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GROWTH AND COMPOSITION OF *Arthrospira (Spirulina) platensis* IN A TUBULAR PHOTOBIOREACTOR USING AMMONIUM NITRATE AS THE NITROGEN SOURCE IN A FED-BATCH PROCESS

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Abstract - NH_4NO_3 simultaneously provides a readily assimilable nitrogen source (ammonia) and a reserve of nitrogen (nitrate), allowing for an increase in *Arthrospira platensis* biomass production while reducing the cost of the cultivation medium. In this study, a 2^2 plus star central composite experimental design combined with response surface methodology was employed to analyze the influence of light intensity (I) and the total amount of added NH_4NO_3 (M_t) on a bench-scale tubular photobioreactor for fed-batch cultures. The maximum cell concentration (X_m), cell productivity (P_X) and biomass yield on nitrogen ($Y_{X/N}$) were evaluated, as were the protein and lipid contents. Under optimized conditions (I = 148 μmol·photons·m^{-2}·s^{-1} and M_t = 9.7 mM NH_4NO_3), X_m = 4710 ±34.4 mg·L^{-1}, P_X = 478.9 ±3.8 mg·L^{-1}·d^{-1} and $Y_{X/N}$ = 15.87 ±0.13 mg·mg^{-1} were obtained. The best conditions for protein content in the biomass (63.2%) were not the same as those that maximized cell growth (I = 180 μmol·photons·m^{-2}·s^{-1} and M_t = 22.5 mM NH_4NO_3). Based on these results, it is possible to conclude that ammonium nitrate is an interesting alternate nitrogen source for the cultivation of *A. platensis* in a fed-batch process and could be used for other photosynthetic microorganisms.
Keywords: *Arthrospira (Spirulina) platensis*; Fed-batch cultivation; Ammonium nitrate; Light intensity; Tubular photobioreactor.

INTRODUCTION

The use of microorganisms in food production and in the environmental field, including the biofixation of CO_2 and bioremediation, is a major focus of recent biotechnological studies. In this context, photosynthetic microorganisms such as cyanobacteria are of particular interest because they generate several compounds with diverse applications (Belay, 1997; Spolaore *et al*., 2006; Vonshak, 1997) in addition to food protein. *A. platensis* is an important source of cellular protein, essential fatty acids, phycocyanin and chlorophyll *a* (Belay, 1997; Spolaore *et al*., 2006, Moraes *et al*., 2011).

In conventional cyanobacteria cultivation, nitrate salts (sodium and potassium nitrates) are the most

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used nitrogen sources, and several studies have demonstrated the feasibility of replacing these conventional nitrogen sources with low-cost alternatives such as urea, ammonium sulfate and ammonium chloride (Carvalho *et al*., 2004; Bezerra *et al*., 2008; Matsudo *et al*., 2012; Ferreira *et al*., 2010; Avila-Leon *et al*., 2012). Depending on the purpose of cultivation (e.g., pigments, carotenoid, fatty acids, biomass), different variables may be controlled in the growth of *A. platensis*. For example, the quantity and quality of the nitrogen source have a significant effect on the biomass yield and quality (Piorreck *et al*., 1984; Danesi *et al*., 2002; Avila-Leon *et al*., 2012). Under alkaline conditions, such as those usually adopted in *S. platensis* production, ammonium salts are in part present in the medium as ammonia, which is volatile and can even be toxic to the microorganism, depending on its concentration (Abeliovich & Azov, 1976; Muro-Pastor & Florencio, 2003; Bezerra *et al*., 2008). Even when urea is used as a nitrogen source, ammonia is formed either by its hydrolysis under alkaline conditions (Danesi *et al*., 2002) or by urease activity (Shimamatsu, 2004). *Spirulina platensis* can be cultivated by batch, continuous, semi-continuous (Reichert *et al*., 2006) and fed-batch processes (Carvalho *et al*., 2004). The latter process, when conducted at both a suitable feeding time and feeding rate to supply the nitrogen source according to *A. platensis* demand, can avoid any inhibitory ammonia concentration in the medium (Bezerra *et al*., 2008; Carvalho *et al*., 2004; Matsudo *et al*., 2009). Ammonium nitrate provides the culture with a readily assimilable nitrogen source (ammonia) and one reserve nitrogen source (nitrate) because ammonia is the preferred type of nitrogen for *S. platensis* (Boussiba, 1989). Therefore, high biomass concentration and productivity are expected when such a nitrogen source is employed in *A. platensis* cultivation using a fed-batch process.

The light intensity is also an important variable in cyanobacteria cultivation. High values of light intensity promote growth parameters such as maximum specific growth rate, whereas low values result in a biomass that is rich in pigments and proteins (Danesi *et al*., 2004).

