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SEMICONTINUOUS CULTIVATION OF THE CYANOBACTERIUM *Spirulina platensis* IN A CLOSED PHOTOBIOREACTOR

C. C. Reichert[1], C. O. Reinehr[2] and J. A. V. Costa[1]*

[1]Departamento de Química, Fundação Universidade Federal do Rio Grande,
Cx. P. 474, CEP 96201-900, Rio Grande - RS, Brazil.
E-mail: dqmjorge@furg.br
[2]Centro de Pesquisa em Alimentação, Universidade de Passo Fundo,
Cx. P. 611, CEP 99070-010, Passo Fundo - RS, Brazil.
E-mail: reinehr@upf.br

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Abstract - The cultivation of photosynthetic microorganisms such as the cyanobacterium *Spirulina platensis* has been studied by researchers in many countries because these organisms can produce products with industrial potential. We studied the specific growth rate (μ_x, day^{-1}) and productivity (P_x, in mg/L/day of *Spirulina platensis* biomass, dry weight basis) of two *S. platensis* strains (LEB-52 and Paracas) growing in aerated semicontinuous culture in two-liter Erlenmeyer flasks for 90 days (2160 h) at 30°C under 2500 lux of illumination in a 12 h photoperiod. Independent of the *S. platensis* strain used we found that low biomass concentrations (0.50 g/L) and high renewal rates (50% v/v) resulted in a high specific growth rate ($\mu_x = 0.111$ day^{-1}) and high productivity ($P_x = 42.3$ mg/L/day). These values are two to four times higher than those obtained in simple batch cultivation and indicate that the semicontinuous cultivation of *S. platensis* is viable.
Keywords: Biomass; Semicontinuous cultivation; *Spirulina platensis*; Renewal rate.

INTRODUCTION

The cyanobacterium *Spirulina platensis* is composed of 50 to 70% protein, 5 to 10% lipid and 10 to 20% carbohydrate (Vonshak, 1997) as well as substances with anticarcinogenic, hypocholesterolemic and antioxidant properties. Because of its high nutrient and complex organic molecule content, *S. platensis* can be used not only in human and animal foodstuffs, but also in fine chemistry. In 1981, the Food and Drug Administration (FDA) decided that since *Spirulina* constitutes a source of proteins and contains several vitamins and minerals it could legally be sold as a food supplement (Estrada et al., 2001; Torres-Durán et al., 1999; Belay et al., 1993).

Strains of *S. platensis* and *Spirulina maxima* typically inhabit some African and Mexican alkaline lakes, which are rich in carbonates and bicarbonates (Richmond, 1990), but these strains can also be grown artificially in synthetic medium in batch, semicontinuous or continuous systems. Despite the fact that continuous systems produce a homogeneous product, they are often economically or technically not viable (Donati & Paludetto, 1999). Semicontinuous cultivation is a viable alternative system, in which a portion of the culture medium is periodically removed and the remaining culture used as the starting point for continuation of the culture, thus ensuring a high inoculum ratio at the time of addition of new medium. The relative amount of fresh medium added is called the "renewal rate" and the biomass concentration at the time of the addition of fresh medium is called the "blend concentration."

The use of semicontinuous cultures, in which the dilution cycles have the same duration as the cell cycles, provides a simple tool for studying the characteristics of cultures maintained in different light-dark cycles and avoids the interference which

*To whom correspondence should be addressed

sometimes occurs under continuous dilution (Otero et al., 1998). Semicontinuous cultivation also has operational advantages, such as maintaining a constant amount of inoculum and allowing the cultured microorganism to be maintained at high specific growth rates (Fábregas et al., 1996). Although semicontinuous cultivation is often used for the culture of cyanobacteria and microalgae, this type of system has not been well-studied for these types of organisms and there is a lack of scientific data on biomass concentration and renewal rate.

The work published in this paper is concerned with the semicontinuous cultivation of two *S. platensis* strains in a closed photobioreactor and the evaluation of the biomass concentration and renewal rate, factors which have the potential to affect the specific growth rate of *S. platensis* and the productivity of the process.

MATERIALS AND METHODS

Microorganism and Cultivation Medium

The cyanobacterium *Spirulina platensis* strains LEB-52 (Costa et al., 2002) and Paracas (Duarte Filho et al., 2002) were used in this study. The strains were grown and maintained on Zarrouk's medium (Zarrouk, 1966), a standard medium for the culture of *Spirulina*. All reagents were of at least analytical grade and were purchased from Merck KGaA (Darmstadt, Germany), Vetec (São Paulo, Brazil) or the Synth Chemical Company (São Paulo, Brazil).

