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Biochemical Engineering Journal 40 (2008) 27–34

Biochemical Engineering Journal

www.elsevier.com/locate/bej

Biomass production and carbon dioxide fixation by *Aphanothece microscopica Nägeli* in a bubble column photobioreactor

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Received 2 March 2007; received in revised form 29 October 2007; accepted 18 November 2007

Abstract

The objective of the present study was to evaluate the growth kinetics of *Aphanothece microscopica Nägeli* under different conditions of temperature, light intensity and CO_2 concentration. The growth kinetics of the microorganism and carbon biofixation were evaluated using a central composite design, considering five different temperature levels (21.5, 25, 30, 35 and 38.5 °C), light intensities (0.96, 3, 6, 9 and 11 klux) and carbon dioxide concentrations (3, 15, 25, 50 and 62%). The results obtained showed the effects of temperature, light intensity and CO_2 concentration ($p < 0.05$) on the photosynthetic metabolism of the microorganism. Response surface methodology was adequate for process optimisation, providing a carbon fixation rate to the order of 109.2 mg L^{-1} h^{-1} under conditions of 11 klux, 35 °C and 15% carbon dioxide, representing an increase of 58.1% as compared to the conditions tested initially.

Keywords: Carbon dioxide sequestration; Photobioreactor; Cyanobacteria

1. Introduction

Innumerable research projects have suggested that the biofixation of CO_2 by cyanobacteria in photobioreactors is a sustainable strategy, since carbon dioxide can be incorporated into the molecular structure of cells in the form of proteins, carbohydrates and lipids by way of photosynthetic reactions. The advantages of these processes are related to the greater photosynthetic efficiency of these systems when compared to higher plants, the resistance of these microorganisms to high carbon dioxide concentrations and the possibility of controlling the culture growth conditions. In addition, the biomass produced by the bioconversion of carbon dioxide allows one to obtain products of high added value, such as, fatty acids, biodiesel, biogas and organic fertilizers, as a function of the microalgal species used and the effluent to be treated [1–3].

According to Lee et al. [4] and Jacob-Lopes et al. [5], only a small fraction of the carbon dioxide injected into the photobioreactors is incorporated into the microalgal biomass. According to these authors, other products of photosynthetic metabolism, such as the formation of extracellular biopolymers, precipitates of chemical species such as carbonates and bicarbonates and volatile organic compounds (VOCs), are more highly represented in the carbon dioxide transformation processes of these systems, substantially increasing the fixation rates.

Aphanothece microscopica Nägeli is a cyanobacterium characteristic of the estuaries in southern Brazil, belonging to the family *choroococaceae* and forming blue-green colonies adapted for floating. It shows a macroscopic, amorphous structure with abundant, firm and rigid mucilage, and non-cylindrical ellipitical adult cells measuring 9.0–9.5 μm × 4.0–4.2 μm, approximately 2.2 times longer than they are wider [6]. Previous studies have shown the potential of applying this microorganism in bioremediation and for the production of single-cell protein (SCP) [7–9].

Thus the objective of the present study was to evaluate distinct operational conditions for photobioreactors, expressed in terms of the temperature, light intensity and carbon dioxide concentration, aimed at maximizing carbon fixation in the cells of the cyanobacterium *Aphanothece microscopica Nägeli*.

2. Materials and methods

2.1. Microorganism and culture medium

Unialgal cultures of *Aphanothece microscopica Nägeli* (RSMan92) were originally isolated from the Patos Lagoon

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doi:10.1016/j.bej.2007.11.013

Nomenclature

C_c	percent carbon in the biomass (%)
(L)	linear effect
M_{CO2}	molecular weight of CO_2
M_C	molecular weight of carbon
(Q)	quadratic effect
R^2	coefficient of determination
R_C	carbon fixation rate (mg L^{-1} h^{-1})
t	residence time in $t = n$ (h)
t_0	residence time in $t = 0$ (h)
t_g	generation time (h)
t_{log}	duration of logarithmic growth phase (h)
X_m	maximum cell concentration (mg L^{-1})
X_0	initial cell concentration (mg L^{-1})
X_1, X_2, X_3	independent variables of the statistical model
Y	variable response

