

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

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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 applications¹. Microalgae can grow in different ways (autotrophic, mixotrophic, or heterotrophic) and should be considered for lipid production.

Some microalgae species can survive under phototrophic or heterotrophic or both conditions.

Furthermore, the CO₂ 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 lipids². Heterotrophic and mixotrophic microalgae grow much faster than phototrophic microalgae and generate a more significant amount of cellular lipid³. Table 1 shows different metabolic conditions (mixo or heterotrophic) for some microalgae species

with their respective lipid content.

According to Tan, et al. the organic load of wastewater can positively affect lipid production by microalgae mixotrophically⁴. Thuy Lan Chi, Mathimani study showed that nutrient scarcity in the stationary phase increased lipid and reduced protein content of the biomass⁵. 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-deprived⁶.

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 microalgae¹.

During optimal growth conditions, *Chlorella vulgaris* can yield 5-58% lipids in dry biomass weight, especially during unfavorable growth conditions⁷, consisting of neutral lipids, glycolipids, hydrocarbons, phospholipids, and small amounts of free fatty acids⁸. 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 times⁹.

In order to reduce microalgal production costs, it is essential to find cheap organic substrates that meet the nutritional needs of lipid production by

microalgae¹⁰. 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 source¹. 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 treatment⁹. 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 cells¹¹; (2) achieving a substantial level of nutrient removal from wastewater, and meeting increasingly stringent discharge and reuse standards¹²; (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 biocomposites¹⁴⁻¹⁶. 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.

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
<i>Botryococcus terribilis</i>	Mixotrophic	9.5 – 25	C16:0, C16:1, C17:0, C18:0, C18:1, C18:2	17
<i>Botryococcus braunii</i>	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
<i>Chlorella protothecoides</i>	Heterotrophic*	47.0 – 50.6	-	19
<i>Chlorella pyrenoidosa</i>	Mixotrophic	30.76	C12:0, C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, C18:3, C20:0, C22:0	10
<i>Chlorella</i> sp.	Mixotrophic	20,94	C16:0, C18:0, C16:1, C18:1 e C18:3	20
<i>Chlorella sorokiniana</i>	Mixotrophic	25 – 35	-	21
		15.07	C14:0, C16:0, C16:1, C18:1, C18:2, C20:1	22
		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
	Mixotrophic	27	C16:0, C16:1, C16:2, C16:3, C18:0, C18:1, C18:2, C18:3	23
<i>Chlorella vulgaris</i>		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
<i>Chlorococcum</i> sp.	Heterotrophic	39-42	C12:0, C14:0, C16:0, C16:1, C16:2, C18:0, C18:1, C18:2, C18:3	26
<i>Scenedesmus obliquus</i>	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
<i>Scenedesmus</i> 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
<i>Scenedesmus incrassulatus</i>	Mixotrophic	50.25	C12:0, C14:0, C16:0, C18:0, C18:1, C18:2, C18:3, C20:0, C22:1	28
<i>Thalassiosira pseudonana</i>	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
<i>Thraustochytrium</i> 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 (myristic 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.

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 methods³² and presented 514.6 mg L⁻¹ CaCO₃ of alkalinity, 8.0 mg L⁻¹ (O₂) of Biochemical Oxygen Demand (BOD₅, 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 x 7.8 mm) column (Phenomenex) at 85°C³³. 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 BF₃/methanol method³⁴. 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). Polimethylsiloxane 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

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).

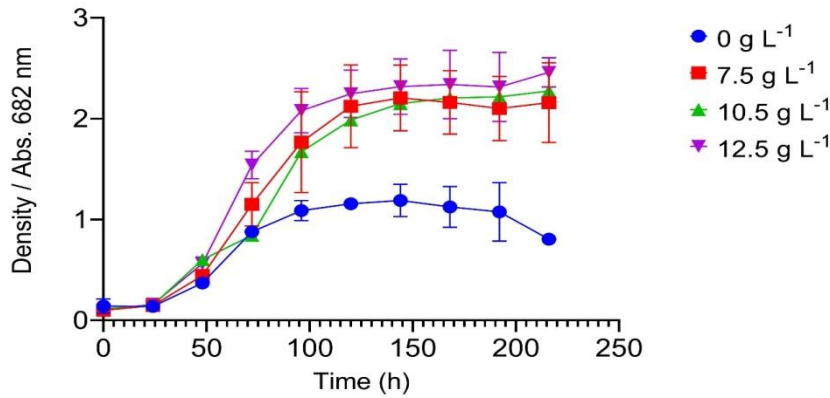


Figure 1: Growth curves of microalgae in sanitary wastewater supplemented with different glycerol concentrations (g L⁻¹). Data are displayed as mean ± standard deviation, n = 3

