UFSM

CIÊNCIA e NATURA

ISSN 2179-460x
Acesso aberto

Ci. e Nat., Santa Maria, v. 44, e48, 2022 • https://doi.org/10.5902/2179460X69474
Submitted: 04/03/2022 • Approved: 12/09/2022 • Published: xx/xx/20xx

Environment

Effects of culture medium recycling in the chemical composition of *Spirulina platensis* biomass cultivated in semi-continuous mode

Efeitos do reciclo de meio cultivo na composição química da biomassa de *Spirulina platensis* cultivada em modo semicontínuo

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ABSTRACT

Commercial microalgae cultivations use the semi-continuous mode, but there are no reports of using this cultivation mode to produce carbohydrate-rich biomass. It was evaluated the biomass productivity and intracellular carbohydrates by Spirulina platensis in semi-continuous cultivation. Besides, the reuse of the culture media after the blend was accomplished in order to obtain nutrient-depleted cultures. In the first stage (Step 1), performed in closed Erlenmeyer's flasks, it was verified that the blend concentration and renewal rate did not influence the intracellular concentration of carbohydrates. However, the interaction of variables influenced cell concentration and carbohydrates yields, being the best results obtained with blend concentration and renewal rate of 0.5 g/L and 60%, respectively. In Step 2, realized in 10 L open mini raceways, the cultures remained viable up to 3 cycles in semi-continuous mode, obtaining mean carbohydrate contents of 41.7% (w/w) and mean carbohydrate yield of 69.3 mg/L d using the media Zarrouk 30% and 70% renewal rate. These values were higher than those observed in experiments in closed bioreactors. Therefore, it was demonstrated that semi-continuous cultivation is viable to obtain biomass for bioethanol production, which represents a possibility of reusing nutrients from the culture medium itself for this purpose.

Keywords: Bioethanol; Carbohydrates; Microalgae; Sustainability; Ultrafiltration

RESUMO

Os cultivos comerciais de microalgas utilizam o modo semicontínuo, mas não há relatos de uso desse modo de cultivo para produzir biomassa rica em carboidratos. Nesse contexto, avaliou-se a produtividade de biomassa e carboidratos intracelulares por Spirulina platensis cultivada em regime semicontínuo. Além disso, foi realizado o reaproveitamento dos meios de cultura após os cortes para obtenção de cultivos com escassez de nutrientes. Na Etapa 1, verificou-se que a concentração de corte e a taxa de renovação não influenciaram a concentração intracelular de carboidratos. Contudo, a interação das variáveis influenciou a concentração celular e os rendimentos de carboidratos, sendo os melhores resultados obtidos com concentração de corte e taxa de renovação de 0,5 g/L e 60%, respectivamente. Na Etapa 2, as culturas permaneceram viáveis por até 3 ciclos em modo semicontínuo, obtendo teores médios de carboidratos de 41,7% (m/m) e rendimento médio de carboidratos de 69,3 mg/L d, utilizando o meio Zarrouk na taxa de renovação de 30% e 70%. Portanto, foi demonstrado que o cultivo semicontínuo é viável para obtenção de biomassa para produção de bioetanol, o que representa uma possibilidade de reaproveitamento de nutrientes do próprio meio de cultivo para essa finalidade.

Palavras-chave: Bioetanol; Carboidratos; Microalgas; Sustentabilidade; Ultrafiltração

1 INTRODUCTION

When compared to first and second-generation, the bioethanol produced from microalgae biomass (third-generation) has advantages including non-competition with food production by arable land, nutrient cycling capacity, low operating cost, and lower energy consumption, this may increase the sustainability of the process (ESCOBAR *et al*., 2009; NGUYEN *et al*., 2019; SU *et al*., 2017). Besides, numerous high value-added bioproducts can be obtained from microalgae in the concept of integrated biorefineries, which can make biofuel production viable (COSTA *et al*., 2019; DE SOUZA *et al*., 2019; PANCHA *et al*., 2019).

Bioethanol production in the context of biorefineries has been reported in the literature (COSTA *et al*., 2019; DEL RÍO *et al*., 2019; REMPEL *et al*., 2019; SONG *et al*., 2019), being necessary to accumulate intracellular carbohydrates in microalgae for a viable production of third-generation bioethanol (BRAGA *et al*., 2018; ZAPAROLI *et al*., 2020). *Spirulina* has been reported due to its ability to produce high protein levels and antioxidants (DE SOUZA *et al*., 2019; BECKER, 2007). On the other hand, *Spirulina* and other species can accumulate high carbohydrate concentrations under growth stress

conditions, such as nutrient depletion, luminosity, among others (MAGRO *et al*., 2018; MARKOU *et al*., 2013; SALLA *et al*., 2016; ZAPAROLI *et al*., 2020).

According to Juneja *et al*. (2013), the available nutrients, temperature, pH, CO_2 levels, amount and intensity of sunlight, and among others, alter the chemical composition of biomass, which determines its viability in biofuels production. Therefore, conditions that increase both biomass concentration and intracellular compounds, such as carbohydrates, should be investigated.