The cultivation of *S. platensis* can be performed in conventional open ponds or closed photobioreactors (Carvalho *et al*., 2013). The utilization of tubular photobioreactors in the cultivation of *A. platensis* is recommended for ammonia compounds (Ferreira *et al*., 2010) because nitrogen loss by off-gassing and water evaporation can be avoided, thereby increasing cellular concentration and reducing the cultivation cost.

A 2^2 plus star central composite design combined with response surface methodology was used to optimize the production of *A. platensis* in a tubular photobioreactor, employing ammonium nitrate as the nitrogen source using a fed-batch process. The analysis measured the influence of light intensity (I) and the total amount of added ammonium nitrate (M_t) on growth and kinetic parameters such as maximum cell concentration (X_m), cell productivity (P_X), biomass yield on nitrogen ($Y_{X/N}$) and on the protein and lipid contents.

MATERIAL AND METHODS

Microorganism Used and Inoculum Preparation

The strain *Spirulina platensis* UTEX 1926, recently re-classified as *Arthrospira platensis* (Nordstedt) Gomont (Silva *et al*., 1996), was obtained from the University of Texas Culture Collection. It was grown in 500 mL-Erlenmeyer flasks containing 250 mL of Schlösser medium (Schlösser, 1982), containing the following nutrients (g L^{-1}): $NaHCO_3$, 13.61; Na_2CO_3, 4.03; NaCl, 1.00; K_2SO_4, 1.00; $NaNO_3$, 2.50; K_2HPO_4, 0.50; $MgSO_4.7H_2O$, 0.20; $CaCl_2.2H_2O$, 0.04. All nutrients were dissolved in distilled water containing (per liter): 6 mL of metal solution (750 mg Na_2EDTA, 97 mg $FeCl_3.6H_2O$, 41 mg $MnCl_2.4H_2O$, 5.0 mg $ZnCl_2$, 2 mg $CoCl_2.6H_2O$, 4.0 mg $Na_2MoO_4.2H_2O$) and 1 mL of micronutrient solution (50.0 mg Na_2EDTA, 618 mg H_3BO_3, 19.6 mg $CuSO_4.5H_2O$, 44.0 mg $ZnSO_4.7H_2O$, 20.0 mg $CoCl_2.6H_2O$, 12.6 mg $MnCl_2.4H_2O$, 12.6 mg $Na_2MoO_4.2H_2O$).

These flasks were maintained on a rotary shaker at 100 rpm, 30 °C and 6.0 klux (72 μmol photons m^{-2} s^{-1}). The resulting suspension was harvested during exponential growth, filtered and washed twice in a physiological solution (0.9% NaCl) to remove absorbed salts, including $NaNO_3$. The cells were then resuspended in the same Schlösser medium without any nitrogen source and used to inoculate the airlift photobioreactor. The initial cell concentration was fixed at 400 mg l^{-1}, expressed as dry weight (Soletto *et al*., 2008).

Photobioreactor

All of the experiments were carried out in a tubular photobioreactor with an airlift system (Figure 1). The photobioreactor used in this study was developed at the Fermentation Technology Laboratory of the Department of Biochemical and Pharmaceutical Technology of São Paulo University.

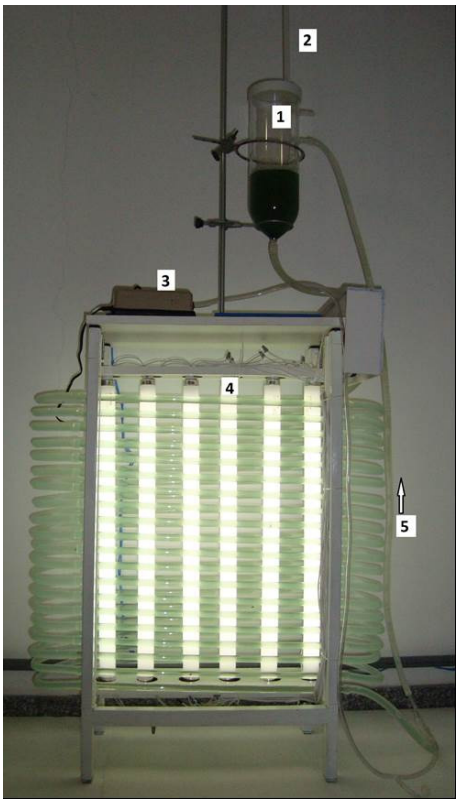

Figure 1: Tubular photobioreactor. (1): Degasser; (2): Condenser tube; (3): Air pump; (4): 20 W Fluorescent lamps; (5): Airlift system (Carvalho *et al*., 2013).

