorella vulgaris	Mixotrophic	12.1 - 15.91	C14:0, C16:0, C16:1, C17:0, C18:0, C18:1, C18:2, C18:3, C20:0, C20:1, C22:0, C22:1, C24:0, C24:1	18
		27	C16:0, C16:1, C16:2, C16:3, C18:0, C18:1,C18:2,C18:3	23
		34	-	24
		13 – 27	C16:0, C16:1, C17:0, C18:0, C18:1, C18:2	17
		20 – 62	-	21
	Mixotrophic	15.11	C14:0, C15:0, C16:0, C16:1, C16:2, C18:0, C18:1, C18:2, C18:3, C19:0, C19:1, C20:0, C21:0, C24:0	25
Chlorococcum sp.	Heterotrophic	39-42	C12:0, C14:0, C16:0, C16:1, C16:2, C18:0, C18:1, C18:2, C18:3	26
Scenedesmus obliquus	Mixotrophic	26,5	C14:0, C16:1n10, C16:1n7, C16:2; C16:3, C14:4n3, C18:0, C18:1n9, C18:2n6, C18:3n3, C18:3n6, C18:4n3	27
Scenedesmus sp.	Mixotrophic	13.11 - 16.24	C14:0, C16:0, C16:1, C17:0, C18:0, C18:1, C18:2, C18:3, C20:0, C20:1, C22:0, C22:1, C24:0, C24:1	18
Scenedesmus *incrassulatus*	Mixotrophic	50.25	C12:0, C14:0, C16:0, C18:0, C18:1, C18:2, C18:3, C20:0, C22:1	28
Thalassiosira pseudonana	Mixotrophic	15.06	C14:0, C15:0, C16:0, C16:1, C16:3n3, C18:0, C18:1n9c, C18:1n9t, C18:2n6c, C18:4n3, C20:5n3, C22:6n3	29
Thraustochytrium sp.	Heterotrophic	33.1 – 48	C16:0, C18:0, C18:1, C20:4, C20:5, C22:5, C22:6	30
		41.87	C14:0, C16:0, C16:1, C22:6	31

C12:0 (lauric acid), C14:0 (miristic acid), C16:0 (palmitic acid), C16:1 (palmitoleic acid), C17:0 (margaric acid), C18:0 (stearic acid), C18:1 (oleic acid), C18:2 (linoleic acid), C18:3 (linolenic acid), C18:4 (stearidonic acid), C19:0 (Nonadecylic acid) C19:1 (nonadecenoic acid) C20:0 (araquidic acid), C20:5 (eicosapentaenoic acid (EPA)),C21:0 (Heneicosanoic acid), C22:0 (behenic acid), C22:1 (erucic acid), C24:0 (lignoceric acid), C24:1 (nervonic acid), C20:4 (arachidonic acid), C22:5 (docosapentaenoic acid), C22:6 (docosahexaenoic acid (DHA)); n is the position of the first unsaturation from the terminal methyl, c is cis and t is trans.

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Materials and Methods

Cultivation

The cultivation of microalgae was done in the sanitary wastewater from the university sewage treatment plant (29° 41' 55.7" S, 52° 26' 29.5" W) collected after the up-flow anaerobic sludge blanket (UASB) reactor. The wastewater analysis was conducted according to standard methods [32] and presented 514.6 mg L^{-1} $CaCO_3$ of alkalinity, 8.0 mg L^{-1} (O_2) of Biochemical Oxygen Demand (BOD_5, oximeter, Hanna, Italy, Edge^{DO} and incubator, JProlab, Brazil, JP1000), 23.35 mg L^{-1} of Chemical Oxygen Demand (COD) (Equipment Quimis, Brazil), 5.19 mg L^{-1} total phosphorus, and 64.9 mg L^{-1} of total Kjeldahl nitrogen.

The predominant microalgae cultivated in the wastewater were studied in four identical tubular photobioreactors with 2.5 L capacity using 24-h artificial light and constant aeration, forming a bubble column. The experiments started with 20% inoculum at 4, 75 x 105 cell mL^{-1} of cell density. The inoculum viability was monitored by cell counting in the Neubauer chamber every 3 days. By microscopy analysis, the predominance specie was determined as Chlorella sp.

The experimental conditions considered the glycerol addition in 3 concentrations (7.5, 10.5, and 12.5 g L^{-1}) and a control without glycerol, both cultivated simultaneously and with the same wastewater batch.

