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Production of *Spirulina* in semicontinuous cultivation using medium recycle

Ana Priscila Centeno da Rosa*, Thaisa Duarte Santos*, Elisângela Martha Radmann*, Fabrício Butierres Santana*,Jorge Alberto Vieira Costa*
*(College of Chemistry and Food Engineering, Federal University of Rio Grande, Brazil)

ABSTRACT
The biomass of cyanobacteria *Spirulina* is rich in bioactive compounds, with main applications in food and pharmaceutical industry. The objective of this study was to evaluate the effect of nutrient consumption on the growth kinetics of *Spirulina* sp. LEB 18 grown in semicontinuous cultivation in raceway bioreactor. Zarrouk medium was used, maintaining the original concentrations of carbon, nitrogen and phosphorus, while the other nutrients varied by 25%, 50%, 75%, and 100%. Chemical determinations were performed in the biomass for each growth cycle. *Spirulina* sp. LEB 18 exhibited cell growth until approximately 130 days of experiment. The maximum specific growth rate (0.184 d^{-1}) and productivity (48.03 mg L^{-1} d^{-1}) were observed in the trial with 50% Zarrouk medium, except for nitrogen, phosphorus and carbon. Nitrogen and phosphorus concentrations reduced up to 100% and 64.8%, respectively. No significant difference ($p < 0.0003$) was observed in the protein concentrations for all tests. The results showed that *Spirulina* sp. LEB 18 can provide high growth rate and productivity, as well as production of proteic biomass when cultivated with lower nutrients concentration in Zarrouk medium, thus reducing production costs.

***Keywords*-**cyanobacteria, lipids, nutrients, protein, raceway

I. INTRODUCTION

The cultivation of photosynthetic microorganisms, in particular microalgae, is extensively studied because it presents an efficient biological system for use of solar energy in the production of organic compounds [1].

Spirulina is used mainly in food [2, 3] and animal feed [4, 5, 6, 7], due to its high content of protein and fatty acids. This cyanobacteria is used for the treatment of diseases such as hypertension, diabetes, and malnutrition [8, 9] and in the production of energy, biofertilizer, medicines, pigments, vitamins, fatty acids and polysaccharides[10, 11, 12, 13, 14].

According to Grobbelaar [15], one of the most important factors to obtain high biomass productivity is the nutritional content of the culture medium. The use of certain nutrients can alter production costs and affect growth and/or biomass composition [16].

Semicontinuous cultivation has operational advantages, such as the use of the same inoculum, and analysis of nutritional and kinetics parameters for a long period [17, 18]. When this process is carried out using medium recycle technologies, the biomass production cost can be reduced.

This study aimed to investigate different nutrient concentrations of Zarrouk medium, except carbon, nitrogen and phosphorus, and to evaluate the effect of the consumption of these nutrients on kinetic growth parameters of *Spirulina* sp. LEB 18, as well as protein and lipids content of its biomass.

II. MATERIAL AND METHODS

2.1 Microorganism and culture medium

The cyanobacteria *Spirulina* sp. LEB 18 isolated from Mangueira Lagoon, South Brazil [19] was used in the experiments. It was maintained in Zarrouk medium [20] with the following composition (g L^{-1}): solution A [$NaHCO_3$ (16.8); $NaNO_3$ (2.5); K_2HPO_4 (0.5)], and solution B [K_2SO_4 (1.0); NaCl (1.0); $MgSO_4.7H_2O$ (0.2); $CaCl_2$ (0.04); $FeSO_4.7H_2O$ (0.01); EDTA (0.08) and trace element solution of H_3BO_3, $MnCl_2.4H_2O$, $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$, $NaMoO_4$, $K_2Cr_2(SO_4)_4.24H_2O$, NH_4VO_3, $NiSO_4.7H_2O$, $Na_2WO_4.2H_2O$, $TiOSO_4.H_2SO_4.8H_2O$, and $CO(NO_3)_2.6H_2O$].