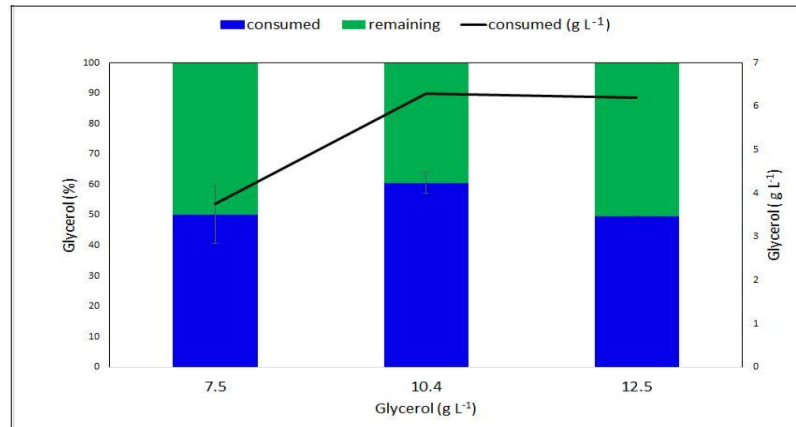


Figure 2: Glycerol consumption (g L⁻¹) in the microalgal cultivation

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 glycerol³⁵.

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.

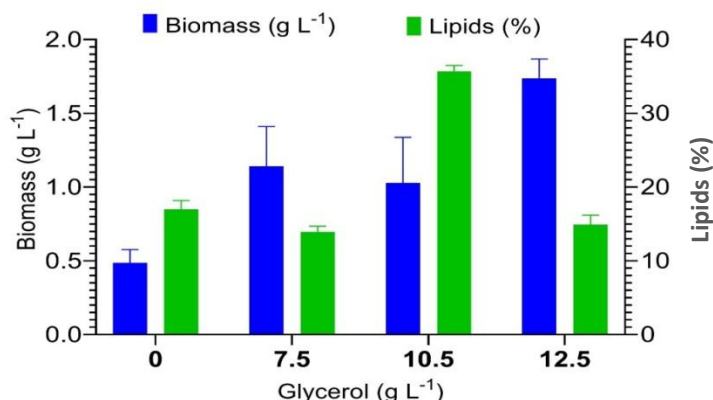


Figure 3: Biomass and lipids yield in relation to glycerol supplementation. Data are displayed as mean \pm standard deviation, $n = 3$.

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, Paranjape³⁶, who supplemented xylose and glycerol to microalgae, the indigenous microalgae could grow more mixotrophically than phototrophically. Liang, Sarkany and Cui²⁴ also supplemented glycerol in

the *C. vulgaris* cultivation, highlighting the use of 1% of glycerol to improve growth. In Rana and Prajapati¹⁰ 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, Tanaka³⁷ reported that glycerol could increase endogenous glycerol kinase overexpression, promoting biomass and metabolite production.

Dario, Balmant³⁸ 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, Fortunato³⁹, 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 medium⁴⁰. 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, Tuo²³, 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 microalgae³⁵. 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 wetlands⁴¹.

The results obtained can be improved if the cultivation is carried out with nutrient control as

performed by Sengmee, Cheirsilp⁴², 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 medium³⁵. 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, Tuo⁴³ 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, Dujjanutat²⁸ 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 Chlorophyceae (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, Singh³⁰, a decrease was found in the content of total polyunsaturated fatty acids by increasing the glycerol concentration. In the study by Rattanapoltee, Dujjanutat²⁸ 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 Prajapati¹⁰ highlighted that with *Chlorella pyrenoidosa* grown in wastewater supplemented with residual glycerol,

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 m³ of wastewater, there were more total lipids from 10.5 g L⁻¹ of glycerol supplementation (Figure 4).