After the cultivation, the medium was centrifuged at 2,500 rpm for 15 min. Biomass was dried by lyophilization (Liotop, K120), and glycerol was determined in the liquid phase.

Glycerol determination

Glycerol concentration in the medium was determined by high-pressure liquid chromatography with a refraction index detector (HPLC/RID, Shimadzu, Japan, LC-20 AT). The mobile phase used was ultrapure water at 0.6 mL min^{-1}, with a Rezex RHM Monosaccharide H^+(300 mm x7.8 mm) column (Phenomenex) at 85°C [33]. The limit of detection (LOD) and limit of quantification (LOQ) were > 1 mg L^{-1} and > 10 mg L^{-1}, respectively.

Lipids determination

The total lipids were determined in relation to dried weight (DW) biomass. The lipidic extract was obtained by Bligh and Dyer's method, using ultrasound for cell disruption, chloroform (Sigma-Aldrich, USA), and methanol (J.T. Baker, USA). Fatty acids were transesterified using BF_3/methanol method [34]. The methyl esters of fatty acids contained in the extract were identified and quantified by gas chromatography with mass spectroscopy (GC/MS, Shimadzu, Japan, QP2010plus) using an external standard of saturated and unsaturated fatty acids (ME10 and ME12 kits, Sigma) and by similarity with spectra library (Wiley). Polimethysiloxane with 5% phenyl (DB5ms, 30 m x 0.25 mm x 0.25 µm) was used at Helium gas (1.0 mL min^{-1}). The furnace programmed temperature initiated with 80°C, increasing with 5 °C min^{-1} until 270 °C (10 min). The samples were diluted and injected with a split ratio of 1:10. The injector, interface, and detector were used at 250, 290, and 300 °C, respectively.

Statistical data analysis

All experiments were performed in triplicate (n = 3) with their values expressed as mean ± standard deviation. Regarding non-parametric statistics, the Kruskal-Wallis test was applied to compare the results of biomass yield and lipid content in different glycerol concentrations during the cultivation. The significance level of the tests was 5% ($p < 0.05$).

Ethical issues

SISGEN register A256CC3.

Results

Chlorella sp. was the predominant species in all experiments with sanitary wastewater collected after the UASB reactor. The control (without glycerol supplementation) presented lower growth ($p < 0.05$) (Figure 1). There was no significant difference among the experiments with glycerol ($p > 0.05$). The optical density (OD) curves showed higher growth in the exponential and more prolonged stationary phase for the medium with glycerol than in the medium without glycerol. In

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the latter, cell death was observed at the end of the experiment.

To quantify the glycerol used by the microbiota rich in microalgae, the analysis of glycerol remaining in the medium after harvesting the biomass was analyzed by HPLC (Figure 2).

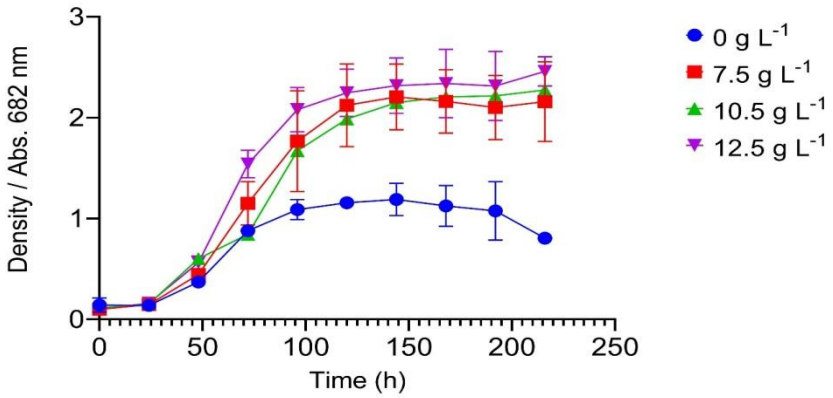

Figure 1: Growth curves of microalgae in sanitary wastewater supplemented with different glycerol concentrations (g L^{-1}). Data are displayed as mean ± standard deviation, n = 3