2.2 Bioreactor and cultivation conditions

The cultivations were performed in 6 L raceway bioreactors, with 5 L working volume and continuous stirring at 18 rpm using rotating blades. The experiments were kept in a thermostatic chamber at 30 °C, 3000 lux, with a photoperiod of 12 h light/dark [21].

Semicontinuous cultivations were performed with initial biomass concentration of 0.15 g L^{-1} and blend concentration of 0.5 g L^{-1}[17]. The cultivation period between each blend concentration was called growth cycle. At the end of each growth cycle, cultures were filtered using quantitative filter paper

(Whatman no. 2) for removal of biomass and reuse of culture medium. Evaporation was controlled by keeping the volume of the cultures with daily replacement of distilled water.

Zarrouk medium was used, maintaining the original concentration of carbon, nitrogen, and phosphorus sources (solution A), while the other nutrients (solution B) varied by 25% (Z-B25), 50% (Z-B50), and 75% (Z-B75) from the original concentration. The medium containing 100% nutrient (Z-100) was also used as the standard.

2.3 Analytical determinations

The biomass concentration and pH measurements were monitored daily. The biomass concentration was determined by measuring the optical density of the cultures at 670 nm in a spectrophotometer (700-Plus Femto, Brazil), using a standard curve that relates the optical density with dry weight of biomass. Digital pHmeter was used to measure pH (Quimis Q.400H, Brazil).

Phosphorus and nitrogen concentrations in the culture medium were evaluated every three days. Phosphorus concentration was determined by colorimetric analysis (PhosVer 3, Hach, USA), and nitrogen was determined using colorimetric method proposed by Cataldo et al. [22].

At the end of each growth cycle, concentrations of protein and lipids in the biomass were evaluated. Protein was determined according to the micro-Kjeldahl method [23]. Lipids were extracted with chloroform/methanol 2:1, and determined gravimetrically [24]

2.4 Determination of kinetic parameters

The following parameters were determined: maximum specific growth rate (μmax, d^{-1}), specific death rate (kd, d^{-1}) [25], mean biomass productivity (P_{mean}, mg L^{-1} d^{-1}) and nitrogen and phosphorus consumption rates (q, mg L^{-1} d^{-1}). The maximum specific growth rate was obtained by linear regression in the logarithmic growth rate. Productivity

(P, mg L^{-1} d^{-1}) was calculated according to the Equation 1, where Xt is the cellular concentration (mg L^{-1}) at time t (d) and X_0 (mg L^{-1}) is the cellular concentration at time t_0 (d).

$$P = \frac{X_t - X_0}{t - t_0} \quad (1)$$

The nitrogen and phosphorus consumption rates were calculated according to the Equation 2, where Ct is the nutrient concentration (mg L^{-1}) at time t (d), and C_0 (mg L^{-1}) is the nutrient concentration at time t_0 (d).

$$q = \frac{C_t - C_0}{t - t_0} \quad (2)$$

2.5 Statistical Analysis

The responses were assessed by analysis of variance (ANOVA) followed by Tukey's test, at 95% ($p \leq 0.05$) confidence level.

III. RESULTS AND DISCUSSION

The experiments were performed by keeping the concentrations of carbon, phosphorus, and nitrogen, since these are the most important nutrients for autotrophic growth of microalgae [26]. The other nutrients varied by 25% (Z-B25), 50% (Z-B50), and 75% (Z-B75) from the original concentration of Zarrouk medium. The original medium (Z-100) was also used. Fig. 1 shows the growth curves for *Spirulina* sp. LEB 18 and nitrogen and phosphorus consumption in the cultures.

Z-B25 exhibited the lowest number of growth cycles (Fig. 1a). The cell death phase started after90 days, with a specific death rate of 0.08 d^{-1}. In this test, the fourth growth cycle showed maximum specific growth rate 50% smaller (0.067 d^{-1}) when compared to the first cycle (0.126 d^{-1}), evidencing that the culture medium formulated with lower concentrations of nutrients is not suitable for biomass production for a long period. The test Z-100 exhibited eight growth cycles (Fig. 1d), and the cell death phase started after 120 days of cultivation.