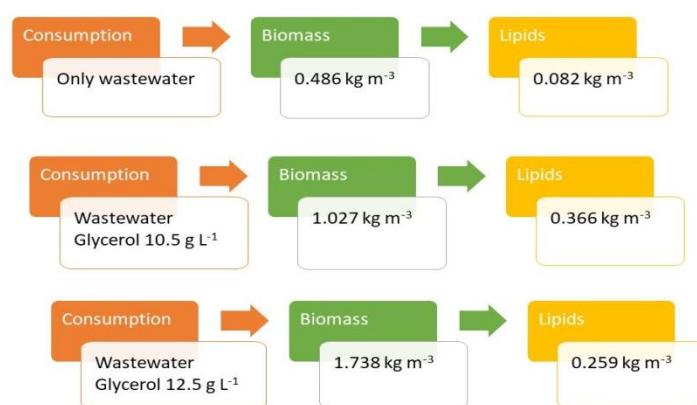


Figure 4: Prospect average biomass and lipids production in 1 m³ of wastewater in 10 days of cultivation

It has been highlighted that the concentration of N and P are also relevant to improving the yield in biomass and lipids⁴⁶. 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 pathway⁴⁷. According to Xue, Chen and Jiang⁴⁸, 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

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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Referencias

1. Ma X, Mi Y, Zhao C, et al. A comprehensive review on carbon source effect of microalgae lipid accumulation for biofuel production. *Sci Total Environ.* 2022;806(Pt 3):151387.
2. Cheng P, Huang J, Song X, et al. Heterotrophic and mixotrophic cultivation of microalgae to simultaneously achieve furfural wastewater treatment and lipid production. *Bioresour Technol.* 2022;349:126888.
3. Hu R, Cao Y, Chen X, et al. WITHDRAWN: Progress on microalgae biomass production from wastewater phycoremediation: Metabolic mechanism, response behavior, improvement strategy and principle. *Chem Eng J.* 2022:137187.[In Press]
4. Tan XB, Yang LB, Zhang WW, et al. Lipids production and nutrients recycling by microalgae mixotrophic culture in anaerobic digestate of sludge using wasted organics as carbon source. *Bioresour Technol.* 2020;297:122379.
5. Thuy Lan Chi N, Mathimani T, Manigandan S, et al. Small scale photobioreactor, outdoor open pond cultivation of *Chlorella* sp. and harvesting at log and stationary growth phase towards lipids and methyl ester production. *Fuel.* 2022;319:123813.
6. Anto S, Pugazhendhi A, Mathimani T. Lipid enhancement through nutrient starvation in *Chlorella* sp. and its fatty acid profiling for appropriate bioenergy feedstock. *Biocatalysis and Agricultural Biotechnology.* 2019;20:101179.
7. Safi C, Zebib B, Merah O, et al. Morphology, composition, production, processing and applications of *Chlorella vulgaris*: A review. *Renew Sustain Energy Rev.* 2014;35:265-78.
8. Dragone G. Challenges and opportunities to increase economic feasibility and sustainability of mixotrophic cultivation of green microalgae of the genus *Chlorella*. *Renew Sustain Energy Rev.* 2022;160:112284.
9. Do CVT, Pham MHT, Pham TYT, et al. Microalgae and bioremediation of domestic wastewater. *Curr Opin Green Sustain Chem.* 2022;34:100595.
10. Rana MS, Prajapati SK. Stimulating effects of glycerol on the growth, phycoremediation and biofuel potential of *Chlorella pyrenoidosa* cultivated in wastewater. *Environ Technol Innov.* 2021;24:102082.
11. Pooja K, Priyanka V, Rao BCS, et al. Cost-effective treatment of sewage wastewater using microalgae *Chlorella vulgaris* and its application as bio-fertilizer. *Energy Nexus.* 2022;7:100122.
12. Wang JH, Zhang TY, Dao GH, et al. Microalgae-based advanced municipal wastewater treatment for reuse in water bodies. *Appl Microbiol Biotechnol.* 2017;101(7):2659-75.
13. De Souza MP, Hoeltz M, Gressler PD, et al. Potential of Microalgal Bioproducts: General