It is made of a metal structure surrounded by 40 glass tubes that are linked by silicone rubber tubes. The glass tubes (0.01 m internal diameter (Carlozzi and Pizani, 2005), 0.015 m wall thickness and 0.5 m length) was positioned with a 2% (1.15 °) incline. At the bottom of the reactor is a "Y" tube that receives air and cell culture, leading the latter to a degasser vessel that is located at the top of the structure. This flask has a glass tube that contributes to reducing the loss of both water and ammonia by evaporation. The working volume was 3.4 L. The illuminated volume (in the tubes illuminated by the fluorescent lamps) corresponds to approximately 57% of the total working volume. The culture flow was 40 L h^{-1}, the temperature was set at 29 ± 1 °C, and the pH was 9.5 ± 0.5 (Sanchez-Luna *et al*., 2007), controlled by the addition of CO_2 from a cylinder (Matsudo *et al*., 2012).

Experimental Design

In fed-batch runs using NH_4NO_3, the Schlösser medium (Schlösser, 1982) lacking its original nitrogen source ($NaNO_3$) was used, employing a feeding time of 6 days for adding NH_4NO_3. The total amount of nitrogen source (added twice a day) and the light intensities were adjusted according to the experimental design (Table 1).

Table 1: Factorial design for fed-batch cultivation of *A. platensis* using different light intensities, concentrations of ammonium nitrate as a nitrogen source and related experimental results (Parts A and B).

Test	X_1 [a]	X_2 [b]	I [c]	M_t [d]	T_C [e]	X_m [f]	P_X [g]	$Y_{X/N}$ [h]	NH_3 [i]	$NO3$ [j]	*Lip* [k]	*Ptn* [l]
Part A. Tests of the initial experimental design												
1	-1	-1	60	7.5	8	3914	439.2	16.73	0.55	*	8.3	23.8
2	+1	-1	180	7.5	10	4834	443.4	21.11	0.02	0.4	5.9	14.8
3	-1	+1	60	22.5	5	1029	125.8	1.00	11.10	12.2	**	**
4	+1	+1	180	22.5	5	960	112.1	0.89	10.4	10.6	17.3	63.2
5	0	-1.414	120	4.4	7	3422	431.7	24.53	0.01	0.5	3.3	12.3
6	-1.414	0	35	15.0	7	2164	252.0	4.20	0.09	9.2	12.5	41.7
7	0	+1.414	120	25.6	4	676	69.1	0.38	10.2	12.2	**	**
8	+1.414	0	205	15.0	8	3926	440.7	8.39	0.44	0.63	8.1	25.8
9	0	0	120	15.0	8	4194	474.2	9.03	0.10	12.4	7.6	25.5
10	0	0	120	15.0	7	3854	493.4	8.22	0.10	11.3	7.0	22.5
11	0	0	120	15.0	8	3938	442.2	8.42	0.06	7.5	9.9	29.3
Part B. Confirmation tests												
12	0.47	- 0.71	148	9.7	9	4699	477.7	15.83	0.07	0.5	10.3	21.4
13	0.47	- 0.71	148	9.7	9	4683	475.9	15.77	0.04	2.6	9.4	18.9
14	0.47	- 0.71	148	9.7	9	4749	483.2	16.01	0.03	1.2	10.5	19.8

[a] X_1 = Codified values for light intensity, [b] X_2 = Codified values for addition of ammonium nitrate (Mt), [c] I = Light intensity values (μmol photons m^{-2} s^{-1}), [d] M_t = total amount of ammonium nitrate added (mM), [e] T_C = Cultivation time (days), [f] X_m = Maximum cell concentration (mg L^{-1}), [g] P_X = Cell productivity (mg L^{-1} d^{-1}), [h] $Y_{X/N}$ = Yield of biomass on nitrogen (mg mg^{-1}), [i] NH_3 = Final concentration of total ammonia (mM), [j] NO_3 = final concentration of nitrate (mM),[k] Lip = lipid biomass content (%),[l] Ptn = protein biomass content (%), * value below the detection limit of the analytical method, ** It was not possible to quantify (cell death).

The addition of NH_4NO_3 was made according to the method proposed by Matsudo *et al*. (2009) for nitrogen addition, using the following equation:

$$M = 1.33 + k \cdot t \tag{1}$$

where M is the amount of NH_4NO_3 added until time t (days). The initial amount of NH_4NO_3 was 1.33 mM, and k corresponded to the quantity of NH_4NO_3 added daily. The total feeding time (t_f) was 6 days. When $t = t_f$, the M corresponded to the total quantity of NH_4NO_3 added (M_t) (Table 1).