Greek letters

β	parameters of the statistical model
μ_{max}	maximum specific growth rate (h^{-1})

estuary, Rio Grande do Sul State, Brazil (32°01′S–52°05′W). Stock cultures were propagated and maintained on synthetic BGN medium [10] with the following composition: $K_2HPO_4{\cdot}3H_2O$ (0.040 g L^{-1}), $MgSO_4{\cdot}7H_2O$ (0.075 g L^{-1}), EDTA (0.001 g L^{-1}), H_3BO_3 (2.860 g L^{-1}), $MnCl_2{\cdot}4H_2O$ (1.810 g L^{-1}), $ZnSO_4{\cdot}7H_2O$ (0.222 g L^{-1}), $Na_2MoO_4{\cdot}2H_2O$ (0.390 g L^{-1}), $CuSO_4{\cdot}5H_2O$ (0.079 g L^{-1}), $CaCl_2{\cdot}6H_2O$ (0.040 g L^{-1}), $NaNO_3$ (150 g L^{-1}) $C_6H_8O_7{\cdot}H_2O$ (0.006 g L^{-1}), ammonium and iron citrate (0.006 g L^{-1}), pH 8.0. The conditions used were 25 °C, 1 klux of light intensity and a photoperiod of 12 h.

2.2. Photobioreactor

The diagram of the experimental apparatus used in this study is shown in Fig. 1. Measurements were made in a bubble column photobioreactor. The system was built in 4 mm thick glass, an internal diameter of 7.5 cm, height of 75 cm and nominal working volume of 3.0 L. The dispersion system for the reactor consisted of a 1.5 cm diameter air diffuser located in the centre of the column. The reactor was continuously illuminated with sixteen 20 W fluorescent lamps connected in parallel, located in a photoperiod chamber. Different numbers of lamps on each side of the photoperiod chamber were combined to give the desired light intensity. Airflow into the photobioreactor was provided via filtered air and pure CO_2 cylinder through Teflon tubing. The CO_2/air mixture was adjusted to achieve the desired concentration of carbon dioxide in the airstream, through three rotameters that measured the flow rates of the carbon dioxide, the air and the mixture of gases, respectively.

2.3. Obtaining and analysis of the kinetic data in an experimental photobioreactor

The experiments were carried out in bioreactors operating with an intermittent regime, fed on 3.0 L synthetic BGN medium. The test conditions were: initial cell concentration of 100 mg L^{-1}, isothermal reactor operating under different temperatures and light intensities and continuous aeration of

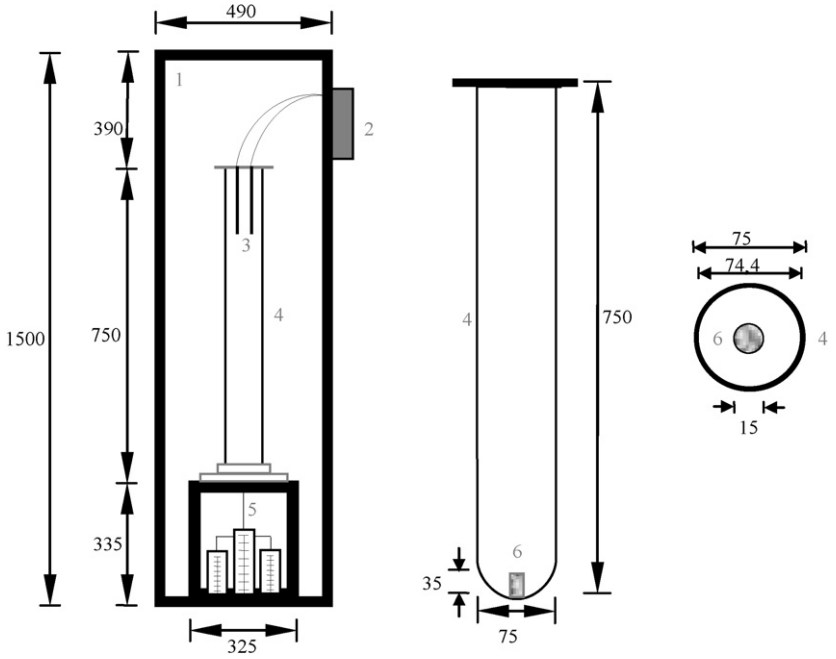

Fig. 1. Photobioreactor diagram. 1: Photoperiod chamber; 2: pH, temperature and CO_2 analyser; 3: pH, temperature and CO_2 sensors; 4: photobioreactor; 5: system controlling the flow rate and mixture of the gases; 6: gas diffuser. All dimensions in mm.