Semi-continuous mode of microalgae cultivation in open raceways has been used for large-scale cultivation. However, in this mode of process, biomass harvesting occurs more frequently, and the growth curve remains in the exponential phase of growth (RADMANN *et al*., 2007), which does not allow carbohydrate accumulation since the media still contains enough nutrients for growth. In order to provide the nutrient depletion required for carbohydrate accumulation in semi-continuous cultures, one possibility would be the recirculation of media obtained after each blend, reducing the costs with the culture medium and the environmental impacts that could be caused by the effluents, such as acidification and eutrophication (COLLA *et al*., 2019; JUNEJA *et al*., 2013).

To the best of our knowledge, there is no data in the literature yet about the use of semi-continuous cultivation of *Spirulina platensis* to obtain carbohydrate-rich cells, nor the recirculation of ultrafiltrated media to reduce costs with nutrients, water consumption and thus reduce environmental impacts. In this sense, this work aimed to cultivate the *Spirulina platensis* in a semi-continuous mode focusing on the obtaining of high cell concentration and intracellular carbohydrates productivities. Besides, the reuse of the culture media was accomplished to obtain nutrient-depleted culture, allowing better use of the nutrients.

2 MATERIAL AND METHODS

2.1 Microorganism and inoculum maintenance

A *Spirulina platensis* strain was used. The Zarrouk medium (ZARROUK, 1966) was used

for inoculum preparation and maintenance with a concentration of 50%. The inoculum was kept in a thermostatic chamber at 30°C, with illumination provided by fluorescent lamps (12 hours light/dark).

2.2 Cultivation conditions

Cultures were grown in a semi-continuous mode in two steps. The first step was accomplished in closed Erlenmeyer flasks of 1.8 L of useful volume, and the second step in acrylic mini raceways with 10 L useful volume.

In Step 1, the culture medium used was Zarrouk at a concentration of 30% (ZARROUK, 1966), performed by the dilution of this media with distilled water. Cultivation was carried out in closed 2 L Erlenmeyer's flasks with a useful volume of 1.8 L, in a thermostatic chamber at 30°C and 12 hours photoperiod (light/dark), air injection provided from 3W (Boyo) compressors. The initial inoculum concentration was 0.2 g/L. The variables studied were the renewal rate (RR) (30% and 60%) and the blend concentration (BC) (0.5 g/L and 1.0 g/L). Response variables were the intracellular carbohydrate concentration and carbohydrate productivity. The biomass concentration (g/L) and the pH were still determined over the tests. The assays were performed in duplicates (n=2). At each blend, the reactor volume was completed with fresh Zarrouk medium at a concentration of 30%.

In Step 2, the initial inoculum concentration was kept at 0.2 g/L, but the experiments were conducted in acrylic mini raceways (20 L) with a useful volume of 10 L, located in a hydroponic greenhouse with a controlled temperature between 20ºC and 30ºC and uncontrolled luminosity with solar illumination, agitation around 0.35 m/s provided by blade rotation (MAGRO *et al*., 2018). The studied variables were the Zarrouk medium concentration (20% and 30%) and the renewal rate (70% and 90%). However, different from the first step, the blend was made after the cultures reached the stationary phase of growth and remained in this phase for two days. Harvesting was performed by centrifugation at 3,500 rpm and the supernatant was subjected to ultrafiltration

on a 50 kDa cut-off polyethersulfone membrane. The ultra-filtered media was used to complete the bioreactor volumes, seeking reuse of residual nutrients. After the first and second cycles of cultivation in semi-continuous mode, the biomass was separated by centrifugation and dried in an air circulation oven (Tecnal, model TE-394/1) at 50°C until to 10% of humidity and then stored at -20°C until the realization of the analytical determinations.

2.3 Analytical determinations

2.3.1 Cell concentration and pH

The determination of biomass concentration during cultivation was performed every 24 hours by measuring optical density in a spectrophotometer (Femto) at 670 nm (RADMANN *et al*., 2007). The pH of the medium was analyzed every 72 hours to determinate the buffering of the medium. The analysis was performed using a digital pH meter.

2.3.2 Quantification of intracellular carbohydrates in biomass

The determination of microalgae intracellular carbohydrate concentration was performed according to Dubois *et al*. (1956). For intracellular carbohydrates to be quantified by the method, it was necessary to prepare a cell extract. For this purpose, a 5 mg/10 mL biomass suspension of distilled water was prepared and then sonicated in an ultrasound probe to rupture the microalgae cell wall. Intracellular carbohydrate determination was performed at each blend during semi-continuous cultivation.