Analytical Methods

The dry cell concentration was determined by optical density at 560 nm using a calibration curve (Leduy and Therien, 1997). The dry weight of *A. platensis* biomass was determined using a digital balance after filtering cells on a 0.8 μm filter and drying at 60 °C overnight. A good linear relationship between the dry weight concentration (DWC) and the OD560 nm was obtained. The ammonia concentration was determined using the phenol-hypochlorite method at 640 nm with a calibration curve (Solorzano, 1969). The nitrate concentration was determined in accordance with the methodology described by Vogel (2002). The total carbonate concentration was determined using the titration method (Pierce and Haenisch, 1948). The pH was determined daily using a potentiometer. At the end of each cultivation, cells were filtered, washed with distilled water and dried at 55 °C. The total lipids of the dried biomass were extracted in a Soxhlet using a 2:1 (v/v) chloroform-methanol mixture in accordance with the method described by Olguín *et al*. (2001). The protein content of the dry biomass was determined using the Kjeldahl method (Association of Official Analytical Chemists, 1984) with a conversion factor of 6.25.

Parameter Calculations

The biomass yield on nitrogen ($Y_{X/N}$) was calculated as the ratio of the produced cell mass to the amount of nitrogen added to the system:

$$Y_{X/N} = V\left(X_m - X_i\right) / m_N \tag{2}$$

where X_m is the maximum cell concentration in the reactor and X_i is the cell concentration in the inoculum, V is the working volume of the reactor, and m_N is the total mass of nitrogen added to the reactor.

The cell productivity (P_X) was calculated as the ratio of the variation in cell concentration (X_m - X_i) to the cultivation time (T_C), which is the time at which X_m was reached:

$$P_X = \left(X_m - X_i\right) / T_C \tag{3}$$

Statistical Analysis

The response surface methodology (RSM) was used in this study to evaluate the effects of light intensity (I) and total ammonium nitrate addition (M_t) on the fed-batch growth of *A. platensis*. This allowed the determination of the influence of the two independent variables on the three response variables selected for this study: X_m, P_X and $Y_{X/N}$. Multivariable regression analyses were performed under the conditions that had preliminarily been selected for the experimental design (Table 1). Such a design was based on the methodology called ‘‘star planning” (Barros-Neto *et al*., 2003), which consists of 2 factors in 5 levels of independent variables (runs 1–8). The central point was repeated 3 times (runs 9–11) to check the reproducibility of the results.

The response equation and the corresponding surface plot were generated in this study according to the following general polynomial equation:

$$y = b_0 + b_1 X_1 + b_2 X_2 + b_{11} X_1^{\,2} + b_{22} X_2^{\,2} + b_{12} X_1 X_2 \tag{4}$$

where y is the predicted response of the dependent variable (X_m, P_X or $Y_{X/N}$), X_1 and X_2 are the codified values of the independent variables I and M_t, respectively, and the parameter b is the polynomial coefficient to be estimated by model fitting using the S-PLUS 2000 program. Values < 0.10 were considered significant for regression analysis, and $p < 0.05$ was considered significant in the analyses of variance (ANOVA).

RESULTS AND DISCUSSION

Comparing $NaNO_3$ and NH_4NO_3 as a Nitrogen Source

The growth curves of all of the runs with NH_4NO_3 did not show a lag phase, as shown in Figure 2, with the exception of run 7, in which there was no growth from the first day of cultivation because of the inhibitory ammonia concentration (≥7.3 mM) (Carvalho *et al*., 2004). The absence of a lag phase was expected, primarily because nitrate was the nitrogen

source used by *A. platensis* during inoculum cultivation. The total carbonate concentration was practically constant due to the daily pH correction using carbon dioxide, and generally remained higher than 8.0 g l^{-1}. This procedure resulted in avoiding any lack of carbon source during the cultivation (Matsudo *et al*., 2012). This control permits us to assume that the variations obtained during the cultivation occurred due to the independent variables studied in this work. Concerning the nitrate concentration, the residual concentration of this nitrogen form was higher in runs that were conducted with higher concentrations of ammonium nitrate, such as runs 3, 4 and 7 (Table 1). This behavior was expected because when both ammonia and nitrate are present in the medium, ammonia is preferentially assimilated (Boussiba, 1989).