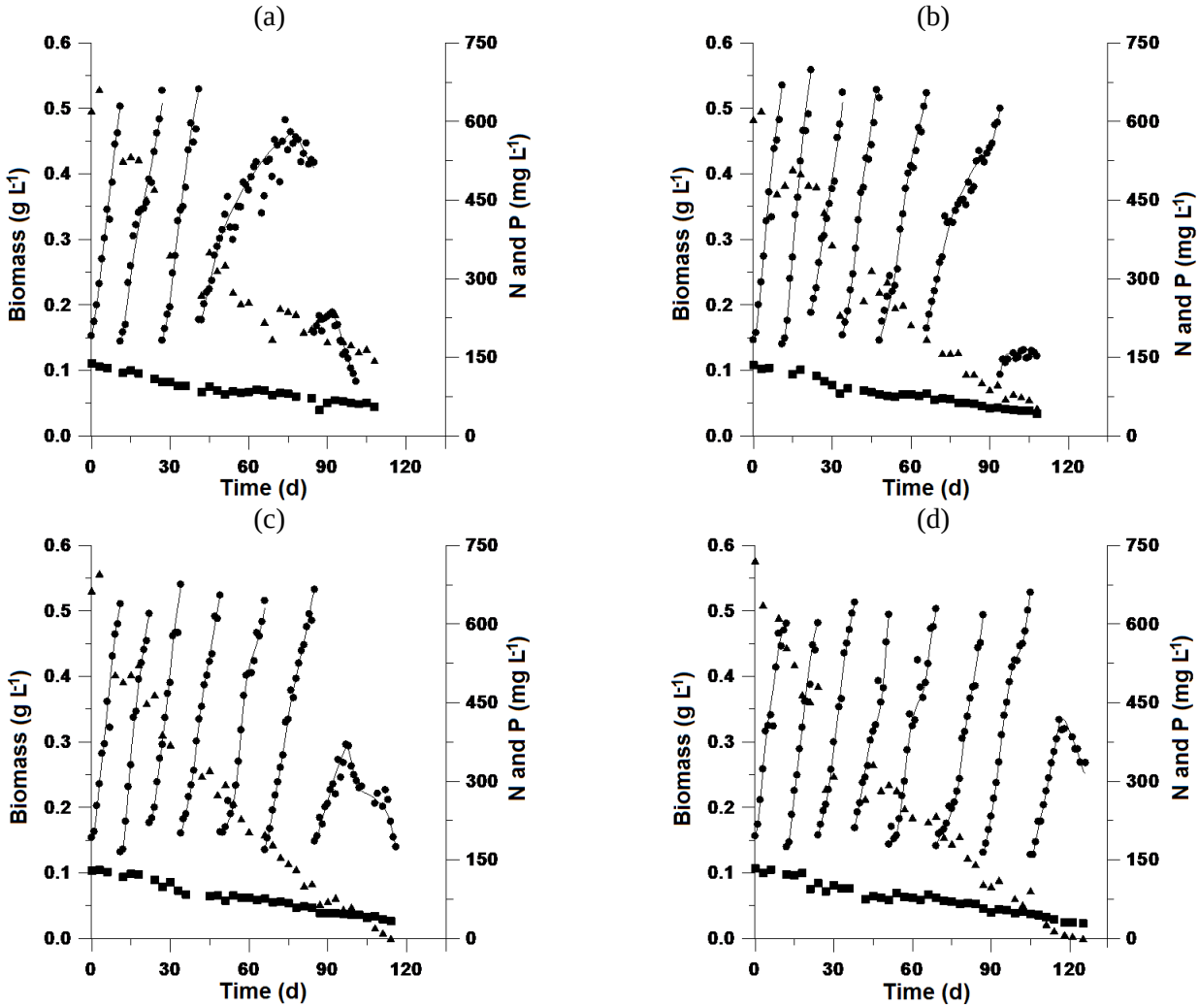

Figure 1- Growth curves (●) of *Spirulina* sp. LEB 18, and concentrations of nitrogen (▲) and phosphorus (■) in the tests Z-B25 (a), Z-B50 (b), Z-B75 (c), and Z-100 (d).