2.4 Growth kinetic parameters

After the growth curves construction, the following parameters were analyzed: final cell biomass concentration (X_f, g/L); biomass productivity (P_{max}, g/L d), obtained for the Equation (1) (where X: biomass concentration (g/L) at time t (d); X_0: biomass concentration (g/L) at time t_0 (d)); maximum specific growth velocity (μ_{max}, d^{-1}), obtained by exponential

regression applied to the logarithmic growth phase, through Equation (2), being μ_{max}: maximum specific growth rate (d^{-1}); X_1: initial biomass concentration (g/L); X_2: final concentration (g/L); t: time (d).

$$\#\phi(x) = \sum^{k} (\#n_i + 1) \tag{1}$$

$$y_j = \underbrace{x_j x_j \ldots x_j}_{n_i} \tag{2}$$

The generation time (d) was calculated using the maximum specific growth velocities, according to Equation (3).

$$\sum^{k} \tag{3}$$

Using the values of intracellular carbohydrate determination, carbohydrate productivity was determined by Equation (4), being X_f: final concentration (g/L); X_0: initial concentration (g/L) in each cycle of cultivation; CHO: carbohydrate content in the biomass in percentage; tc: cultivation time (d) of each cycle of cultivation in semi-continuous cultivation.

$$\pi = 11 \quad 11 \tag{4}$$

2.5 Data processing and statistical analysis

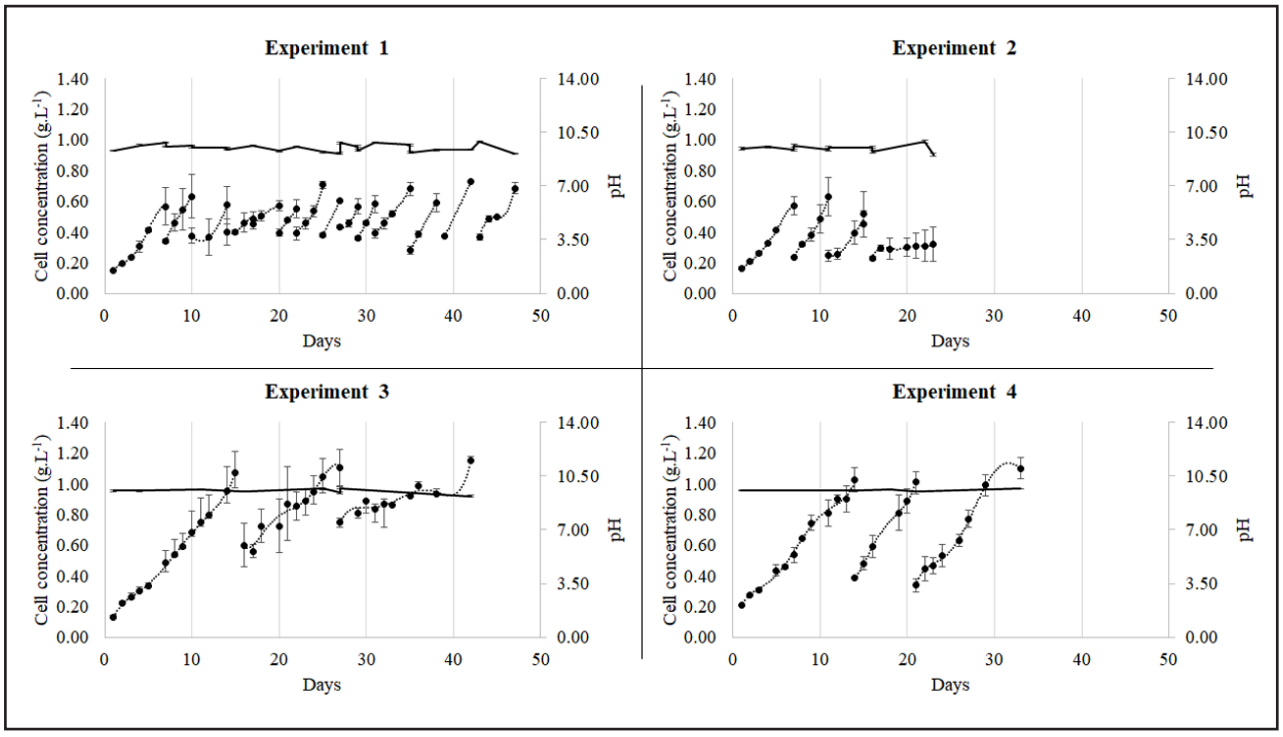
The effect of variables on intracellular carbohydrate content and carbohydrate productivities in the cultures was evaluated through the Experimental Design module of *Statistica* Software 5.5, which estimates the main effects and the interaction of the analyzed variables. Standard error was calculated based on tests performed in

duplicate, with a 95% confidence level.

3 RESULTS AND DISCUSSION

The results of biomass concentration (g L^{-1}) and pH over the cultivation time (h) of the first step assays performed in 1.8 L Erlenmeyer's flasks are shown in Figure 1. The pH of the assays remained in the range of 8.3 and 11.0 (Figure 1), ideal for *Spirulina* cultivation (COSTA *et al*., 2002). After the beginning of cultivation, the pH of the culture medium gradually increases and, consequently, there is an increase in the carbon source available to microalgae through the conversion of bicarbonate to carbonate, which leads to an increase in biomass productivity (TOURANG *et al*., 2019).