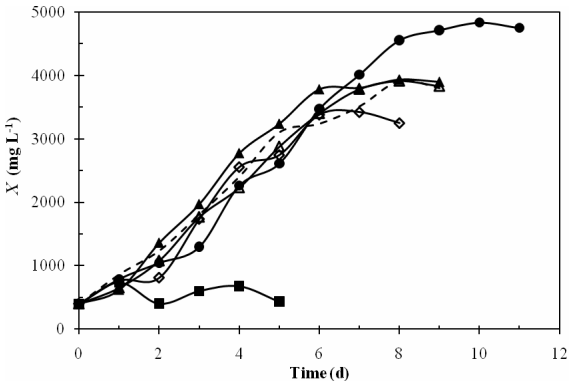

Figure 2: *A. platensis* growth at different concentrations of NH_4NO_3 and light intensities. (●): Run 2 (7.5 mM, 180 μmol photons m^{-2} s^{-1}); (∆): Run 3 (22.5 mM, 60 μmol photons m^{-2} s^{-1}); (◊): Run 5 (4.4 mM, 120 μmol photons m^{-2} s^{-1}); (■): Run 7 (25.6 mM, 120 μmol photons m^{-2} s^{-1}); (▲): Run 11 (15.0 mM, 120 μmol photons m^{-2} s^{-1}).

The central point runs (runs 9 - 11; *I = 120* μmol photons m^{-2} s^{-1} and *Mt* = 15 mM) provided a mean value of X_m = 3995.33 mg L^{-1} in 7 to 8 days. This X_m value is slightly higher than the value obtained under the same cultivation condition (tubular photobioreactor, light intensity, microorganism and pure CO_2) but employing the original culture medium, with $NaNO_3$ as a nitrogen source (X_m = 3209 mg L^{-1}) (Bezerra *et al*., 2013). Additionally, the cultivation time was the same (7 days). These results indicate that it would be feasible to replace the original nitrogen source with NH_4NO_3. Nevertheless, the unsatisfactory results in the runs with the lowest (run 5, Table 1) and highest (run 7, Table 1) total amounts of ammonium nitrate indicate a need to optimize the addition of this nutrient. In fact, higher values of X_m were obtained when the nitrogen supply was in the range of 7.5 mM ≤ Mt ≤ 15 mM and the light intensity was in the range of 60 μmol photons m^{-2} s^{-1} ≤ I ≤ 180 μmol photons m^{-2} s^{-1}.

Regarding the biomass yield on nitrogen ($Y_{X/N}$), in the central point runs of this study (runs 9 - 11; *I* = 120 μmol photons m^{-2} s^{-1} and *Mt* = 15 mM), a mean value of $Y_{X/N}$ = 8.55 mg mg^{-1} was obtained. This value is higher than that obtained under the same cultivation conditions (tubular photobioreactor, light intensity, microorganism and pure CO_2) but employing the original culture medium with $NaNO_3$ as nitrogen source ($Y_{X/N}$ = 4.5 mg mg^{-1}) (Bezerra *et al*., 2013). It is important to note that this parameter is calculated by accounting for the total amount of nitrogen added to the system (Equation (2)) without considering the residual nitrogen remaining at the end of the cultivation.

When using NH_4NO_3 as a nitrogen source, the concentration of ammonia inside the reactor must be maintained at low levels (less than 1.6 mM) due to the well-known inhibitory effect that ammonia can exert on *A. platensis* growth (Carvalho *et al*., 2004). As a consequence, practically all of the nitrogen added during cell growth is converted to biomass. Conversely, when using only nitrate as the nitrogen source, higher concentrations (greater than 10 mM) must be maintained in the culture medium to prevent growth limitation (Faintuch, 1989), and the efficiency of the conversion of total nitrogen added to cells thus greatly decreases.

The use of ammoniacal nitrate is justified to obtain an increase in the biomass production of *A. platensis* as a consequence of the adequate supply of nitrogen to the culture system. The constant presence of nitrate prevents the nitrogen deficiency found in cultivations with ammonia, and the presence of ammonia, which is readily assimilated by cyanobacteria, requires less nitrate and thus helps reduce the costs of the culture medium. Moreover, when the cells use ammonia for growth, energy is saved because less nitrate is converted to ammonia by nitrate reductase (Hatori and Myers, 1996).

Optimization of *A. platensis* Growth Using Ammonium Nitrate as a Nitrogen Source

Influence of Independent Variables on the Growth of *A. platensis*

Depending on the concentration of the nitrogen source and the light intensity evaluated in this work, different values of maximum cell concentration (X_m), cell productivity (P_X) and biomass yield on nitrogen ($Y_{X/N}$) were obtained in a tubular photobioreactor (Table 1).

Because different reactor conditions lead to distinct operational conditions, the relationship between M_t and I must be evaluated simultaneously to optimize *A. platensis* growth.