Table 1
Values of the independent variables for the different levels of the design

Independent variable	Symbol	Level				
		−1.68	−1	0	1	+1.68
Temperature (°C)	X_1	21.5	25	30	35	38.5
Light intensity (klux)	X_2	0.96	3	6	9	11
CO_2 concentration (%)	X_3	3	15	25	50	62

3 L min^{-1} of air supplemented with CO_2. The cell concentration was monitored every 12 h during the microbial growth phases. Residence times of up to 156 h were considered for all the experiments.

Response surface methodology was used to determine the optimal conditions for carbon fixation as a function of three experimental factors (temperature, light intensity and concentration of carbon dioxide enriched air). This methodology is widely used for controlling the effects of parameters in many processes. Its usage decreases the number of experiments, reducing time and material resources. Furthermore, the analysis performed on the results is easily carried out and experimental errors are minimized. The statistical method measures the effects of changes in operating variables and their mutual interactions on the process by way of an experimental design. The three steps used in the experimental design included statistical design experiments, estimation of coefficient using a mathematical model and an analysis of model applicability [11].

A five level, central composite design was used to evaluate the relationship between the culture conditions (independent variables) and the carbon fixation rate (dependent variable). Three replicates at the central point were used to estimate the experimental error.

The experimental design and the statistical analyses were carried out using the Statistica 7.0 software (Statsoft, USA). Table 1 shows the levels of the experimental variables used:

For a 3-factor system, the statistical model is defined by Eq. (1):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

The process was optimised from an analysis of the experimental design initially proposed, carried out in triplicate.

The calculated cell biomass values were used to calculate the maximum cell concentration (X_m, mg L^{-1}), maximum specific growth rate (μ_{max}, h^{-1}) and generation time (t_g, h). The carbon fixation rate was calculated from the elemental analysis of the biomass, as shown in Eq. (2) [12]:

$$R_C = C_c \times \left(\frac{X_m - X_0}{t - t_0}\right) \times \left(\frac{M_{CO_2}}{M_c}\right) \quad (2)$$

2.4. Analytical methods

The cell concentration was evaluated gravimetrically by filtering a known volume of culture medium through a 0.45 μm filter and drying at 60 °C for 24 h. The measurements of light intensity incident on the reactor were carried out on the external column surface using a digital luximeter (Minipa MLM 1010). The temperature was controlled using thermostats, and measured using a polarographic sensor (Mettler Toledo lnPro5000 series). The flow rates of the carbon dioxide, air and CO_2 enriched air were determined using rotameters (AFSG 100 Key Instruments). The elemental composition of the *Aphanothece microscopica Nägeli* cells was determined using a Perkin Elmer 2400 CHNS/O element analyser. Two-milligram samples of biomass were oxidised at 1000 °C and the resulting gases were determined using a thermal conductivity probe for carbon, nitrogen and hydrogen. The standard used was acetanilide, with a composition of 71.09% carbon, 11.84% oxygen, 6.71% hydrogen and 10.36% nitrogen.

Table 2
Kinetic parameters for *Aphanothece microscopica Nägeli*

Condition				Kinetic parameter			
X_1 (°C)	X_2 (klux)	X_3 (%)	μ_{max} (h^{-1})	R^2	t_g (h)	t_{log} (h)	X_m (mg L^{-1})
35.0	3	15	0.027	0.99	25.67	60	1672
35.0	9	15	0.034	0.94	20.38	72	3000
35.0	9	50	0.025	0.97	27.72	72	600
35.0	3	50	0.022	0.99	31.50	72	500
25.0	3	15	0.010	0.97	69.31	36	270
25.0	9	15	0.013	0.90	53.31	108	455
25.0	3	50	0.023	0.98	30.13	108	600
25.0	9	50	0.023	0.98	30.13	96	905
21.5	6	25	0.024	0.99	28.88	84	730
38.5	6	25	0.003	0.98	231.1	36	230
30.0	11	25	0.030	0.98	23.10	72	2300
30.0	0.96	25	0.008	0.94	86.64	72	360
30.0	6	62	0.023	0.98	30.13	72	1135
30.0	6	3	0.021	0.97	33.01	48	1600
30.0	6	25	0.028	0.99	24.75	108	1760