For the tests Z-B50 and Z-B75 (Fig. 1b and 1c), no significant ($p>0.15$) differences were observed for the maximum specific growth rates from the third growth cycle.

Table 1 shows the maximum specific growth rate or death rate, mean productivity, and nitrogen and phosphorus consumption rate for each growth cycle. The maximum specific rates ($p<0.04$) were observed in the first (0.163 d^{-1}) and second (0.184 d^{-1}) growth cycle of the test Z-B50, and in the second cycle of the test Z-B25 (0.152 d^{-1}).

Although the medium was not renewed every growth cycle, the test Z-B50 showed seven growth cycles, with specific growth rate of 0.122 d^{-1}, and productivity of 30.04 mg L^{-1} d^{-1}. The highest productivity (48.03 mg L^{-1} d^{-1}) was observed in the third growth cycle ($p <0.0002$) for Z-B50 (Table 1).In the test with original medium (Z-100), the maximum specific growth rates were statistically similar ($p> 0.37$) from the third growth cycle; however, at the beginning of this cycle, the nitrogen and phosphorus concentrations in the culture medium decreased 54% and 29%, respectively, when compared to the initial concentration.

Table 1 - Maximum specific growth rate (μ_{max}, d^{-1}), mean biomass productivity (P_{mean}, $mgL^{-1}d^{-1}$), and nitrogen and phosphorus consumption rates (q_N and q_P, $mg.L^{-1}.d^{-1}$) for *Spirulina* sp. LEB 18 in the tests Z-B25, Z-B50, Z-B75 and Z-100.

Tests	Growth cycles / nº of days	$\mu_{máx}$ (d^{-1})	P_{mean} (mg L^{-1} d^{-1})	q_N (mg L^{-1} d^{-1})	q_P (mg L^{-1} d^{-1})
Z-B25	1 / 11	0.126	31.87	7.93	1.54
	2 / 16	0.152	23.93	3.63	1.10
	3 / 14	0.120	27.40	14.38	1.39
	4 / 44	0.067	5.43	1.48	0.26
	5 / 16	-0.080*		2.72	0.74
Z-B50	1 / 11	0.163	35.37	6.35	1.18
	2 / 11	0.184	38.00	3.63	0.40
	3 / 12	0.082	48.03	18.59	3.64
	4 / 14	0.118	25.87	4.99	0.15
	5 / 18	0.096	20.97	4.27	0.50
	6 / 28	0.088	12.00	3.22	0.61
	7 / 14	-0.030*		2.99	0.77
Z-B75	1 / 11	0.143	32.40	14.51	0.92
	2 / 11	0.143	33.10	4.53	3.26
	3 / 12	0.108	30.30	8.77	0.24
	4 / 10	0.099	24.20	5.29	0.22
	5 / 17	0.133	20.73	4.23	0.33
	6 / 19	0.105	20.93	5.21	0.91
	7 / 31	-0.120*		3.47	0.87
Z-100	1 / 12	0.140	27.03	13.83	0.72
	2 / 12	0.123	28.50	6.12	1.39
	3 / 14	0.098	25.40	7.13	0.84
	4 / 13	0.082	25.07	6.35	1.48
	5 / 18	0.090	20.00	3.32	0.08
	6 / 18	0.067	19.57	7.25	1.01
	7 / 18	0.112	22.07	0.68	0.51
	8 / 21	-0.070*		4.55	0.97

* specific death rate

The nutrients consumption and the non-replacement of nutrients in the culture medium affected the productivity for all tests. For the tests Z-B25, Z B50, Z B75 and Z-100, the productivity decreased by 83, 66, 35 and 18%, respectively, when compared to the first cycle.

Semicontinuous cultivations of *Spirulinaplatensis* with replacement of the culture medium was performed by Reichert et al.[17] in closed photobioreactors, and the maximum specific growth rate was 0.111 d^{-1}, with 50% renewal rate. Radmann et al. [21] found, for 12 cycles, maximum specific growth rate of 0.116 d^{-1} and productivity of 40.0 mg L^{-1} d^{-1} when using blend cellular concentration of 0.4 g L^{-1}, renewal rate of 40% and diluted Zarrouk medium (50%). Although the maximum specific growth rate was similar to the present results, in this study the culture medium was not renewed during cultivation.