As shown in Table 1, when the total amount of ammonium nitrate added (M_t) was equal to or greater than 22.5 mM (runs 3, 4 and 7), the resulting X_m, P_X and $Y_{X/N}$ were low, with X_m reaching 676 mg l^{-1} when M_t = 25.6 mM (run 7), irrespective of the light intensity employed. This fact likely occurred because of the increasing ammonia concentration during cell growth, reaching 10 mM by the end of the cultivation (Table 1), a concentration that is considered to inhibit the growth of cyanobacteria (Abeliovich and Azov, 1976; Carvalho *et al*., 2004). In fact, such high values of ammonia concentrations led to cell death in runs 3 and 7. In run 4, despite high concentrations of ammonia during cultivation (22.5 mM), cell death was not observed, likely because of the high light intensity employed (180 μmol photons m^{-2} s^{-1}). The best X_m results were obtained using 7.5 mM ≤ Mt ≤ 15 mM (runs 1, 2, 8 and central runs). In fact, X_m = 4834 mg l^{-1} was obtained in run 2, which was performed with M_t = 7.5 mM NH_4NO_3 and I = 180 μmol photons m^{-2} s^{-1}. Although the lowest addition of ammonium nitrate (4.4 mM) led to a decrease in X_m (3422 mg l^{-1}; run 5, Table 1), this is not a very considerable decrease compared with the X_m obtained when the highest M_t value was used (X_m = 676 mg l^{-1}; run 7, Table 1). These findings suggest that cell growth is more strongly influenced by high ammonia concentrations than it is by low ammonia concentrations.

A clear difference was also observed in the results of X_m for the same value of light intensity (e.g., runs 1 and 3, Table 1), thus demonstrating that the main parameter that influences cell growth is the total amount of ammonium nitrate added. Conversely, as stressed above, the light intensity may be an important variable for attenuating ammonium toxicity because a higher light intensity (up to a saturating point) results in greater cell growth and, consequently, a higher assimilation of ammonia, which rapidly reduces the concentration of this nitrogen source (run 4).

The achievement of the stationary phase either at the lower (≤ 60 μmol photons m^{-2} s^{-1}) or higher (≥ 180 μmol photons m^{-2} s^{-1}) light intensities, even associated with the suitable nitrogen concentrations, could have been the consequence of the shading effect (Vonshak *et al*., 2000).

The analysis of multiple regression applied to the X_m data (Table 1, Part A) showed that this parameter was a satisfactory function of both codified values of I and M_t, as represented in Equation (5):

$$X_m = 3995 + 417.9X_1 - 1330X_2 - 440.9X_1^2 - 939.0X_2^2 \quad (5)$$

($p < 0.002$; adjusted $R^2 = 0.87$)

The adjusted model (Equation (5)) generated an adjusted determination coefficient (R^2) of 0.87, i.e., it explained 87% of the variability in the maximum cell concentration. This value is considered satisfactory for our purposes and is comparable to the values typically reported in the literature for bioprocesses (0.8) (Viswanathan and Kulkarni, 1995; Taragano and Pilosof, 1999; Fratelli *et al*., 2005). Moreover, the analysis of variance of the regression revealed that it is statistically significant (p < 0.002). The corresponding response surface is shown in Figure 3. The value of X_m, as estimated by the model at the optimal point (4565 mg L^{-1}) corresponds to X_1 = 0.47 and X_2 = -0.71, which are both inside the planned experimental range, as indicated in Figure 3. Such an optimal value of X_m was only 2.9% smaller than the experimental value obtained in the confirmation runs (4710 ± 34.4 mg l^{-1}, Table 1, part B), thus confirming the suitability of the model. In fact, the comparison between the values calculated by the model and the experimental values of X_m showed a satisfactory relationship, thus validating the model for estimating the optimal conditions for the biomass production of *A. platensis* using ammonium nitrate as a nitrogen source.

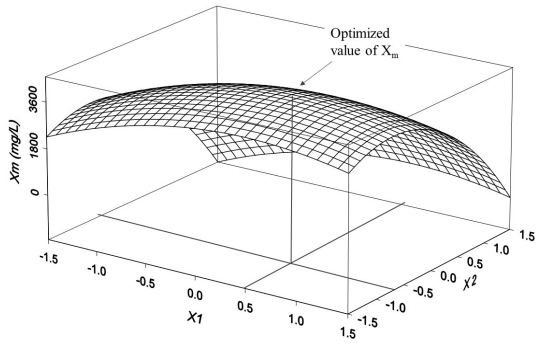

Figure 3: Response surface for maximum cell concentration (X_m) as a function of the codified values of light intensity (X_1) and the total amount of ammonium nitrate added (X_2). The arrow indicates the optimized X_m.