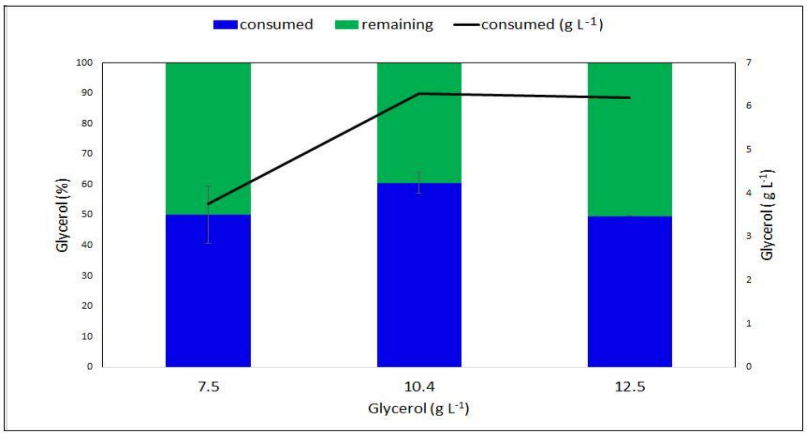

Figure 2: Glycerol consumption (g L^{-1}) in the microalgal cultivation

The biomass and lipids yields presented a significant variation of the medians ($p < 0.05$). The highest yield was obtained with supplemented medium (Figure 3), indicating that glycerol supplementation positively affected the microalgae growth and stimulated lipids accumulation due to triacylglycerols constituted of esterified glycerol [35]. At 10.5 g L^{-1}, a higher concentration of lipids was observed in the biomass (> 2-fold), and at 12.5 g L^{-1}, the biomass yield increased 3-fold compared to the culture in sanitary wastewater without glycerol supplementation. In this sense, the same amount of consumed glycerol led to different compositions.

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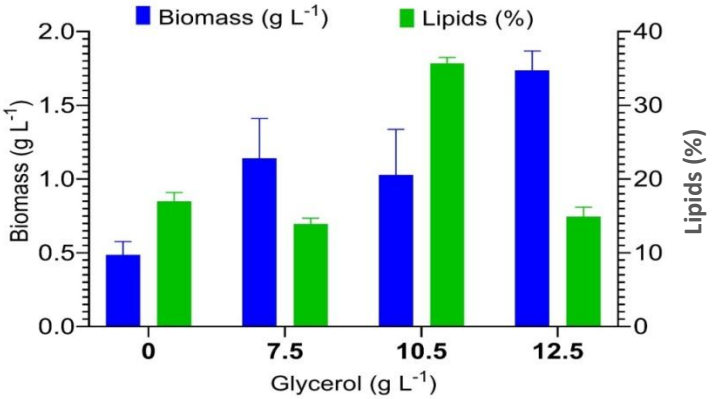

Figure 3: Biomass and lipids yield in relation to glycerol supplementation. Data are displayed as mean ± standard deviation, n = 3.

The condition with more biomass production was not the condition that presented more lipid content. With 12.5 g L^{-1} of glycerol supplementation, more biomass was produced, whereas, at 10.5 g L^{-1}, more lipids were produced. The biomass production rate was obtained from 0.10 to 0.17 g L^{-1} day^{-1} with glycerol supplementation, which was more than twice the yield in the control. On the other hand, the lipid yield showed a different behavior concerning the fatty acids profile (Table 2).

Table 2: Fatty acids profile (%) of the experiment with and without glycerol

Fatty acids	Control (without glycerol)	Glycerol (g L^{-1})		
		7.5	10.5	12.5
C14:0	1.26	2.60	1.16	2.21
C15:0	0.57	0.33	0.89	1.67
C16:0	43.19	40.26	33.81	36.28
C16:1	2.22	2.76	5.92	3.53
C16:2	0.00	0.00	0.00	1.66
C17:0	0.76	3.56	2.05	0.90
C18:0	6.35	7.91	7.16	8.00
C18:1	12.21	9.60	19.79	22.97
C18:2	22.93	19.99	21.09	15.90
C18:3	10.52	12.45	7.38	6.63
C20:0	0.00	0.52	1.15	1.18
Monounsaturated	14.42	12.36	25.70	26.50
Polyunsaturated (≥ 4)	nd	nd	nd	nd
Total unsaturated	47.87	44.80	54.17	50.69
Total saturated	52.13	55.20	45.83	49.31