3. Results and discussion

3.1. Preliminary tests

Fig. 2 shows representative growth curves for the cyanobacterium *Aphanothece microscopica Nägeli* under the different photosynthetic culture conditions. A comparison of the growth curves shows the lack of an adaptation phase for this microorganism, reaching the logarithmic growth phase as from 12 h of cultivation, followed by a stationary phase, and, under some conditions, a declining phase. According to Guerrero et al. [13], the absence of an adaptation phase in microalgal growth curves is characteristic of culture media with high carbon and inorganic nutrient availability. For all the cultures, after a maximum residence time of 5 days, the growth curves already indicated characteristics of the stationary phase, the maximum cell densities being obtained in this period. Similar results were obtained by Yue and Chen [14] and Hsueh et al. [15] in the photosynthetic cultivation of the microalgae *Chlorella* ZY-1 and *Nannochlopsis oculta*, obtaining positive growth rates in residence times below 120 h.

Table 2 shows the growth kinetics of *Aphanothece microscopica Nägeli*. An analysis of these results shows that the maximum specific growth rates were obtained at 35 °C, 9 klux and 15%

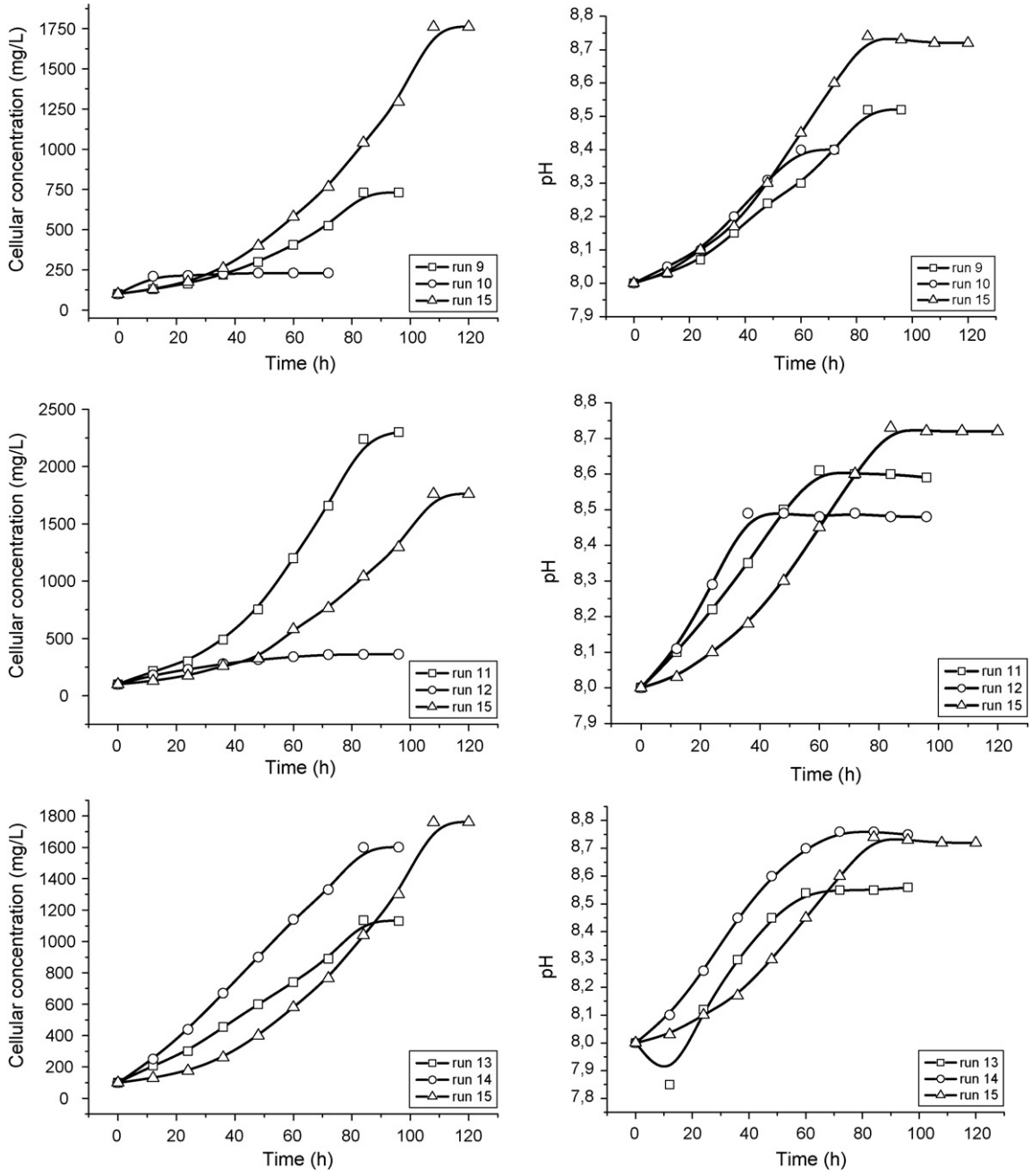

Fig. 2. Representative growth and pH curves as a function of temperature, light intensity and CO_2 concentration. Effect of temperature (run 9, 10 and 15); Effect of light intensity (run 11, 12 and 15) and effect of carbon dioxide concentration (run 13, 14 and 15).

CO_2, not differing significantly, according to the Tukey test ($p < 0.05$), from the cultures grown at 30 °C, 11 klux and 25% CO_2. The maximum values obtained for μ_{max} were similar to those obtained by other authors for the species *Euglena gracilis* and *Anabaena variabilis*, cultivated using photosynthetic metabolism [16,17]. However the maximum concentration of carbon dioxide enrichment used for these cultures was 13% (v/v), suggesting that the *Aphanothece microscopica Nägeli* is more tolerant to gases enriched with high levels of CO_2, since high growth rates were obtained with up to 62% (v/v) of carbon dioxide. With respect to the generation times, a variation between 23.1 and 231.1 h was observed, as a function of the different cultivation conditions, indicating a dependence of cell growth on the conditions of temperature, light intensity and concentration of carbon dioxide enriched air in the operation of the photobioreactor. The