Influence of ammonium sulphate feeding time on fed-batch *Arthrospira* (*Spirulina*) *platensis* cultivation and biomass composition with and without pH control

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ABSTRACT

Previous work demonstrated that a mixture of NH_4Cl and KNO_3 as nitrogen source was beneficial to fed-batch *Arthrospira* (*Spirulina*) *platensis* cultivation, in terms of either lower costs or higher cell concentration. On the basis of those results, this study focused on the use of a cheaper nitrogen source mixture, namely $(NH_4)_2SO_4$ plus $NaNO_3$, varying the ammonium feeding time (T = 7–15 days), either controlling the pH by CO_2 addition or not. *A. platensis* was cultivated in mini-tanks at 30 °C, 156 µmol photons $m^{-2} s^{-1}$, and starting cell concentration of 400 mg L^{-1}, on a modified Schlösser medium. T = 13 days under pH control were selected as optimum conditions, ensuring the best results in terms of biomass production (maximum cell concentration of 2911 mg L^{-1}, cell productivity of 179 mg $L^{-1} d^{-1}$ and specific growth rate of 0.77 d^{-1}) and satisfactory protein and lipid contents (around 30% each).

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1. Introduction

Arthrospira (*Spirulina*) *platensis* is a photosynthetic microorganism of industrial interest because of its high contents of amino acids, polyunsaturated fatty acids, vitamins, and pigments, such as beta-carotene and chlorophyll (Ciferri, 1983). *A. platensis* is considered one of the most valuable sources of proteins; up to 74% of its biomass can be constituted by this fraction (Cohen, 1997). One of the most abundant protein present in this cyanobacterium biomass is the phycocyanin, which is a nitrogen reserve involved in photosynthetic reactions (Ciferri, 1983). The most commonly found polyunsaturated fatty acids in *A. platensis* are the palmitic (34–42%), linoleic (19–26%), gamma-linolenic (16–25%) and oleic (3–8%) ones (Piorreck et al., 1984).

A. platensis production is relatively cheap and simple, since it can grow quickly with only inorganic nutrients and light, it is resistant to unfavorable environmental conditions and easily harvested. *A. platensis* biomass is useful, for example, as nutritional supplement for humans and animals (Cohen, 1997), as source of active principles in pharmaceutical and cosmetic industries (Belay et al., 1993), and as adsorbent material for heavy metals (Solisio et al., 2008). For the production of this cyanobacterium, the most widely

used systems are the outdoor ponds that allow obtaining large amounts of biomass at low costs. Nowadays, its commercial production is carried out almost exclusively in open ponds (Vonshak and Richmond, 1988), which do not require rigid control of the environmental conditions.

The cultivation of *A. platensis* depends greatly on nutrient availability, especially the nitrogen source. Although nitrates are commonly used in media (Paoletti et al., 1975; Schlösser, 1982), many studies have reported the benefits of using cheaper sources, such as ammonium salts (Bezerra et al., 2008; Sassano et al., 2007) and urea (Matsudo et al., 2009; Sánchez-Luna et al., 2007). Ammonium salts, mainly NH_4Cl and $(NH_4)_2SO_4$, are readily assimilated by *A. platensis* (Carvalho et al., 2004) and reduce the chances of culture contamination by other microorganisms (Borowitzka, 1999). On the other hand, ammonia can be toxic to cyanobacteria and microalgae when present in high concentrations in the medium (Abeliovich and Azov, 1976; Belkin and Boussiba, 1991). Because of its flexibility in supplying nutrients during cultivation, the fed-batch process has recently become one of the most promising for cell growth and metabolites production. For this reason, it is particularly suited to processes where nutrient levels must be adequately controlled. The fed-batch addition of ammonia-based nitrogen sources was shown to effectively prevent any inhibiting effect (Abeliovich and Azov, 1976; Belkin and Boussiba, 1991) on *Arthrospira* sp. cultivation. Moreover, probably due to off-gassing,

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the sole use of ammonium salts as nitrogen source in open tank cultivations usually leads to nitrogen deficient cultures and, consequently, to low final cell concentration. Rodrigues et al. (2010) suggested the simultaneous use of nitrates and ammonium salts as a way to avoid the difficulties encountered in *A. platensis* cultivation with only one nitrogen source: whereas the nitrate ensures high biomass yields, the ammonium salt allows reducing the production costs.