Figure 1 – Biomass and pH concentration as a function of time for Step 1 experiments



Source: The authors (2022)

Legend: a) Experiment 1 - BC: 0.5 g/L; RR: 30%; b) Experiment 2 - BC: 0.5 g/L; RR: 60%; c) Experiment 3 - BC: 1.0 g/L; RR: 30%; d) Experiment 4 - BC: 1.0 g/L; RR: 60%. BC: concentration in the culture when reached a predetermined level, named "blend concentration"; RR: a portion of the medium removed and substituted by fresh media, named "renewal rate". Full line: results of pH. Dotted line: results of cell concentration

Table 1 – Specific growth rate (μ_x) and generation time (t_d) for the experiments of Step 1

Cycle	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
	μ_x (d^{-1})**	t_d (d)**	μ_x (d^{-1})**	t_d (d)**	μ_x (d^{-1})**	t_d (d)**	μ_x (d^{-1})**	t_d (d)**
1	0.278 ±0.032	2.491 ±0.291	0.205 ±0.029	3.414 ±0.477	0.111 ±0.006	6.235 ±0.360	0.109 ±0.002	6.369 ±0.128
2	0.206 ±0.093	3.371 ±1.866	0.236 ±0.061	3.599 ±0.790	0.047 ±0.054	13.012 ±2.279	0.138 ±0.004	5.292 ±0.168
3	0.142 ±0.029	4.865 ±1.024	0.160 ±0.019	4.203 ±0.492	0.026 ±0.003	25.884 ±2.920	0.1244 ±0.024	5.674 ±1.093
4	0.135 ±0.020	5.129 ±0.801	*	*	0.075 ±0.037	10.399 ±5.096	*	*
5	0.079 ±0.014	8.797 ±1.645	*	*	*	*	*	*
6	0.166 ±0.078	4.188 ±2.265	*	*	*	*	*	*
7	0.219 ±0.024	3.512 ±0.346	*	*	*	*	*	*
8	0.154 ±0.004	2.981 ±0.012	*	*	*	*	*	*
9	0.131 ±0.036	5.353 ±1.530	*	*	*	*	*	*
10	0.238 ±0.029	2.907 ±0.362	*	*	*	*	*	*
11	0.139 ±0.004	5.022 ±0.159	*	*	*	*	*	*
12	0.243 ±0.001	2.848 ±0.017	*	*	*	*	*	*
13	0.101 ±0.006	6.922 ±0.444	*	*	*	*	*	*
14	0.162 ±0.022	4.792 ±0.707	*	*	*	*	*	*

Source: The authors (2022)
Experiment 1: BC 0.5 g/L; RR 30%; Experiment 2: BC 0.5 g/L; RR 60%; Experiment 3: BC 1.0 g/L; RR 30%; Experiment 4: BC 1.0 g/L; RR 60%.
* Not determined values
** Mean values (n=2) ± standard deviation

μ_x: specific growth rate; t_d: generation time; BC: blend concentration; RR: renewal rate

Experiment 1 (BC: 0.5 g/L; RR: 30%) resulted in a higher number of cycles of cultivation (14) (Figure 1), the cultures reaching the blend concentration in approximately 3 days. In Experiment 2 (BC: 0.5 g/L and RR: 60%), only 3 growth cycles were possible as a result of the higher dilution promoted by the RR of 60% in comparison with Experiment 1.

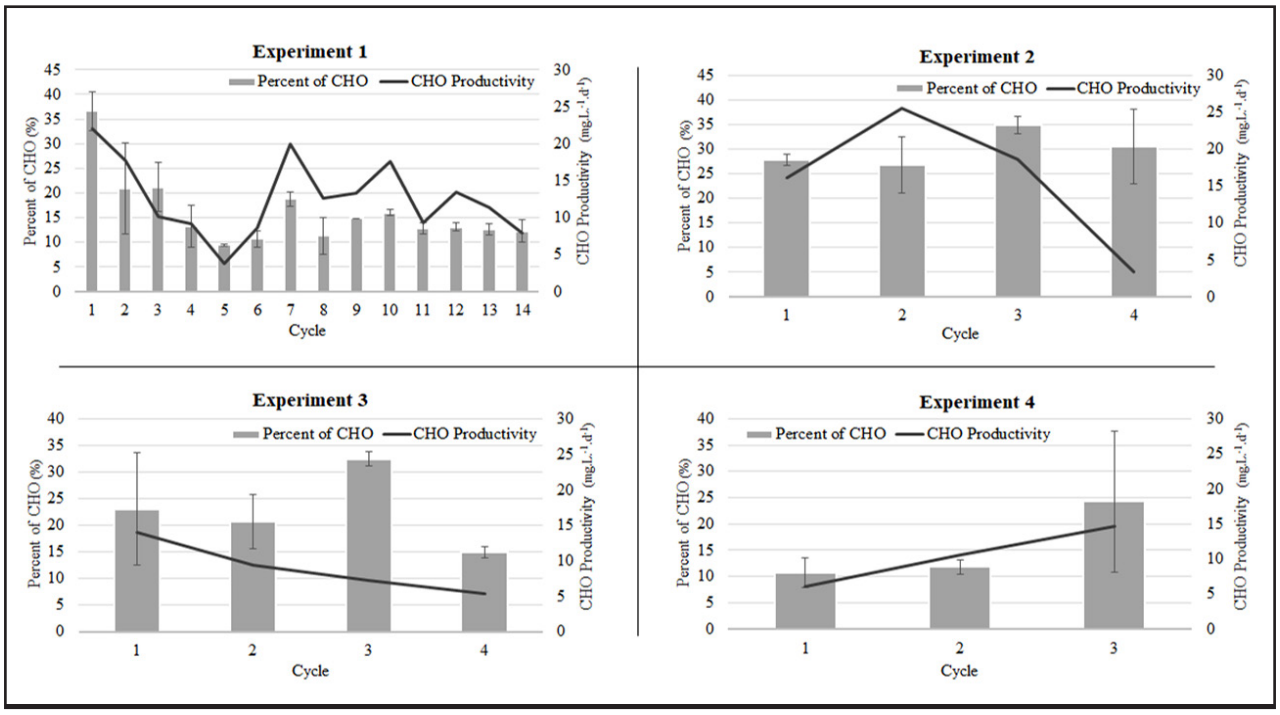
Experiment 2 presented cell death in the fourth cycle of semi-continuous cultivation (identified by the yellowish coloration and the formation of lumps and sedimentation), probably caused by the effect of decreased cell concentration due to the high renewal rate (60%) and light availability. The hypothesis is that the cells presented sensitivity to light once after each blend, the cell concentration of the culture returned to 0.2 g/L. Excessive lighting can affect biomass production and CO_2 fixation capacity, resulting in the effect known as photoinhibition (HO *et al*., 2012).