logarithmic growth phases, determined by linear regression of the data, indicated exponential growth between 35 and 108 h. The maximum cell density of 3.0 g L^{-1}, was obtained by cultivating in air enriched with 15% carbon dioxide at a temperature of 35 °C, with a light intensity of 9 klux. Of the conditions evaluated, the results suggest these experimental conditions as the most adequate to produce biomass by the cyanobacterium *Aphanothece microscopica Nägeli*.

Cell growth associated with carbon dioxide assimilation from the medium coincided with an increase in pH values. Fig. 2 shows the representative variation in pH of the culture medium as a function of residence time. The pH range observed in the culture medium varied from 6.0 to 9.9, indicating that the species HCO_3^- was the predominant form of carbon in the cultures.

The gradual increase in pH of the culture media, independent of the conditions, indicated that cultivation occurred in an alkaline pH range. Reductions in the initial pH value of the culture medium were only observed when cultivated with carbon dioxide concentrations equal or greater than 50%. On the other hand, the highest pH values (9.9) were found for the cultures with the highest cell densities (3.0 g L^{-1}), suggesting predominance of the photosynthetic metabolism of the microorganism under the experimental conditions used. According to Arnon [18] and Zuber [19], the increase in pH in photosynthetic cultures is an indication of the consumption of inorganic carbon due to cell growth. These authors reported that the increment in pH in the culture medium could be attributed to two main mechanisms: firstly the transport of hydroxide ions to outside the cell occurs by way of a reaction catalysed by the enzyme carbon anhydrase during the conversion of bicarbonate ions inside the cell to provide CO_2 for the photosynthetic reaction, raising the pH of the culture medium. A second potential mechanism would be the increase in pH due to activity of the enzyme ribulose 1,5-bisphosphate carboxylase, whose activity is considerably dependent on pH, increasing with increase in pH. This enzyme is present in the photosynthetic apparatus of the cyanobacteria, where the H^+ ions are sequestered to the inside of the tylacoid membrane with a simultaneous transfer of Mg^{2+} to the environment. These light energy induced fluxes result in an increase in pH and in the Mg^{2+} concentration, activating the RubisCO enzyme and resulting in efficient carbon dioxide fixation.