The control of culture pH around optimum values is a way to improve growth (Jiménez et al., 2003; Ogbonda et al., 2007) as much as ammonia and inorganic carbon uptake during cultivation (Azov, 1982). In a medium containing carbonate and bicarbonate, the latter is preferentially assimilated by cyanobacteria (Miller and Colman, 1980) and its formation is ensured at pH in the range 6–10; below this range, CO_2 prevails, while beyond it, carbonate does. During the incorporation of two bicarbonate ions, the cyanobacteria consume one in the form of carbon dioxide and release the other in the form of carbonate, leading to the progressive pH increase (Miller and Colman, 1980). The use of carbon dioxide to control the pH as well as to supply the carbon source (Soletto et al., 2008; Watanabe and Hall, 1995) in *A. platensis* cultivation, among many other cyanobacteria and microalgae, has the additional benefit of combining valuable biomass production and CO_2 emission reduction.

The objective of this work was to study the effects of ammonium feeding time and pH control on growth and biomass composition of *A. platensis*. To this purpose, such a cyanobacterium was grown at $30 \pm 1\ °C$ and 156 ± 6 μmol photons m^{-2} s^{-1} using a mixture of nitrogen sources [$(NH_4)_2SO_4$ plus $NaNO_3$] that is cheaper than the one suggested by Rodrigues et al. (2010). Five different ammonium feeding times ($T = 15, 13, 11, 9$ and 7 days) were studied in order to establish which one of them would best supply nitrogen to cultivations. The effect of pH control on *A. platensis* growth was observed through the daily addition of pure CO_2 until pH 8.7. Finally, the influence of T and pH control on the culture was also evaluated in terms of biomass composition, i.e. chlorophyll, protein, lipid, and carbohydrate contents.

2. Methods

2.1. Microorganism, media and inoculum preparation

The strain *Arthrospira* (*Spirulina*) *platensis* (Nordstedt) Gomont was obtained from the University of Texas Culture Collection and maintained in standard media (Paoletti et al., 1975; Schlösser, 1982).

The medium of Paoletti et al. contained (g L^{-1}): 2.57 KNO$_3$; 0.50 K$_2$HPO$_4$; 0.92 NaCl; 15.2 NaHCO$_3$; 8.89 Na$_2$CO$_3$; 1.88 Na$_2$SO$_4$; 0.05 CaCl$_2$·2H$_2$O; 0.25 MgSO$_4$·7H$_2$O; 1.0 mL L^{-1} Fe–EDTA solution (29.8 EDTA–Na$_2$; 24.9 FeSO$_4$·7H$_2$O); and 1.0 mL L^{-1} of micronutrient solution (2.85 BaCl$_2$·2H$_2$O; 0.19 CoCl$_2$·6H$_2$O; 0.17 MnCl$_4$·H$_2$O; 0.14 SeCl$_2$·2H$_2$O; 0.30 SnCl$_2$·2H$_2$O; 0.29 LiCl; 0.18 CuSO$_4$·5H$_2$O; 0.71 NiSO$_4$·5H$_2$O; and 0.12 NaMoO$_4$·2H$_2$O).

The medium of Schlösser contained (g L^{-1}): 13.6 NaHCO$_3$; 4.03 Na$_2$CO$_3$; 0.50 K$_2$HPO$_4$; 2.50 NaNO$_3$; 1.00 K$_2$SO$_4$; 1.00 NaCl; 0.20 MgSO$_4$·7H$_2$O; 0.04 CaCl$_2$·2H$_2$O; 6.0 mL L^{-1} of P-IV metal solution (0.75 Na$_2$EDTA·2H$_2$O; 0.097 FeCl$_3$·6H$_2$O; 0.041 MnCl$_2$·4H$_2$O; 0.005 ZnCl$_2$; 0.002 CoCl$_2$·6H$_2$O; 0.004 Na$_2$MoO$_4$·2H$_2$O); and 1.0 mL L^{-1} of CHU micronutrient solution (0.020 CuSO$_4$·5H$_2$O; 0.044 ZnSO$_4$·7H$_2$O; 0.020 CoCl$_2$·6H$_2$O; 0.012 MnCl$_2$·4H$_2$O; 0.012 Na$_2$MoO$_4$·2H$_2$O; 0.620 H$_3$BO$_3$; 0.050 Na$_2$EDTA·2H$_2$O).

For the purpose of experimentation, medium of Paoletti et al. (1975) and Schlösser (1982) had their initial nitrate concentration reduced to 20%, and were supplemented with 15.0 mM NH$_4$Cl and 7.50 mM (NH$_4$)$_2$SO$_4$, respectively. In the runs in which pH was controlled, a 1:33 NaCO$_3$/NaHCO$_3$ ratio was used in the medium of

Schlösser (1982) modified as above, in order to start cultivations at pH 8.7.