With a blend concentration of 1.0 g/L and a renewal rate of 30% or 60% (Experiments 3 and 4), the growth behavior was similar, with 4 cycles of cultivation. The maximum specific growth rates were lower than the observed in Experiments 1 and 2 (Table 1), probably related to the fact that the specific growth rate tends to decrease with increasing biomass concentrations due to the shading effect.

Comparing the patterns of growth culture of these experiments with those of literature, it can be verified that the values found of maximum specific growth rates varied a lot depending on the blend concentrations and renewal rates studied. Radmann *et al*. (2007) obtained maximum specific growth velocities of 0.1377 d^{-1} under conditions of renewal rate (40%) and blend concentration of 0.40 g/L. The differences may be related to the cultivation conditions, as well as the size and type of the reactors used.

Semi-continuous cultures with shorter growth cycles tend to produce higher concentrations of biomass and productivity, since the higher the renewal rate, the lower the biomass concentration and the greater the availability of light (MOREIRA *et al*., 2016). This phenomenon was also observed in our study because when the renewal rate was high, the cell concentrations obtained were lower. This is due to the greater effect of dilution, which resulted in higher specific speeds because there is no shading. Results of intracellular carbohydrate composition of biomass and carbohydrate productivities after each blend of the experiments of Step 1 are shown in Figure 2.

Figure 2 – Number of cycles performed, carbohydrate concentration (CHO) and carbohydrate yield for semi-continuous *Spirulina* mode crops in Step 1



Source: The authors (2022)

Legend: (Experiment 1: BC: 0.5 g/L; RR: 30%; Experiment 2: BC: 0.5 g/L; RR: 60%; Experiment 3: BC: 1.0 g/L; RR: 30%; Experiment 4: BC: 1.0 g/L; TR 60%). BC: blend concentration; RR: renewal rate; CHO: carbohydrates

Experiment 1 presented above 30% CHO at the end of the first growth cycle (Figure 2). After the third cycle, there was a reduction of less than 15%. Carbohydrate productivity in cycle 1 reached 0.022 g/L/d but reduced to 0.006 g/L/d in the last cycle. The experiment with the highest carbohydrate productivity was Experiment 2, carried out at 0.5 g/L blend concentration and 60% renewal rate. This behavior may be related to the cellular stress caused by the increase of luminosity obtained after the blend in this treatment (LEE *et al*., 2012). In the discontinuous cultivation performed by Zhang *et al*. (2015), the maximum productivity in *Spirulina platensis* cells was achieved with a luminosity of 200 µmol photons $m^{-2}.s^{-1}$, and the light intensity above this value did not increase the biomass productivity. On the other hand, after cycle 2, the cells of Experiment 2 could not adapt to the reduction of cell concentration (0.2 g/L) and, due to the increase in luminosity, there was lump formation and cell sedimentation.

Consequently, a reduction in productivity was observed.

Da Rosa *et al*. (2015) reached 28.2% of carbohydrates in a semi-continuous system with the addition of monoethanolamine. Thus, the carbohydrate levels obtained in our study are in agreement with the values found for *Spirulina platensis* in the work mentioned. Experiment 2 was the one with the highest value (30%), followed by Experiments 3, 1, and 4, with 22.76%, 17.45%, and 15.54% of intracellular carbohydrates, respectively (Figure 2). In other studies of our research group, higher intracellular contents of carbohydrates were obtained in discontinuous cultivation mode.