An analysis of the elements in the *Aphanothece microscopica Nägeli* cells at the end of cultivation showed that 1 g of biomass could contain between 0.48 and 0.52 g of carbon as a function of the cultivation conditions used. These values were used to estimate the carbon fixation rates (Table 3).

Table 4 shows the results for the effects of and the interactions between the factors of temperature, light intensity and carbon dioxide concentration, as also the coefficients of the model. An analysis of this table shows that in the range evaluated, the carbon fixation rate was controlled by the factors of light intensity (L), temperature (Q), interaction between temperature (L) and carbon dioxide concentration (L), and by the carbon dioxide concentration (L), in this order of importance. The other factors and interactions showed a lower proportion of statistical importance.

Table 3
Coded matrix of the effects of temperature, light intensity and CO_2 concentration on the carbon fixation rate

Run	Temperature (X_1)	Light intensity (X_2)	CO_2 (X_3)	R_C (mg L^{-1} h^{-1})
1	+1	−1	−1	24.80
2	+1	+1	−1	45.78
3	+1	+1	+1	11.84
4	+1	−1	+1	11.36
5	−1	−1	−1	3.45
6	−1	+1	−1	6.30
7	−1	−1	+1	8.88
8	−1	+1	+1	16.30
9	−1.68	0	0	12.78
10	+1.68	0	0	4.61
11	0	+1.68	0	39.07
12	0	−1.68	0	6.15
13	0	0	+1.68	21.01
14	0	0	−1.68	30.44
15	0	0	0	26.20
16	0	0	0	25.96
17	0	0	0	26.04

Table 4
Coefficients of the model estimated by linear regression

Factor	Effects	Standard error	$t(2)$	p	Coefficients	Estimates per interval	
						−95%	+95%
Mean	26.22	0.07	372.47	0.00000	26.22	25.92	26.53
X_1 (L)	6.60	0.06	99.88	0.00010	3.30	3.16	3.44
X_1 (Q)	−13.39	0.07	−183.95	0.00003	−6.69	−6.85	−6.53
X_2 (L)	12.75	0.06	192.85	0.00002	6.37	6.23	6.51
X_2 (Q)	−3.55	0.07	−48.78	0.00042	−1.77	−1.93	−1.61
X_3 (L)	−7.00	0.06	−105.86	0.00008	−3.50	−3.64	−3.35
X_3 (Q)	−1.34	0.07	−18.52	0.00290	−0.67	−0.83	−0.51
X_1(L) × X_2(L)	2.79	0.08	32.37	0.00095	1.39	1.21	1.58
X_1(L) × X_3(L)	−15.70	0.08	−181.72	0.00003	−7.85	−8.03	−7.66
X_2(L) × X_3(L)	−3.98	0.08	−46.08	0.00047	−1.99	−2.17	−1.80

Eq. (3) represents the statistical model for the variable response carbon fixation rate:

$$Y = 26.2 + 3.3X_1 - 6.7X_1^2 + 6.4X_2 - 1.77X_2^2 - 3.5X_3 - 0.67X_3^2 + 1.4X_1X_2 - 7.85X_1X_3 - 2.0X_2X_3 \quad (3)$$

Contour curves (Fig. 3) show the variation in the carbon fixation rate as a function of the factors studied. Thus an increase in carbon fixation rate is obtained not only by fixing the light intensity at the highest level, that is, carrying out the cultivation under high light intensities, but also by fixing the temperature in the central region and the carbon dioxide concentration at the lowest level (reduced proportions of CO_2 enrichment in the air entering the photobioreactor).