Cell productivity (P_X) as a function of the independent variables presented a behavior similar to that found for maximum cell concentration, which can be ascribed to the low difference in the cultivation time (T_C), both in runs with low cell growth (runs 3, 4 and

7, Table 1) and in runs with relatively high cell growth (runs 1, 2, 8-11, Table 1). P_X was shown to be a quadratic function of X_1 and X_2 (Figure 4), with negative values of the corresponding quadratic coefficients, as shown in Equation (6):

$$P_X = 469.9 - 144.7X_2 - 66.36{X_1}^2 - 114.4{X_2}^2 \quad (6)$$

($p <$ 0.001; adjusted $R^2 = 0.88$)

From the data shown in Figure 4, it is possible to infer that x_1 (light intensity) had less influence on P_X than x_2 (total amount of ammonium nitrate) did.

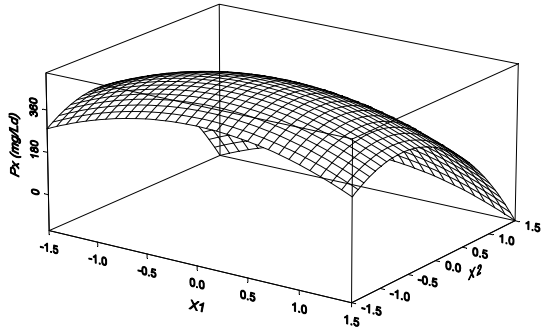

Figure 4: Response surface for cell productivity (P_X) as a function of the codified values of light intensity (X_1) and the total amount of ammonium nitrate added (X_2).

The equation obtained in the regression analysis to describe the biomass yield on nitrogen was as follows:

$$Y_{X/N} = 8.56 + 1.27X_1 - 8.76X_2 - 0.99{X_1}^2 + 2.09{X_2}^2 - 1.12X_1X_2 \quad (7)$$

($p < 0.001$; adjusted $R^2 = 0.99$)

Similar to X_m and P_X, $Y_{X/N}$ was a quadratic function of both x_1 and x_2. As shown in Figure 5, it is possible to infer that x_1 (light intensity) had less influence on $Y_{X/N}$ than x_2 (total amount of ammonium nitrate) did. Nevertheless, it is worth noting that only in this variable did the interaction effect between independent variables occur (b_{12} = -1.12). Figure 5 shows that higher $Y_{X/N}$ values were obtained in the runs carried out at low values of M_t, which is in agreement with the results obtained by Carvalho *et al*. (2004), who cultivated *A. platensis* in open ponds using ammonium chloride as the nitrogen source.

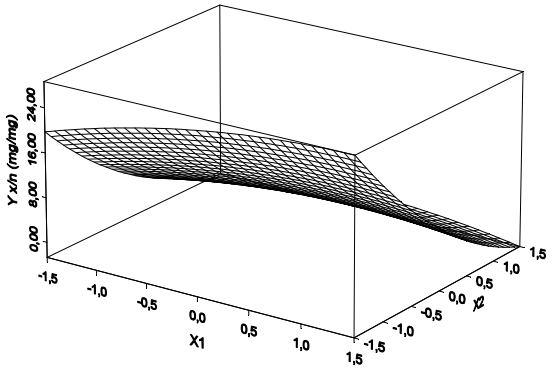

Figure 5: Response surface for biomass yield on nitrogen ($Y_{X/N}$) as a function of the codified values of light intensity (X_1) and the total amount of ammonium nitrate added (X_2).

Influence of Independent Variables on the Protein and Lipid Contents

The highest content of proteins in the biomass was obtained in run 4 (63.2%), in which the values of X_m (960 mg L^{-1}) and P_X (112.1 mg L^{-1} d^{-1}) were lower due to the high total amount of NH_4NO_3 added to the reactor (22.5 mM NH_4NO_3). Despite the higher X_m obtained in run 2, the protein content in the biomass composition was very low (14.8%; Table 1). In fact, in run 2, the lower protein content indicated that there was not enough nitrogen for its accumulation in the form of protein. Thus, the cyanobacterium grew and accumulated energy, most likely in the form of carbohydrates (Sassano *et al*., 2010).

Regarding the regression of protein content in the biomass, only values of $X_2 \leq 0$ were considered because $X_2 > 0$ led to cell death in most runs. Equation (8) represents such a regression:

$$Ptn = 25.77 - 5.25X_1 + 12.72X_2 + 3.99{X_1}^2 + 2.26{X_2}^2 \quad (8)$$

$p <$ 0.05; adjusted $R^2 = 0.90$

It is interesting to note that, in contrast with the X_m and $Y_{X/N}$ values, the highest protein values were obtained when high levels of total ammonium nitrate were employed in cell cultivation (Figure 6). The protein content in runs 12-14 showed an average value of 20.0 ± 1.3%, while the average value of the lipid content in the biomass was 10.1 ± 0.6%, similar to the values observed in runs 9 to 11 (25.8 ± 3.4% for protein content and 8.2 ± 1.5% for lipid content).