Cultivation Conditions

Semicontinuous cultivation was carried out in two-liter Erlenmeyer flasks containing an initial volume of 1.8 litres of Zarrouk's medium and an initial *S. platensis* biomass concentration of 0.15 g/L of either strain LEB-52 or the Paracas strain. Aeration was accomplished using diaphragm pumps to produce a flow rate of 20 L/h of air (Costa et al., 2002) and 40W fluorescent lamps (Osram, Brazil) provided an illuminance of 2500 lux. The whole apparatus (Figure 1) was maintained in an unsterile chamber at 30°C with a 12 h light/dark photoperiod (Vonshak et al., 1982). When the *S. platensis* biomass concentration in the culture reached a predetermined level (0.50 or 0.75 g/L, called the "blend concentration," X_1) a portion of the medium (25 or 50% (v/v), the "renewal rate," X_2) was removed and the same amount of fresh medium added, each experiment being with the duration of about 90 days (2160 h). Each day samples were collected aseptically and the biomass concentration calculated by measuring the optical density at 670 nm using a Varian Cary 100 spectrophotometer and a calibration curve relating optical density to *S. platensis* dry weight biomass. The number of times that fresh medium was added (called the "dilution cycle," N), which varied according to the specific conditions of each run, was also recorded.

A standard batch cultivation was performed for each strain using the same basic conditions as those described above except that no fresh medium was added and the runs were for only 42 days (1000 h).

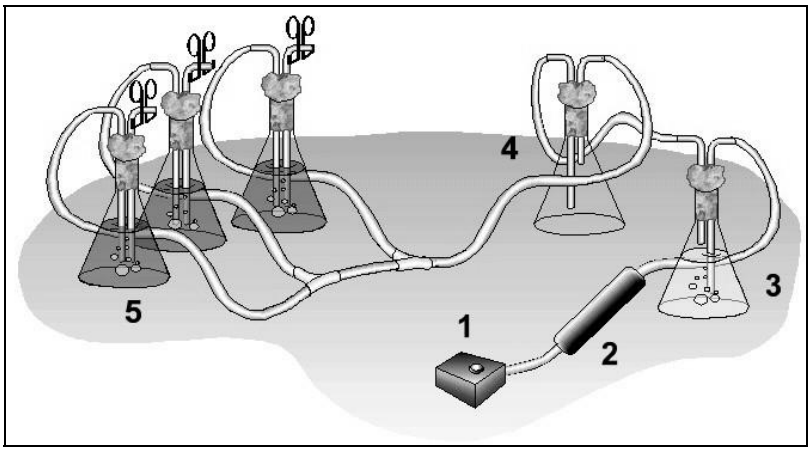

Figure 1: Diagram of the apparatus used to cultivate *Spirulina platensis*. (1) diaphragm pump, (2) air filter, (3) humidifier, (4) droplet filter, (5) photobioreactors.

Experimental Design

A complete 2^3 factorial design (Table 1) was used in this study; the variables were blend concentration (X_1, 0.50 and 0.75 g/L), renewal rate (X_2, 25 and 50% v/v) and strain (LEB-52 and Paracas).

The specific growth rate (μ_x, day^{-1}) was obtained by regression of the exponential logarithmic growth phase, while productivity (P_x) was calculated from the quantity of *S. platensis* biomass formed per liter per day (mg/L/day, dry weight basis). These values were calculated separately for each dilution cycle (N) and averaged (with standard deviations) for each separate run.

The relationships between blend concentration (X_1), renewal rate (X_2) and strain (X_3) and μ_x and P_x were evaluated using analysis of variance (ANOVA) at a significance level of 5%.

RESULTS AND DISCUSSION

The results of the semicontinuous and batch cultivation experiments are shown in Table 1, where the values of μ_x and P_x are given as the average values for the run ± the standard deviation. There were large differences in the number of dilution cycles for each of the runs because of the different blend concentrations and renewal rates used.