The carbon fixation rate varied from 3.45 to 45.78 mg L^{-1} h^{-1} under the different culture conditions. These results are higher than those obtained by Kajiwara et al. [12] when cultivating the cyanobacterium *Synechoccus* (PCC 7942) in photobioreactors at 30 °C with 8 klux and air enriched with carbon dioxide at 5%, obtaining maximum carbon fixation rates to the order of 25 mg L^{-1} h^{-1} and Yun et al. [20], who obtained rates of 23.4 mg L^{-1} h^{-1} for the microalga *Chlorella vulgaris* (UTEX 259) under conditions of 27 °C, 15% of CO_2 and 110 μE m^{-2} s^{-1}.

These results reflect the adaptation of the microorganism under study to the experimental conditions, suggesting the importance of optimising the photobioreactor operational conditions in order to obtain higher rates of biological carbon fixation.

Different authors have reported the importance of the environmental conditions on the growth of cyanobacteria. Parameters such as temperature, light intensity, pH and carbon dioxide concentration have been indicated as being of fundamental importance in the development of these microorganisms [21]. Thus it was shown that cultivation conditions in the temperature range from 30 to 35 °C, with 9–11 klux and 15–25% CO_2, favoured cell growth, associated with carbon fixation. These results agreed with those of Munoz and Guieysse [22], who reported that the efficiency of systems using microalgae normally decreased at low and higher temperatures, justifying the low cell performance of the cultures incubated at 21.5 and 38.5 °C, when compared to those incubated at 30 °C under the same conditions of light intensity and CO_2 concentration. According to Grossman et al. [23], the optimum temperature range for the development of cyanobacteria is from 25 to 35 °C.

With respect to light intensity, it was shown that the maximum intensity used was insufficient to promote the phenomenon of cell photoinhibition, since even at 11 klux high growth rates were recorded. On the other hand, at 0.96 klux there was a pronounced reduction in cell growth, suggesting limitation of the energy required for the sequence of photosynthetic reactions that would result in carbon fixation under these conditions.

The effect of different carbon dioxide concentrations in the gases entering the photobioreactor was analysed by Yue and Chen [14], who found photosynthetic activity for concentrations of up to 70% (v/v) enrichment of the air with CO_2, which is usually the main source of carbon in photosynthetic cultures of cyanobacteria. According to Cuaresma et al. [24], the way in which cyanobacteria adapt themselves to use CO_2 as the carbon source is related to the carbon concentration mechanisms (CCM). The photosynthetic mechanism developed

Table 5
Analysis of variance (ANOVA) for the quadratic model

Source of variation	Sum of squares	Degrees of freedom	Mean squared	$F_{calculated}$
Regression	1938.8	9	215.42	2.83[a]
Residues	532.66	7	76.09	
Lack of fit	532.63	5	106.52	
Pure error	0.03	2	0.015	
Total	2741.44	16		

[a] Statistical significance ($p < 0.1$).