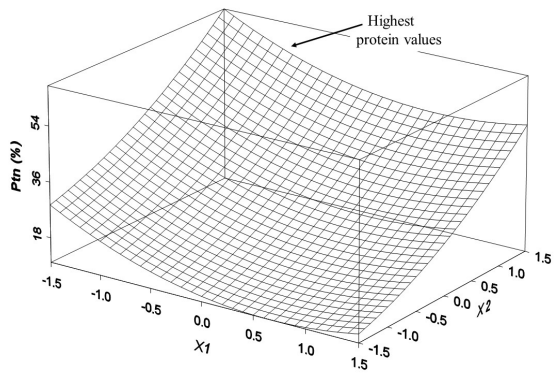

Figure 6: Response surface for biomass protein content (*Ptn*) as a function of the codified values of light intensity (X_1) and the total amount of ammonium nitrate added (X_2). The arrow indicates the highest protein value.

These protein content values are lower than expected for *A. platensis* (50 – 60%), according to Vonshak (1997b). Bezerra *et al*. (2008), who studied the effect of light intensity and ammonium chloride feeding time on the cultivation of *A. platensis*, achieved an average protein content of 35.9% in cultivations conducted under optimized conditions for that independent variable. Increased levels of nitrogen may favor protein accumulation in the biomass. In this study, the addition of optimized amounts of nitrogen that were sufficient for cell growth is likely to be one of the reasons for the reduced protein content, in contrast with the results from Matsudo *et al*. (2012), who obtained a protein content as high as 56% and employed urea as a nitrogen source under a continuous process.

Conversely, by calculating the protein productivity (i.e., multiplying the values of cell productivity and the fraction corresponding to protein in the biomass), one can conclude that, even with a lower protein content, the overall protein productivity was much higher in the central point runs (runs 9-11, P_{PTN} = 120.5 ± 9.2 mg $L^{-1}d^{-1}$) than it was in run 4 with the highest protein content (70.8 mg $L^{-1}d^{-1}$).

No effect of the light intensity on the biomass lipid content was detected. However, this variable was a positive linear function of the total ammonium nitrate addition (M_t). The maximum value of this dependent variable (17.3%) was obtained in run 4, where M_t = 22.5 mM. Similarly, the lowest lipid content was obtained in run 5 (4.4 mM NH_4NO_3), with a value of 3.3% (Table 1), due to the lack of nitrogen. Evaluating different levels of nitrogen sources in the cultivation of *A. platensis*, Piorreck *et al*. (1984) observed that the lipid content decreased under conditions of nitrogen limitation. The lipid content obtained in most of the runs is in the range expected for this photosynthetic microorganism. According to Cohen (1997), cyanobacteria are generally poor in lipids, commonly containing 6 – 13%, half of which are fatty acids.

CONCLUSIONS

This study demonstrated that light intensity (I) and the total amount of added ammonium nitrate (M_t) influence the growth of *A. platensis*. Values of I = 148 μmol photons m^{-2} s^{-1} and M_t = 9.7 mM NH_4NO_3 were the best combination for obtaining high values of X_m, with an average value of 4710 ± 34.4 mg L^{-1}. Such a value is very close to that estimated by the model for the optimal point (4565 mg L^{-1}). Under such conditions, the mean values of P_X and $Y_{X/N}$ were 478.9 ± 3.8 mg L^{-1} d^{-1} and 15.87 ± 0.13, respectively. It was possible to confirm the suitability of the multivariable regression analysis as a tool for obtaining optimized experimental conditions for X_m using ammonium nitrate as a nitrogen source. The best conditions found for cell growth were not the same as those for maximizing protein content. However, considering the overall protein productivity, the optimized conditions for cell growth were also appropriate for producing high-quality single cell protein. Our results show that ammonium nitrate is an interesting alternative nitrogen source for the cultivation of *A. platensis* in a fed-batch process and that it can be used for other photosynthetic microorganisms.

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NOMENCLATURE

I	Light intensity (μmol·photons·m^{-2}·s^{-1})
M_t	Total amount of NH_4NO_3 added (mM)
X_m	Maximum cell concentration (mg·L^{-1})
X_i	Initial cell concentration (mg·L^{-1})
P_X	Cell productivity (mg·L^{-1}·d^{-1})

$Y_{X/N}$	Yield of biomass on nitrogen ($mg \cdot mg^{-1}$)
V	Reactor volume (L)
T_C	Cultivation time (days)
X_1	Codified values of I (dimensionless)
X_2	Codified values of M_t (dimensionless)

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