A. platensis was grown in 500 mL-Erlenmeyer flasks containing 200 mL of standard media on a rotary shaker at 100 rpm, $30 \pm 1\ °C$, and 6.0 ± 0.5 klux $(72 \pm 6$ μmol photons m^{-2} s^{-1}). The resulting suspension was harvested during the exponential growth phase, filtered, and washed thrice with physiological solution (0.9% NaCl) to remove adsorbed salts. The recovered cells were then suspended again in the modified media and used to inoculate the cultivation tanks.

2.2. Experiments and cultivation conditions

Experiments were carried out in 68 cm-long mini-tanks (Belay, 1997) made of PVC foils, each having total area of 0.123 m^2. The cultivation volume was 5 L, which was maintained through the daily addition of distilled water to replace water loss by evaporation. Paddle wheels ensured culture mixing at 18 rpm. Temperature was maintained at $30 \pm 1\ °C$ by submersible electronic thermostats (Aristos, São Paulo, SP, Brazil), and light intensity was regulated at 13 ± 0.5 klux $(156 \pm 6$ μmol photons m^{-2} s^{-1}) by fluorescent lamps and a light meter, model 250A (Li-Cor, Lincoln, NE, USA).

Four preliminary runs were performed under different conditions to select the best medium for the growth and inoculum level. Run A was performed on the medium proposed by Paoletti et al. (1975) but containing only 20% of KNO$_3$, supplemented with 15.0 mM NH$_4$Cl and inoculated with a cell concentration of 50 mg L^{-1} dry weight (Rodrigues et al., 2010), whereas runs B and C were carried out on the medium of Schlösser (1982) modified in the same way (20% of NaNO$_3$), supplemented with 7.50 mM (NH$_4$)$_2$SO$_4$, and inoculated with 50 and 400 mg L^{-1} dry weight, respectively. Ammonium salts were added throughout 17 days in the form of solution, the starting pH value was set at 9.5, and no pH control was done. Run D was carried out on modified Schlösser (1982) medium exactly as run C but with a 1:33 NaCO$_3$/NaHCO$_3$ ratio, in order to support pH control by daily addition of CO_2 until pH 8.7.

The medium of Schlösser (1982), modified through the reduction of NaNO$_3$ amount and with the addition of 7.50 mM of (NH$_4$)$_2$SO$_4$, and a starting cell concentration of 400 mg L^{-1} dry weight resulted to be the best conditions for *A. platensis* growth and were adopted in runs 1–10, carried out varying the ammonium feeding time from 7 to 15 days. As shown in Table 1, no pH control was done in runs from 1 to 5, whereas runs 6–10 were performed under the same conditions but adjusting the pH by daily addition of CO_2 until pH 8.7.

2.3. Nitrogen source addition and pH control

The addition of nitrate salts was made entirely at the beginning of the runs. In order to avoid any inhibitory effect of ammonia, ammonium salts were added by daily pulse feeding at flow rate exponentially increasing according to the experimental schedule of Table 1 and to the equation:

$$m_t = m_i e^{kt} \tag{1}$$

where m_i and m_t are the amounts of ammonium salts added per unit reactor volume at the start ($t = 0$) and until the instant "t", respectively.

The initial concentrations of NH$_4$Cl and (NH$_4$)$_2$SO$_4$ (m_i) were 1.70 and 0.85 mM, respectively, and the total feeding time (T) was varied from 7 to 17 days. When $t = T$, $m_t = m_T$, the latter being the total amount of ammonium salt added in the reactor. The choice of this flow rate pattern was suggested by the intention of supporting an exponential growth of biomass (Carvalho et al.,

Table 1
Experimental conditions (pH control and ammonium feeding time, T) and main results of *A. platensis* cultivation (cultivation time, T_C; maximum cell concentration, X_m; cell productivity, P_X; maximum specific growth rate, μ_m; yield of biomass on nitrogen, $Y_{X/N}$) and biomass contents (chlorophyll, CLH; protein, PTN; lipid, LIP; carbohydrate, CBH; ashes, A).