The reduction in phosphorus concentrations at the end of the cultivation was 47.6; 60.1; 54.0; and 64.8% for the tests Z-B25, Z B50, Z B75, and Z-100, respectively. According to these results, *Spirulina* was able to remove up to 324 mg L^{-1}

phosphate. Rose and Dunn [27] found complete removal of this nutrient in continuous cultivation of *Spirulina*, but the medium was tannery effluent, which contains lower phosphate concentration (20 mg L^{-1}).

Chlorella vulgaris and *Scenedesmus rubescens* have also been used to study the nutrient consumption in the medium. These microalgae were grown in culture medium with initial phosphorus and nitrogen concentrations of 3 mg L^{-1}. After 5 days of cultivation, phosphorus and nitrogen were consumed, respectively, at a consumption rate of 0.53 mg L^{-1} d^{-1} and 0.56 mg L^{-1} d^{-1} (*C. vulgaris*), and 0.55 mg $L^{-1}.d^{-1}$ and 0.57 mg.$L^{-1}.d^{-1}$ (*S. rubescens*) [28] In this study, in the first growth cycle (Table 1), the phosphorus consumption rate ranged from 0.72 to 1.54 mg L^{-1} d^{-1}, and the nitrogen consumption rate ranged from 6.35 to 14.51 mg L^{-1} d^{-1} for all tests.

Fig. 2 shows the mean protein and lipids concentrations in the biomass of each growth cycle of *Spirulina* sp. 18 LEB. The protein and lipid concentrations were determined in the biomass at the end of each growth cycle, and the mean value was obtained at the end of each culture. The mean protein contents in the cultivations were 43.71 ± 16.15; 43.96 ± 9.41; 44.37 ± 4.60; and 48.84 ± 4.09% for the tests Z-B25, Z-B50, Z-B75, and Z-100, respectively (Fig. 2).

No significant differences ($p < 0.0003$) were observed for the protein contents of all tests, showing that the dilution of nutrients in the Zarrouk medium, except carbon, nitrogen and phosphorus, did not alter the protein concentration. The results are higher than those obtained by Markou et al. [29], in semicontinuous cultivation of *Spirulina*, who found protein contents ranged from 22.07 to 41.28% for phosphate concentrations of 10 and 40 mg L^{-1}, respectively.

The variation in protein in the biomass decreased with increasing the nutrient concentration in the medium.The smallest variation between cycles was 4.09% in the test Z-100.Lower values were found in *Muriellopsis* biomass (47.8 ± 0.4%) grown with initial concentration of 0.02 g L^{-1} $NaNO_3$ in semicontinuous culture with daily replacement of medium [30].