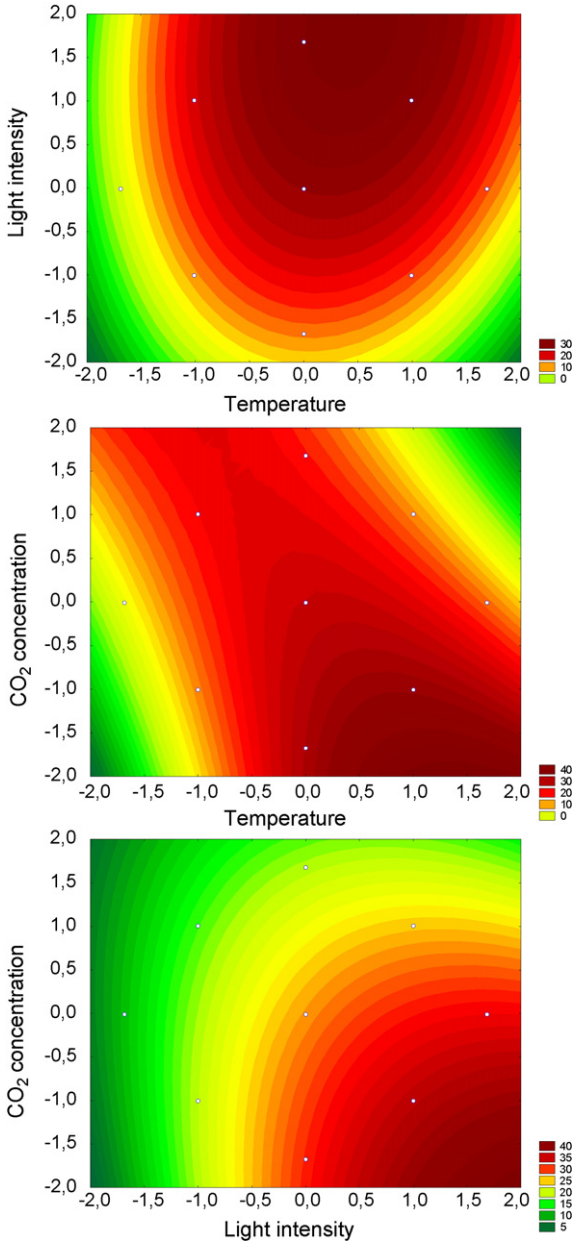

Fig. 3. Contour curves for the variable carbon fixation rate.

by these organisms allowed for the transport of free CO_2 and of bicarbonate ions through a fine plasmatic membrane, being accumulated in the cell as a carbon reservoir, making it possible to assimilate inorganic carbon in the form of bicarbonate or free carbon dioxide. With respect to cell growth and carbon fixation rate, the best results were obtained with air enriched with 15% CO_2, reduced efficiency of the photobioreactor being observed with flow rates below or above 0.45 L_{CO2} min^{-1}.

The model was validated from the *F* distribution (Table 5), which suggested the existence of a quadratic relationship between the variables, indicating that the proposed model fitted the experimental data. The statistical model obtained explained a maximum of 99.99% of the variation.

Table 6
Kinetic parameters for process optimization

Kinetic variable	Value[a]
μ_{max} (h^{-1})	0.04
t_g (h)	17.3
t_{log} (h)	120
X_m (mg L^{-1})	5100
R_C (mg L^{-1} h^{-1})	109.2

[a] Mean of three replicates are shown.

3.2. Process optimisation

As from the statistical model and an analysis of the contour diagrams, which suggested a displacement of the operational conditions to the region of high light intensities (11 klux), maintaining the conditions of temperature at 35 °C and injection of air enriched with 15% CO_2, cultures were carried out under these conditions in order to optimise the fixation of carbon by the cyanobacterium *Aphanothece microscopica Nägeli*. Table 6 shows the kinetic parameters obtained for the process under these conditions. A significant increase in the kinetic variables of growth and carbon fixation was observed, obtaining carbon fixation rates 58.1% higher than those obtained under the best conditions initially evaluated (9 klux, 35 °C, 15% of CO_2), showing the importance of the factors studied and the adjustment of the methodology used.

4. Conclusions

The carbon dioxide concentration, temperature and light intensity are determinant factors in the carbon fixation process by the cyanobacterium *Aphanothece microscopica Nägeli*.

Under operational conditions of 11 klux, 35 °C and 15% CO_2, the microorganism performed better, obtained maximum specific growth rates and carbon fixation rates of 0.04 h^{-1} and 109.2 mg L^{-1} h^{-1}, respectively.

Response surface methodology was adequate to determine the effects of the factors and optimise the process.

Thus the results obtained in the present study suggest the potential of applying this type of process to obtain carbon credits. Nevertheless one must remember that the definition of the ideal process conditions only helps in terms of the magnitude of the variables involved in the system, with the objective of applying the process under complex real conditions. In addition, the carbon fixed in the biomass only represents a fraction of the total carbon dioxide transformed by the system.

Acknowledgements

Funding for this research was provided by the Fundação de Amparo a Pesquisa no Estado de São Paulo – FAPESP (Brazil) and by the ALFA Programme, II-0259-FA-FC - POLYLIFE (European Union). The authors are grateful to Dra. Maria Isabel Queiroz (Fundação Universidade Federal do Rio Grande) for providing the microalgal cultures.

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