Nd = not detected

Discussion

Microalgae growth

Microalgae in the wastewater supplemented with glycerol may have stimulated a mixotrophic metabolic pathway. According to Leite, Paranjape [36], who supplemented xylose and glycerol to microalgae, the indigenous microalgae could grow more mixotrophically than phototrophically. Liang, Sarkany and Cui [24] also supplemented glycerol in the *C. vulgaris* cultivation, highlighting the use of 1% of glycerol to improve growth. In Rana and Prajapati [10] experiments, the *C. pyrenoidosa* harvesting increased by 22.86% using 5 g L^{-1} of glycerol supplementation in BG11 media compared to only BG11. In the present study, it was possible to increase the biomass from 0.72 g L^{-1} (control) to 2.46 with 12.5 g L^{-1} supplementation. Therefore, it was observed that

the concentration of supplemented glycerol could influence the biological systems in the photobioreactors. Muto, Tanaka [37] reported that glycerol could increase endogenous glycerol kinase overexpression, promoting biomass and metabolite production.

Dario, Balmant [38] addressed the importance of the C/N ratio increment, where nitrogen deprivation is associated with using the carbon source mixotrophically, leading to a higher accumulation of carbohydrates and lipids. Furthermore, glycerol may increase the growth ratio as it is instantly available to be converted into energy by the cell. Villanova, Fortunato [39], in their study with *Phaeodactylum tricornutum*, also showed that the use of glycerol by the microalgae cell is a response to nitrogen limitation in the mixotrophic cultivation, which leads to the accumulation of triacylglycerol.

The glycerol consumption reached 6.3 g L^{-1} in 10 days of cultivation. Considering that after 10 days, the system was in the stationary phase, it can be concluded that more glycerol can be converted into biomass and metabolites by extending the cultivation period. Additionally, it can be hypothesized that more glycerol could be supplemented; however, large glycerol amounts could lead to increase viscosity of the medium [40]. With distilled glycerol, a higher yield was obtained with glycerol concentrations above 5 g L^{-1}. This result is not in line with the use of crude glycerol by Ren, Tuo [23], who found low biomass yield when using 10 and 15 g L^{-1} glycerol supplementation. With crude glycerol, there is a higher color contribution, lower pH, and the production of other residual molecules in the medium that could be harmful to microalgae [35]. Crude glycerol required some levels of purification to allow the supplemented concentrations used in the present study. Furthermore, as some glycerol may remain in the medium after harvesting, the residual glycerol can be reused for microalgae cultivation or further treated by biological systems, such as constructed wetlands [41].

The results obtained can be improved if the cultivation is carried out with nutrient control as performed by Sengmee, Cheirsilp [42], for pure strains of *Chlorella*. Thus, there is a possibility of increasing glycerol assimilation, with a consequent increase in lipid content. However, it is important to use wastewater to reduce production costs.

Experiments using *Chlorella vulgaris* presented better results with 10 g L^{-1} of pretreated glycerol reaching 2.92 g L^{-1} of biomass with a standard medium [35]. Researchers found differences in the yield when crude, pretreated, or pure glycerol was used and suggested that bacteria used glycerol in the wastewater. In addition, Ren, Tuo [43] observed that in the condition with higher crude glycerol content (> 5.0 g L^{-1}), the pH decreased, and the biomass could be degraded, since the total organic carbon in the wastewater increased. On the other hand, Rattanapoltee, Dujjanutat [28] found improved lipid production at higher glycerol concentrations using *Scenedesmus incrassulatus*. This finding suggests that both the species and the condition of the glycerol waste are determinants, since they used glycerol derived from biodiesel production using cooking oil waste with NaOH as the catalyst.

There were changes in the fatty acids profile, in terms of the total saturated and the total unsaturated (mono and polyunsaturated) molecules that differ with glycerol supplementation. In all the tested conditions, the most common fatty acids were C16:0, C18:0, C18:1, C18:2, and the C18:3 and C16:0 content decreased by adding more glycerol to the medium. These acids are among the majority in Chlorophyceaen (C16:0, C18:0, C18:1, and C18:2), and C16:0 was also highlighted by other authors [44, 45]. In the experiment with glycerol supplementation conducted by Gupta, Singh [30], a decrease was found in the content of total polyunsaturated fatty acids by increasing the glycerol concentration. In the study by Rattanapoltee, Dujjanutat [28] with *Scenedesmus*, palmitic acid (C16:0), oleic acid (C18:1), and linoleic acid (C18:2) were the primary fatty acids. They reported the presence of arachidic acid (C20:0) for the experiments with 5 and 20 g L^{-1} of crude glycerol. Rana and Prajapati [10] highlighted that with *Chlorella pyrenoidosa* grown in wastewater supplemented with residual glycerol,

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the lipid profile of the biomass showed fatty acids from C12 to C22 with the highest fraction (33.38%) of oleic acid (C18:1). In the current study, fatty acids from C14 to C20 were identified with a maximum of 3 unsaturated fatty acids (C18:3 between 6-13%), which decreased as the glycerol supplementation in the culture increased.