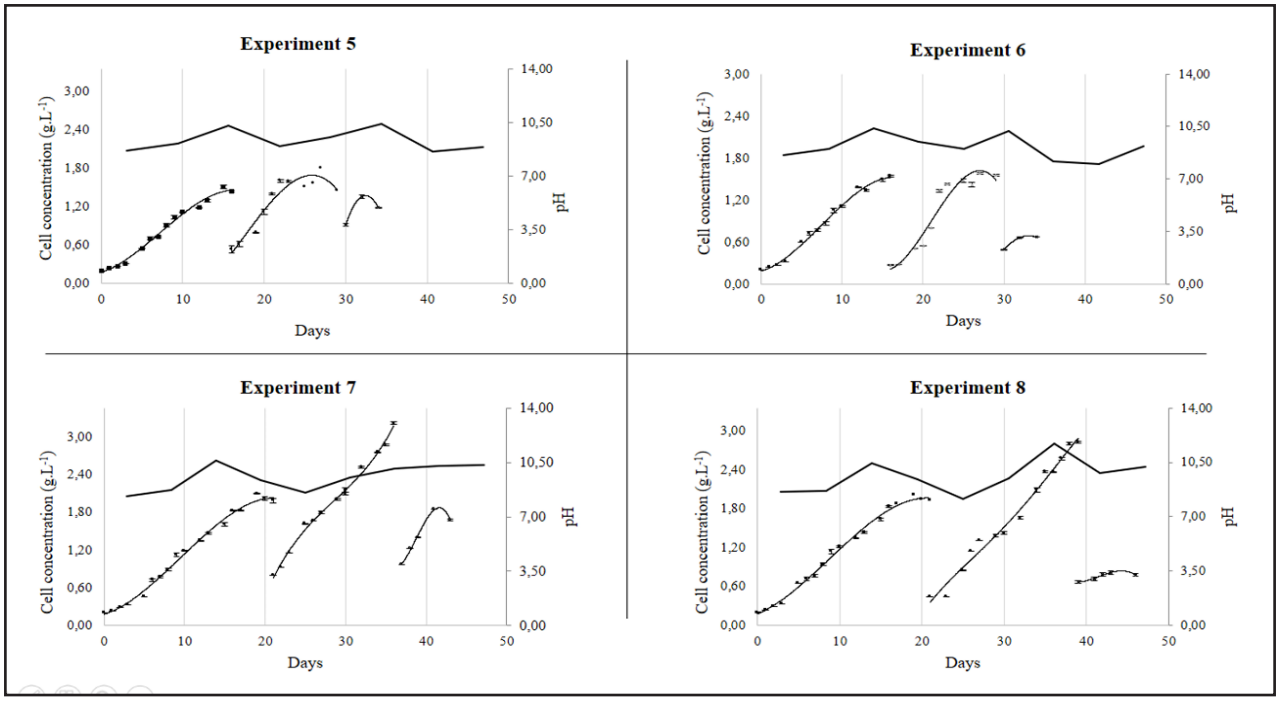
The analysis of the effects of variables on the carbohydrate yield and carbohydrate content obtained in the biomass resulted in no significance to the main effects of the variables ($p=0.2811$ and 0.3117 to the influence of RR and $p=0.0742$ and 0.2597 to the influence of BC in CHO productivity (mg/L d) and CHO (%) in the biomass). However, the interaction of the variables had a significant effect ($p=0.0224$) on carbohydrate content, being the higher values of CHO contents obtained using the blend concentration of 0.5 g/L and 60% renewal rate, probably related to the cellular stress caused by the abrupt increase of brightness after the blend in Experiment 2. However, no influences of the interaction of variables were obtained on carbohydrates productivities ($p=0.6678$).

As the individual effects of the variables were not significant, it was decided to perform the cultivation of Step 2, using mini raceways with Zarrouk medium concentration (30% and 20%) and increasing the renewal rate (70% and 90%). With this strategy of cultivation, the authors aimed to verify the resilience of the cultures operating in semi-continuous mode, since with the reduction in the concentration of Zarrouk media (30% to 20%), associated with an increase in the renewal rate, fewer nutrients would be available to the microalgae cells. Besides, the media removed at each blend was ultra-filtrated and recirculated to the tank.

Figure 3 presents the results of biomass concentration (g/L) and pH over the

cultivation time (d) to the Experiments of Step 2, performed on 10 L mini-raceways

Figure 3 – Concentration of biomass and pH as a function of time for the experiments of Step 2



Source: The authors (2022)

The pHs varied in the cultures ranged from 8.2 to 10.7 in experiments. In general, pH values increased and tended to stabilize around 10 during cultivation. Recycling of culture media can affect pH. Tourang *et al*. (2019) observed a stable pH throughout the analyzed period, with a slight increase when the growth cycles showed steady behavior. However, these authors also report a reduction in pH when the culture medium was added to the bioreactor with either a new or recycled medium, a behavior that was also observed in our assays (Figure 3). The pH increase in photosynthetic cultures occurs due to the biological activity of cells, which reduces the dissolved inorganic carbon content due to consumption during cell growth (CUARESMA *et al*., 2006).

The generation time (td) in the Experiments of Step 2 ranged from 4 to 9.8 days (Table 2). Experiment 6, conducted with a 90% renewal rate and 20% Zarrouk medium concentration, showed adequate growth in the first two cycles, and the

third cycle (after 72 h) showed cell death.