- Perspectives and Main Challenges. Waste Biomass Valorization. 2018;10(8):2139-56.
14. Vargas-Estrada L, Longoria A, Okoye PU, et al. Energy and nutrients recovery from wastewater cultivated microalgae: Assessment of the impact of wastewater dilution on biogas yield. Bioresour Technol. 2021;341:125755.
 15. González-Balderas RM, Felix M, Bengoechea C, et al. Development of composites based on residual microalgae biomass cultivated in wastewater. European Polymer Journal. 2021;160:110766.
 16. Amaral ET, Rizzetti TM, De Souza MP, et al. Opportunities and challenges in algal biofuel. In: Pandey VC, editor. Algae and Aquatic Macrophytes in Cities. Elsevier; 2022.
 17. Cabanelas ITD, Arbib Z, Chinalia FA, et al. From waste to energy: Microalgae production in wastewater and glycerol. Applied Energy. 2013;109:283-90.
 18. Choi HJ, Yu SW. Influence of crude glycerol on the biomass and lipid content of microalgae. Biotechnol Biotechnol Equip. 2015;29(3):506-13.
 19. Feng X, Walker TH, Bridges WC, et al. Biomass and lipid production of *Chlorella protothecoides* under heterotrophic cultivation on a mixed waste substrate of brewer fermentation and crude glycerol. Bioresour Technol. 2014;166:17-23.
 20. Wang X, Qin ZH, Hao TB, et al. A combined light regime and carbon supply regulation strategy for microalgae-based sugar industry wastewater treatment and low-carbon biofuel production to realise a circular economy. Chem Eng J. 2022;446:137422.
 21. Paranjape K, Leite GB, Hallenbeck PC. Effect of nitrogen regime on microalgal lipid production during mixotrophic growth with glycerol. Bioresour Technol. 2016;214:778-86.
 22. Cheah WY, Show PL, Juan JC, et al. Enhancing biomass and lipid productions of microalgae in palm oil mill effluent using carbon and nutrient supplementation. Energy Convers Manag. 2018;164:188-97.
 23. Ren H, Tuo J, Addy MM, et al. Cultivation of *Chlorella vulgaris* in a pilot-scale photobioreactor using real centrate wastewater with waste glycerol for improving microalgae biomass production and wastewater nutrients removal. Bioresour Technol. 2017;245(Pt A):1130-8.
 24. Liang Y, Sarkany N, Cui Y. Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. Biotechnol Lett. 2009;31(7):1043-9.
 25. Gupta PL, Choi HJ, Lee SM. Enhanced nutrient removal from municipal wastewater assisted by mixotrophic microalgal cultivation using glycerol. Environ Sci Pollut Res Int. 2016;23(10):10114-23.
 26. Ummalyma SB, Sukumaran RK. Cultivation of microalgae in dairy effluent for oil production and removal of organic pollution load. Bioresour Technol. 2014;165:295-301.
 27. Xu S, Elsayed M, Ismail GA, et al. Evaluation of bioethanol and biodiesel production from *Scenedesmus obliquus* grown in biodiesel waste glycerol: A sequential integrated route for enhanced energy recovery. Energy Convers Manag. 2019;197:111907.
 28. Rattanapoltee P, Dujjanutat P, Muanruksa P, et al. Biocircular platform for third generation biodiesel production: Batch/fed batch mixotrophic cultivations of microalgae using glycerol waste as a carbon source. Biochem Eng J. 2021;175:108128.
 29. Baldisserotto C, Sabia A, Guerrini A, et al. Mixotrophic cultivation of *Thalassiosira pseudonana* with pure and crude glycerol: Impact on lipid profile. Algal Research. 2021;54:102194.
 30. Gupta A, Singh D, Barrow CJ, et al. Exploring potential use of Australian thraustochytrids for the bioconversion of glycerol to omega-3 and carotenoids production. Biochemical Engineering Journal. 2013;78:11-7.
 31. Chen CY, Lee MH, Leong YK, et al. Biodiesel production from heterotrophic oleaginous microalga *Thraustochytrium* sp. BM2 with enhanced lipid accumulation using crude