Standard EN14214 deals with quality parameters for biodiesel, including the composition of methyl ester of C18:3, which must be a maximum of 12%, and the presence of polyunsaturated (≥ 4 unsaturated content), which must be a maximum of 1%. These limits are not exceeded by the microalgal biomass obtained in the study experiments, suggesting that the fatty acid profile of the lipids obtained may be suitable for biodiesel production.

The condition with a higher concentration of biomass and lipids also had higher total monounsaturated fatty acids. In a production prospect from 1 m^3 of wastewater, there were more total lipids from 10.5 g L^{-1} of glycerol supplementation (Figure 4).

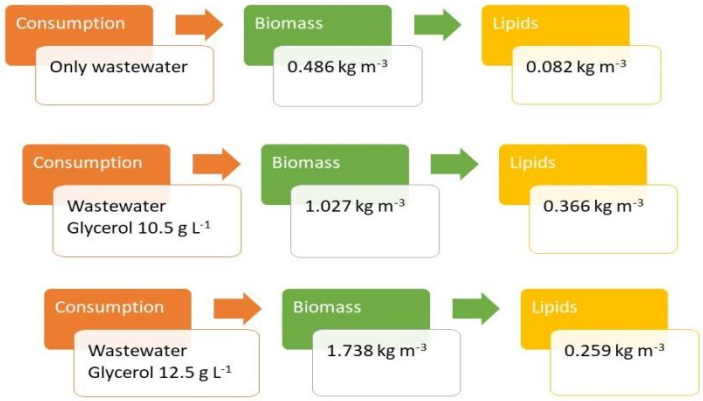

Figure 4: Prospect average biomass and lipids production in 1 m^3 of wastewater in 10 days of cultivation

It has been highlighted that the concentration of N and P are also relevant to improving the yield in biomass and lipids [46]. Glycerol diffuses into the cell and is then used as an osmoregulatory molecule. ATP initially phosphorylates glycerol, and glycerolphosphate is then oxidized to a triose phosphate, an intermediate in the Embden-Meyerhoff-Parnas (EMP) glycolysis pathway. Microalgae have glycerol kinase (EC2.7.1.30), sn-glycerol-3-phosphate Nicotinamide adenine dinucleotide (NAD) oxidoreductase (EC 1.1.1.8), and triose phosphate (E.C.: 5.3.1.1) to convert glycerol into glyceraldehyde-3-phosphate and glycerate, which are intermediates in the EMP pathway of glycolysis forming pyruvate that enters the Tricarboxylic acid (TCA) cycle. Glyceraldehyde-3-phosphate can also be formed by reducing 3-phosphoglycerate, a key intermediate in the Calvin-Benson photosynthesis cycle. As expected for gluconeogenesis, sn-glycerol 3 phosphate inhibits the reversal of the glycolytic pathway [47]. According to Xue, Chen and Jiang [48], the knowledge of glycerol metabolism in the microalgae cell is limited. The glycerol supplementation should be controlled. With 12.5 g L^{-1} of glycerol, the lipid content was minor.

Conclusions

Glycerol supplementation as a nutritional supplement in microalgae cultivation proved viable, since it did not present an inhibitory effect on microalgal growth. Regarding the biomass content, glycerol generated a higher yield, and the lipid yield was doubled compared to the control (without glycerol). With glycerol supplementation, the microalgae produced more lipids. The glycerol concentrations influenced the fatty acid content, which showed a more viable fatty acid profile, suggesting its potential use as a biodiesel feedstock. Considering the production of biomass, lipids, and fatty acids yield to obtain biodiesel, it is concluded that the best glycerol supplementation

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studied was 10.5 g L^{-1}. For further discussion and bioeconomic analysis, it is crucial to assess residual glycerol from biodiesel industries in southern Brazil. In this region, there is a logistical potential for pilot-scale investigations, and evaluation of the minimal required pretreatment of glycerol to ensure biomass and lipid yield by *Chlorella* sp. or other species that adapt well to effluents.

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Conflict of interest

The authors declare they have no financial interests.

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