Table 2 – Specific growth rate (μ_x) and generation time (t_d) for the experiments performed for Step 2

Cycle	Experiment 5		Experiment 6		Experiment 7		Experiment 8	
	μ_x (d^{-1})**	t_d (d)**	μ_x (d^{-1})**	t_d (d)**	μ_x (d^{-1})**	t_d (d)**	μ_x (d^{-1})**	t_d (d)**
1	0.119 ±0.0006	5.827 ±0.027	0.142 ±0.0018	4.898 ±0.063	0.126 ±0.0007	5.511 ±0.029	0.093 ±0.0013	7.484 ±0.101
2	0.118 ±0.0007	5.884 ±0.036	0.177 ±0.0022	3.923 ±0.049	0.087 ±0.0013	7.969 ±0.116	0.070 ±0.0011	9.884 ±0.157
3	*	*	*	*	0.162 ±0.0013	4.292 ±0.035	*	*

Source: The authors (2022)

Experiment 5: Zarrouk 20%; RR 70%; Experiment 6: Zarrouk 20%; RR 90%; Experiment 7: Zarrouk 30%; RR 70%; Experiment 8: Zarrouk 30%; TR 90%.
* Not determined values
** Mean values (replicates of analysis) ± standard deviation
μ_x: specific growth rate; t_d: generation time; RR: renewal rate

The use of Zarrouk 30% in Experiments 7 and 8 allowed in higher final concentrations in each cycle (Figure 3) compared to cultures with Zarrouk 20% (Figures 3a and 3b). However, Experiment 7 showed yellowish coloration, which may indicate cell death and bacterial development.

Experiment 8 reached higher biomass concentrations than Experiments 5 and 6 because it was cultivated in a 30% Zarrouk medium. However, due to the 90% renewal rate, the culture presented lower concentrations after the first blend. In cycle 3, this experiment showed no growth, obtaining behavior similar to Experiment 6, which indicates the non-adaptation of microalgae to the high renewal rate (90%). As in other experiments, the culture turned yellow at the end of cycle 2 and did not return to green in cycle 3. In general, experiments of Step 2 presented cell growth behavior similar to that found in the literature (BRAGA *et al*., 2019) during the first and second cycles, in which a new culture medium was used.

It was possible to cultivate the microalgae with recirculation of residual culture medium from the semi-continuous cultivation blend. However, the specific growth rates were lower, possibly due to the scale increase condition and the light conditions of the greenhouse. Table 3 presents the carbohydrate and protein contents and carbohydrate yields obtained during semi-continuous *Spirulina platensis* cultivation in 10 L mini raceways (Step 2).

Table 3 – Carbohydrate and protein content (%) and carbohydrate yield (mgCHO.L/d) in the experiments of Step 2 realized in mini raceways of 10 L (useful volume)

Experiment	RR (%)	Zarrouk concentration (%)	Cycle	Carbohydrates (%)	Protein (%)	CHO Productivity (mgCHO.L/d)
5	70	20	1	32.06 ± 0.85	44.80 ± 1.27	26.65
			2	14.89 ± 1.05	55.80 ± 0.85	11.38
			3	43.25 ± 0.58	32.21 ± 1.89	29.19
6	90	20	1	30.63 ± 0.67	41.38 ± 0.33	25.85
			2	18.78 ± 1.50	51.80 ± 0.92	18.51
			3	13.58 ± 1.89	59.30 ± 0.67	6.79
7	70	30	1	13.96 ± 0.70	57.48 ± 0.54	12.10
			2	58.63 ± 0.62	22.36 ± 1.04	80.62
			3	52.36 ± 0.34	25.42 ± 0.93	115.19
8	90	30	1	14.06 ± 1.34	51.22 ± 1.12	12.19
			2	42.36 ± 0.66	18.59 ± 0.87	55.07
			3	38.72 ± 0.84	23.45 ± 0.66	13.55

Source: The authors (2022)

RR: renewal rate; CHO: carbohydrate
*Mean values (replicates of analysis) ± standard deviation

Experiments 6 showed higher carbohydrate yield in cycle 1 due to cellular stress caused by low nutrient concentration (20%) and a high renewal rate (90%). However, the microorganism did not adapt to environmental conditions in cycle 2, which resulted in a reduction in carbohydrate productivity. According to Table 3, the renewal rate of 70% was better for carbohydrate yield in the third cultivation cycle independently of the Zarrouk concentration (20 or 30%), reaching productivity above 115 mg.CHO.L/d (Experiment 7).

Due to the higher concentration of nutrients in Experiments 7 and 8, *Spirulina* did not suffer cell stress in cycle 1 and, therefore, the CHO contents were lower. However, in cycle 2 the levels are reversed since the blend in the experiments created a stressful environment, which led the microalgae to produce more carbohydrates. This factor is associated with high cell concentration to higher carbohydrate productivity, such as 0.081 g/L d and 0.055 g/L d in the second cycle of Experiments 7 and 8, respectively.

Experiment 7 showed the highest carbohydrate yield values, from 0.081 g/L d in cycle 2 to 0.115 g/L d in cycle 3, demonstrating the viability of carbohydrate production in 30% Zarrouk cultures in a semi-continuous process operated with media recirculation. Except for Experiment 6, all experiments presented higher CHO content in cycle 3, where there was medium reuse, which is due to nutrient scarcity. However, low cell growth reduces productivity, which makes it impossible to reuse the medium for long periods.