- glycerol as alternative carbon source. *Bioresour Technol.* 2020;306:123113.
32. APHA. Standard Methods For the Examination of Water and Wastewater. American Public Health Association, Water Environment Federation, 2012.
 33. Monosik R, Magdolen P, Stredansky M, et al. Monitoring of monosaccharides, oligosaccharides, ethanol and glycerol during wort fermentation by biosensors, HPLC and spectrophotometry. *Food Chem.* 2013;138(1):220-6.
 34. Martini FA, Rubert A, de Souza MP, et al. Periphytic biomass composition and exploitation from algae turf scrubber system. *SN Appl Sci.* 2019;1(7):9.
 35. Ma X, Zheng H, Addy M, et al. Cultivation of *Chlorella vulgaris* in wastewater with waste glycerol: Strategies for improving nutrients removal and enhancing lipid production. *Bioresour Technol.* 2016;207:252-61.
 36. Leite GB, Paranjape K, Abdelaziz AEM, et al. Utilization of biodiesel-derived glycerol or xylose for increased growth and lipid production by indigenous microalgae. *Bioresour Technol.* 2015;184:123-30.
 37. Muto M, Tanaka M, Liang Y, et al. Enhancement of glycerol metabolism in the oleaginous marine diatom *Fistulifera solaris* JPC DA0580 to improve triacylglycerol productivity. *Biotechnol Biofuels.* 2015;8(1):4.
 38. Dario PP, Balmant W, Lírío FR, et al. Lumped intracellular dynamics: Mathematical modeling of the microalgae *Tetradismus obliquus* cultivation under mixotrophic conditions with glycerol. *Algal Research.* 2021;57:102344.
 39. Villanova V, Fortunato AE, Singh D, et al. Investigating mixotrophic metabolism in the model diatom *Phaeodactylum tricornutum*. *Philos Trans R Soc Lond B Biol Sci.* 2017;372(1728):20160404.
 40. Ferreira GF, Pessoa JGB, Ríos Pinto LF, et al. Mono- and diglyceride production from microalgae: Challenges and prospects of high-value emulsifiers. *Trends Food Sci Technol.* 2021;118:589-600.
 41. de Souza Celente G, Colares GS, da Silva Araujo P, et al. Acute ecotoxicity and genotoxicity assessment of two wastewater treatment units. *Environ Sci Pollut Res Int.* 2020;27(10):10520-7.
 42. Sengmee D, Cheirsilp B, Suksaroge TT, et al. Biophotolysis-based hydrogen and lipid production by oleaginous microalgae using crude glycerol as exogenous carbon source. *Int J Hydrogen Energy.* 2017;42(4):1970-6.
 43. Ren H, Tuo J, Addy MM, et al. Cultivation of *Chlorella vulgaris* in a pilot-scale photobioreactor using real centrate wastewater with waste glycerol for improving microalgae biomass production and wastewater nutrients removal. *Bioresour Technol.* 2017;245:1130-8.
 44. Amaral MS, Loures CCA, Pedro GA, et al. An unconventional two-stage cultivation strategy to increase the lipid content and enhance the fatty acid profile on *Chlorella minutissima* biomass cultivated in a novel internal light integrated photobioreactor aiming at biodiesel production. *Renew Energy.* 2020;156:591-601.
 45. Sheng Y, Mathimani T, Brindhadevi K, et al. Combined effect of CO₂ concentration and low-cost urea repletion/starvation in *Chlorella vulgaris* for ameliorating growth metrics, total and non-polar lipid accumulation and fatty acid composition. *Sci Total Environ.* 2022;808:151969.
 46. Rehman M, Kesharvani S, Dwivedi G, et al. Impact of cultivation conditions on microalgae biomass productivity and lipid content. *Mater Today Proc.* 2022;56(1):282-90.
 47. Perez-Garcia O, Escalante FM, de-Bashan LE, et al. Heterotrophic cultures of microalgae: metabolism and potential products. *Water Res.* 2011;45(1):11-36.
 48. Xue LL, Chen HH, Jiang JG. Implications of glycerol metabolism for lipid production. *Prog Lipid Res.* 2017;68:12-25.