The growth curve for *S. platensis* strain LEB-52 in semicontinuous culture run 8 (X_1 = 0.75 g/L, X_2 = 50%,) is shown in Figure 2. During run 8 strain LEB-52 maintained a high specific growth rate (μ_x = 0.073 ± 0.006 day^{-1}) with little variation throughout allowing the cells to remain in the exponential growth phase for most of the run, thereby producing one of the highest P_x values (41.6 mg/L/day). This was not the case during batch cultivation in which strain LEB-52 reached the end of the exponential phase (the "plateau phase") at a biomass concentration of between 0.8 and 1.0 g/L after 21-25 days (500 to 600 h) of cultivation with no batch culture reaching 1.2 g/L. Similar results were obtained by Costa et al. (2002) and Costa et al. (2000) for batch experiments.

For the semicontinuous cultivations, specific growth rate was lowest (μ_x = 0.050 day^{-1}) for run 2 (X_1 = 0.75 g/L, X_2 = 50%, Paracas strain) and highest (0.111 day^{-1}) for run 7 (X_1 = 0.50 g/L, X_2 = 25%, strain LEB-52) (Table 1). Independent of the strain used, the highest specific growth rates (μ_x) were obtained with a blend concentration (X_1) of 0.50 g/L and a renewal rate (X_2) of 50% (v/v) with the biomass concentration for these parameters varying between 0.25 and 0.50 g/L. It is known that the specific growth rate tends to decrease with higher biomass (cell) concentrations, due to the shadowing effect (Gitelson et al., 1996; Vonshak et al., 1982). Vonshak et al. (1982) showed that during *Spirulina* production the photosynthetic potential of the *Spirulina* cells decreased for biomass concentrations between 0.4 and 1.0 g/L because under the conditions used most of the *Spirulina* cells were shaded from light by neighboring cells. Even at biomass concentrations close to 0.5 g/L (considered ideal for maximal photosynthetic efficiency) about 80% of the cells were without light at certain times so, consequently, low biomass concentrations and high specific growth rates are expected in systems where light is a limiting factor.

Table 1: Semicontinuous and batch cultivation of *S. platensis* in Zarrouk's medium at 30ºC.

Run	Blend concentration (X_1, g/L)	Renewal rate (X_2, %)	Strain (X_3)	Dilution cycles (N)	Maximum specific growth rate* (μ_{max}, day^{-1})	Biomass productivity* (P_x, mg/L/day)
Semicontinuous culture						
1	0.50	25	Paracas	17	0.065 ± 0.006	29.2 ± 3.9
2	0.75	25	Paracas	10	0.050 ± 0.009	34.3 ± 2.9
3	0.50	50	Paracas	12	0.101 ± 0.011	37.0 ± 4.6
4	0.75	50	Paracas	7	0.070 ± 0.014	36.4 ± 3.4
5	0.50	25	LEB-52	22	0.095 ± 0.017	42.3 ± 6.0
6	0.75	25	LEB-52	10	0.052 ± 0.012	33.2 ± 3.6
7	0.50	50	LEB-52	13	0.111 ± 0.019	41.7 ± 6.7
8	0.75	50	LEB-52	8	0.073 ± 0.006	41.6 ± 6.9
Batch culture						
B1	-	-	Paracas	-	0.031 ± 0.005	19.9 ± 2.5
B2	-	-	LEB-52	-	0.034 ± 0.004	22.6 ± 3.1

*Mean values for the run ± standard deviation

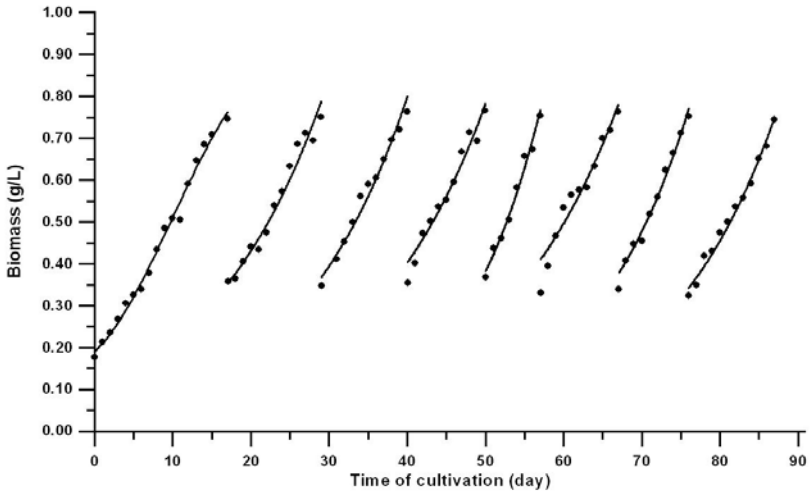

Figure 2: Biomass concentration as a function of cultivation time for *Spirulina platensis* strain LEB-52. Run 8 with a blend concentration (X_1) of 0.75 g/L and a renewal rate (X_2) of 50%.