Higher carbohydrates accumulation is achieved when the culture medium a low amount of nutrients, which occurs in the stationary phase of *Spirulina* growth when this microalga enter in a physiological stress situation that increases their metabolic processes to produce reserve carbohydrates to prepare the cell for nutrient depletion (MAGRO *et al*., 2018).

3.1 Theoretical use of biomass for bioethanol production

A theoretical production of bioethanol was made considering the experiment that presented the best results of cell growth, carbohydrates accumulation, and resilience to the

conditions of media recirculation in a semi-continuous mode of *Spirulina* cultivation. The simulation of bioethanol production is presented in Table 4. It was considered the data of biomass concentration [X] observed at the moment of the cut and the volume removed to calculate the mass of biomass obtained. Considering the percent of carbohydrates in the biomass in each cycle, the amount of carbohydrates available to be converted in bioethanol was calculated. After, the authors used the efficiencies of saccharification (83%) and fermentation (82%) obtained for Rempel *et al*. (2019) for the same biomass (obtained in discontinuous mode) to calculate the mass of ethanol that could be obtained. Finally, the authors calculated the theoretical yield of ethanol that could be obtained per g of biomass and the estimated production of ethanol per L of *Spirulina* culture per day, in each cycle. It is clear from Table 4, that the biomass obtained in cycle 1 is poor in carbohydrates, which would generate the lowest yield in ethanol. However, considering the second and third cycles of cultivation, in which the chemical composition of the biomasses obtained was similar, the yields of ethanol that could be generated per g of biomass were high. It is important to be mentioned, that the culture media was recycled in the second and third cycles, not involving more costs with nutrients.

Table 4 – Theoretical generation of bioethanol considering values of efficiency of saccharification and fermentation of Rempel *et al*. (2019) in the condition of production of biomass of 70% of renewal rate and using Zarrouk concentration in the first cycle of 30% with recirculation of media ultra-filtrated in the second and third cycles

Cycle	[X] (g/L)	V (L)	t (day)	M (g)	CHO (%)	M_{CHO} (g)	Ethanol (g)*	$Y_{Et/Spi}$	P ($mg_{Et}/L_{Spi\_culture}$.day)
1	2.02	7	21	14.1	13.9	1.97	0.68	0.048	4.78
2	3.0	7	16	21.0	58.6	12.3	4.27	0.203	40.6
3	1.85	7	4	12.9	53.4	6.9	2.40	0.186	38.6

Source: The authors (2022)

Legend: [X] Biomass concentration in the moment of cut (g/L). based on Figure 3c; V (L): volume withdrawn of the mini-raceway in each cut; t (day): time of duration of each cycle, based on Figure 3c; M (g): mass of biomass obtained in each cut; CHO (%): carbohydrates in biomass (%) (based on Table 3); M_{CHO} (g): mass

of carbohydrates obtained in each cut; * Ethanol that could be obtained considering data of efficiency of saccharification (82%) and fermentation (83%) obtained using the same microalga as source of carbohydrates and a stoichiometric coefficient of transformation of reducing sugars to ethanol of 0.511, according to Rempel *et al.* 2020.; $Y_{Et/Spi}$: conversion factor of *Spirulina* in ethanol ($g_{ethanol}/g_{Spirulina}$); P ($g_{Et}/L_{Spi\_culture}$. day): estimated production of ethanol per L of *Spirulina* culture per day, (being t the duration of each cycle)

It was demonstrated that the semi-continuous mode of cultivation can be used to production of biomass of *Spirulina* with high content of carbohydrates if the reuse of media is done. Normally, this method is more appropriate for the accumulation of proteins in cells, as shown in the first step of this work, in which the reuse is not accomplished.

3 CONCLUSIONS

Blend concentration and renewal rate studied did not influence the increase of microalgae carbohydrate concentrations in closed bioreactors. The highest intracellular carbohydrate concentration (about 30%) was obtained in Experiment 2, with a blend concentration of 0.5 g/L and a renewal rate of 60%. However, there was no significant influence of the variables on carbohydrate productivity.

Recirculation of media in 10 L acrylic mini raceways demonstrated that it is possible to maintain viable cultures for up to 3 cycles since it was obtained mean values for carbohydrate content and productivity, of 41.65% and 69.3 mg/L d, respectively, at 30% Zarrouk medium concentration and a 70% renewal rate (Experiment 7), which are higher than those observed in the closed bioreactor experiments realized in our study. Therefore, we demonstrate that semi-continuous production processes can be used to obtain viable biomass for bioethanol production, allowing the reuse of nutrients from the crop itself for this purpose.

ACKNOWLEDGEMENTS

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) - Brasil - Finance Code 001 and Conselho Nacional

de Desenvolvimento Científico e Tecnológico (CNPq) - Brasil.

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How to quote this article

GONÇALVES, G. C.; NAZARI, M. T.; MAGRO, F. G.; MARGARITES, A. C. F.; COLLA, L. M. Effects of culture medium recycling in the chemical composition of *Spirulina platensis* biomass cultivated in semi-continuous mode. **Ciência e Natura**, Santa Maria, v. 44, e48, 2022. Available in: https://doi.org/10.5902/2179460X69747.