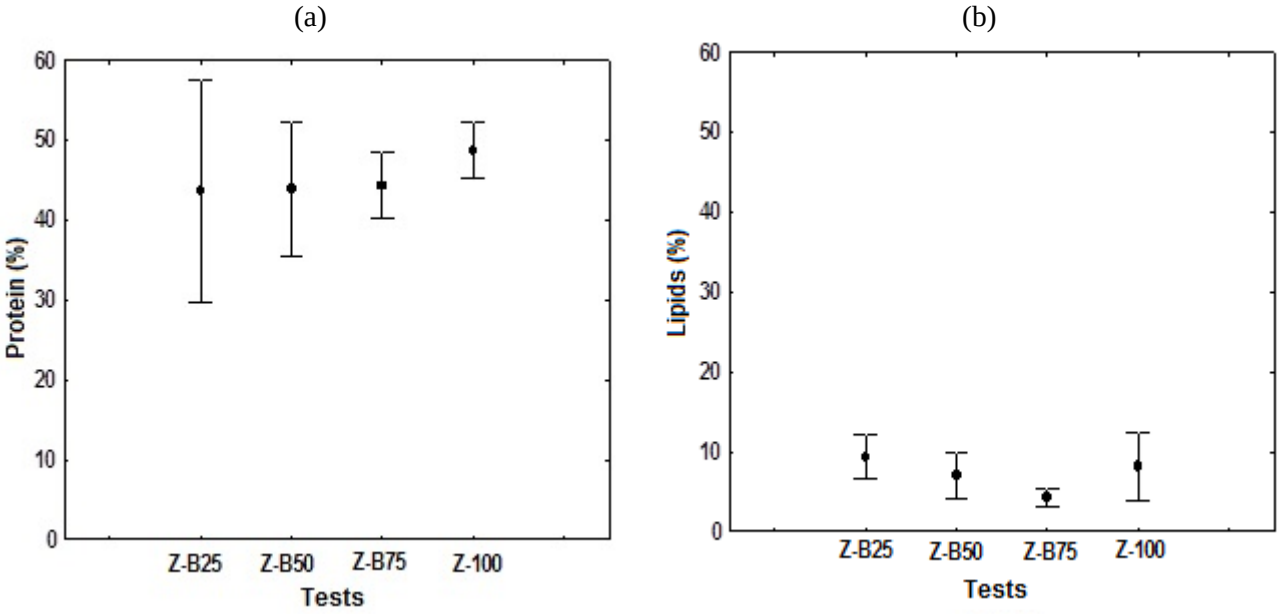

Figure2 - Mean and standard deviation of protein (a) and lipids (b) concentrations for the tests Z-B25, Z-B50, Z-B75 and Z-100.

According to Vargas et al. [31], differences in protein concentration between each cycle may be due to the period when the biomass is collected, variations may occur up to 100% between the lag, exponential, and stationary phase. These differences may be due to operational variables such as agitation of the bioreactor, height of culture medium, photoperiod and illuminance, which are important for the biomass composition, as reported by Grobbelaar [15].

In the last growth cycles of *Spirulina* sp. LEB 18, although nitrogen reductions of up to 80% of the initial concentration were observed, protein biomass has been produced. According to Sassano et al. [16], the microalgae can degrade phycocyanin under minimum nitrogen concentrations, which is used as a reserve source of nitrogen.

Markou [32] obtained 4.9% lipids in semicontinuous cultivation of *Spirulina platensis* in closed photobioreactor, using Zarrouk medium containing 9 mg L^{-1} phosphorus, and the highest lipids content (7.9%) was found for the lower phosphorus concentration (3 mg L^{-1}).

The tests Z-B50 and Z-B75 lasted 48 and 49 days, respectively, until the 4th growth cycle. At the end of this time, the protein concentration was 46.10 ± 2.59% and 46.24 ± 2.20%, respectively. Therefore, to keep the biomass with a high protein concentration over a period of about 50 days, the Zarrouk medium containing the solution B diluted at 50% can be used in the cultivation.

The results show that the semicontinuous cultivation with medium recycle may be used to produce *Spirulina*, using 50% of the nutrients generally used for this cyanobacteria, except nitrogen, phosphorus and carbon. In addition to the cost reduction potential, the proposed system has the advantage of being performed in raceway reactor. This reactor has lower power consumption and lower construction and operation costs [33].

IV. CONCLUSION

The semicontinuous cultivation of *Spirulina* sp. LEB 18 remained viable up to 100 days, using Zarrouk medium formulated with lower nutrients concentration and without renewal of the medium. High growth rate and productivity can be reached, with lower production costs of the biomass with high protein content.

The maximum specific growth rate (0.184 d^{-1}) and productivity (48.03 mg L^{-1} d^{-1}) were obtained when Zarrouk medium containing the solution B diluted at 50% (Z-B50) was used. The biomass exhibited the highest protein concentration (48.81 ± 4.09%) in the test Z-100, while the highest lipids content (9.38 ± 3.35%) was obtained in the test Z - B25. In contrast, in the test Z-B50, *Spirulina* sp. LEB 18 showed 46.10 ± 2.59% protein for a period of approximately 100 days.

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