Productivity (P_x) varied between 19.9 and 42.3 mg/L/day, depending on the run (Figure 3). P_x and μ_x varied as a function of cultivation time for run 5 (X_1 = 0.50 g/L, X_2 = 25% (v/v), strain LEB-52) and 7 (X_1 = 0.50 g/L, X_2 25% (v/v), strain LEB-52). Run 5 had stable P_x and μ_x values for about 71 days (1700 h) before these values started to decline, while for run 7 specific growth rate and productivity increased with time up to 50 days (1200 h), after which both these values tended to drop. The other runs also gave similar results, showing the usefulness of semicontinuous cultivation during which productivity increases as a function of time and consequently high productivity can be maintained for a much longer time than during batch cultivation. Otero et al. (1998) state that when medium is removed from a culture and new medium added poisonous substances are produced from the microorganisms in the culture and development of the culture becomes more difficult. It thus appears best to allow semicontinuous cultivation to proceed until ideal cultivation conditions are no longer found in the culture.

The significance levels and estimated effects obtained using ANOVA for the factorial design in the semicontinuous cultivation are given in Table 2, where it can be seen that, except for the effect of blend concentration on productivity, the three factors studied (blend concentration (X_1), renewal rate (X_2) and strain (X_3)) showed significant ($p<0.05$) interaction. The interaction factors for blend concentration and strain both showed significant ($p<0.05$) interaction with specific growth rate, while the third-order interaction factor showed a significant ($p<0.05$) effect on productivity.

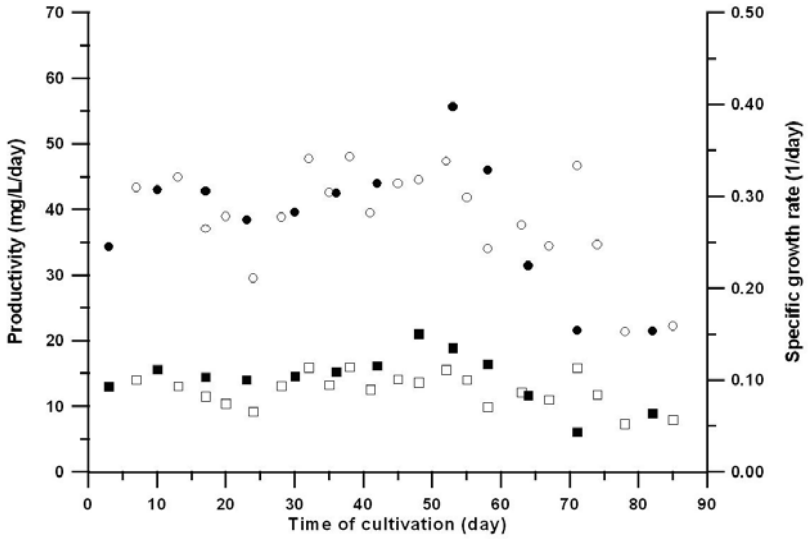

Figure 3: Biomass productivity (P_x) and specific growth rate (μ_x) as a function of cultivation time for *Spirulina platensis* strain LEB-52. Run 5 with a blend concentration (X_1) of 0.50 g/L and a renewal rate (X_2) of 25% (○) P_x and (□) μ_x. Run 7 with X_1 = 0.50 g/L and X_2 = 50% (●) P_x and (■) μ_x.

Table 2: Statistical results (*P*-values) and effects obtained by the analysis of variance of the 2^3 factorial design used in the semicontinuous cultivation of *S. platensis*.

Variable[1]	Specific growth rate (μ_{max})		Biomass productivity (P_x)	
	***P*-value**	**Effect**	***P*-value**	**Effect**
X_1	<0.0001*	-0.0314	0.3664	-1.199
X_2	<0.0001*	0.0234	0.0013*	4.431
X_3	0.0008*	0.0112	0.0001*	5.503
$X_1.X_2$	0.3918	-0.0028	0.5334	0.826
$X_1.X_3$	0.0074*	-0.0088	0.0122*	-3.394
$X_2.X_3$	0.1453	-0.0047	0.6983	-0.514
$X_1.X_2.X_3$	0.1324	0.0049	0.0074*	3.640

[1]Variable: X_1= blend concentration; X_2 = renewal rate; X_3: = Strain; μ_{max} = specific growth rate; P_x = biomass productivity.
*Statistically significant at a confidence level of 95%

There was a significant ($p<0.0001$) positive relationship between renewal rate and specific growth rate (i.e., growth rate increased at higher renewal rates), as discussed above in relation to the shadowing effect. When the blend concentration was 0.50 g/L the growth rate of strain LEB-52 was significantly ($p<0.0001$) higher than that of the Paracas strain, while at the higher blend concentration of 0.75 g/L the growth rates of both strains were statistically the same ($p=0.7539$), as shown in Figure 4. The productivity of both strains increased when the renewal rate was 50% (v/v) ($p=0.0013$), with the productivity of strain LEB-52 being significantly ($p<0.0001$) higher than that of the Paracas strain.

Fábregas et al. (1996) studied the semicontinuous cultivation of the microalga *Chlorella* and found that the specific growth rate increased as the renewal rate decreased. These authors also found that when renewal rates were 40 and 50% (v/v) the percentage of nitrogen transformed in the cells was less than 100%, indicating that nitrogen was non-limiting. This increase in specific growth rate was also observed for all cultivation conditions during our experiments, indicating that there was a higher replacement of nutrients which aided in cell growth.

The results presented here are in agreement with the studies by Fábregas et al. (1995) on semicontinuous cultivation of the microalga *Tetraselmis suecica*, where a daily renewal rate of 50% (v/v) resulted in an increase in productivity of more than 100%, from about 100 mg/L/day to 216 mg/L/day.

Comparison of the semicontinuous and batch experiments showed that higher specific growth rates (μ_x) and productivity (P_x) were obtained for semicontinuous cultivation ($p<0.01$), probably because during semicontinuous cultivation the cells were better adapted to the culture medium (in which better growth conditions prevailed) and remained in the exponential phase for 83 days (2000 h). Travieso et al. (2001) studied the semicontinuous cultivation of *S. platensis* in a tubular reactor for seven days with the daily removal of a portion of the old medium and addition of fresh medium, and obtained a significant increase in productivity as the renewal rate increased from 5 to 20% (v/v) although productivity began to decline from 25% onwards. Our bioreactors were different from those used by Travieso et al. (2001), but in spite of this there was still a two fold increase in productivity and up to four times higher specific growth rate values than in the batch cultivations.

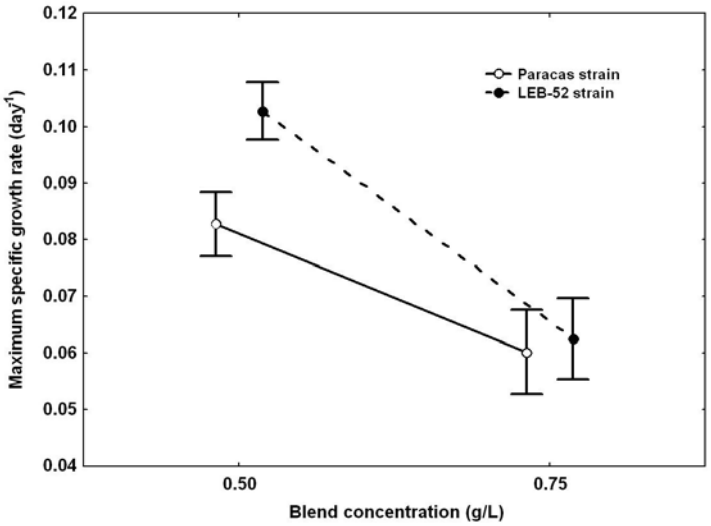

Figure 4: Specific growth rate (μ_x) of *Spirulina platensis* strains LEB-52 and Paracas as a function of blend concentration (X_2).

CONCLUSIONS

Our results indicate that blend concentration and renewal rate affected other factors; higher growth rates and productivites were obtained with lower blend concentrations (0.50 g/L) and higher renewal rates (50%). During our study the maximum specific growth rate (0.111 day^{-1}) was four times that in batch cultivation, while the highest productivity (42.3 mg/L/day) was twice that seen in batch cultivation.

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