Run	Experimentation conditions						Main results					
	pH control[a]	T (d)	T_C (d)	X_m (mg L^{-1})	P_X (mg L^{-1} d^{-1})	μ_m (d^{-1})	$Y_{X/N}$ (mg mg^{-1})	CLH (mg g^{-1})	PTN (%)	LIP (%)	CBH (%)	A (%)
A[b,c]	Free	17	18	2105	114	0.71	7.48	–	–	–	–	–
B[c]	Free	17	19	2613	135	0.75	8.93	–	–	–	–	–
C	Free	17	19	2252	97	0.30	7.70	–	–	–	–	–
D	CO_2	17	18	2338	108	0.32	7.99	–	–	–	–	–
1	Free	15	16	2597	138	0.60	8.88	5.3	26	25	40	9
2	Free	13	14	2637	160	0.76	9.01	5.1	24	26	45	5
3	Free	11	12	1920	127	0.62	6.56	5.3	25	27	40	8
4	Free	9	11	2011	147	0.48	5.87	4.9	33	17	43	7
5	Free	7	10	1820	142	0.50	6.22	4.6	38	16	41	5
6	CO_2	15	16	3000	162	0.59	10.2	4.6	22	26	44	7
7	CO_2	13	14	2911	179	0.77	9.95	4.6	28	30	35	7
8	CO_2	11	12	2516	176	0.64	8.60	4.1	30	25	35	9
9	CO_2	9	11	2287	172	0.56	7.82	4.3	29	20	43	7
10	CO_2	7	11	2099	154	0.55	7.17	4.7	30	14	50	6

– analysis not done.
[a] pH control, in which runs had pH let run free or reduced once a day to 8.7 by CO_2 addition.
[b] Runs carried out on modified Paoletti medium, whereas all other runs were carried out on modified Schlösser medium.
[c] Runs starting with 50 mg L^{-1}, whereas all other runs started with 400 mg L^{-1}.

2004), and the initial concentration of each ammonium salt was based on the total amount of nitrogen actually needed in the cultivation (Rodrigues et al., 2010).

The decrease to 1:33 of $NaCO_3/NaHCO_3$ ratio with respect to that of the original medium of Schlösser (1982) (1:4) allowed reducing the starting pH from 9.5 to 8.7 and made daily pH adjustment by the addition of pure CO_2 easier. Carbon dioxide solubilization in the medium was ensured by circulation of CO_2 and cell suspension in an external hose system (runs D and 6–10).

2.4. Analytical methods

Dry cell mass concentration was determined, as described by Leduy and Therien (1977), by optical density measurement at 560 nm using a calibration curve. The pH was determined by a potentiometer, model 710-A (Orion, Beverly, MA, USA). The concentration of total ammonia was determined by the same potentiometer, but using a selective ammonium ion electrode, model 95–12 (Orion), after preliminary pH adjustment to 13 with NaOH 1.5 M (Carvalho et al., 2004), on medium samples free of cells. Calibration curves were prepared every 2 days, as instructed by the manufacturer of the equipment. The concentration of chlorophyll "a" was determined on cell samples taken on the day corresponding to the maximum cell concentration. For this purpose, it was carried out a methanol-mediated extraction of the biomass pigment, the optical density was measured at 665 nm (Vonshak, 1997), and the concentration values were obtained using a calibration curve.

After harvesting at the end of cultivation, recovered cells were centrifuged, washed with distilled water in order to remove all adsorbed salts, and dried at 55 °C for 12 h (Pelizer et al., 1999). The lipid and protein contents of biomass were analyzed in powdered samples according to Olguín et al. (2001) and the AOAC (2007), respectively. The ash content was determined by calcination of the samples at 450 °C for 6 h in a muffle furnace, and that of carbohydrates was calculated based on lipids, proteins, and ashes percentages.

2.5. Kinetic parameters calculation

The specific growth rate (μ_X), expressed in d^{-1}, was calculated by derivation of experimental growth curves as suggested by

LeDuy and Zajic (1973), and its maximum value found in the exponential growth phase was referred to as μ_m.

The cell productivity (P_X), expressed in mg L^{-1} d^{-1}, was calculated as the ratio of the variation in cell concentration (X_m–X_i), X_m being the maximum value of cell concentration in the reactor and X_i that in the inoculum, respectively, to the cultivation time (T_C):

$$P_X = \frac{X_m - X_i}{T_C} \qquad (2)$$

The yield of biomass on nitrogen ($Y_{X/N}$), expressed in mg cells mg^{-1} N, was calculated as the ratio of the produced cell mass to the amount of nitrogen added to the system:

$$Y_{X/N} = \frac{V(X_m - X_i)}{m_N} \qquad (3)$$

V being the tank working volume (5 L) and m_N the total added mass of nitrogen sources expressed as nitrogen.

2.6. Statistical analysis

Comparison of results of runs 1–10 was made by two-way analysis of variance (ANOVA), performed by the program MINITAB 15, and the statistical significance was evaluated by estimation of the descriptive level (p), being the results considered statistically significant when $p < 0.05$ (confidence level > 95%).

3. Results and discussion

3.1. Preliminary tests

Previous study demonstrated that the simultaneous use of potassium nitrate and ammonium chloride in the well-known medium of Paoletti et al. (1975) ensured better *A. platensis* growth compared to the use of separate nitrogen sources (Rodrigues et al., 2010); therefore, one of the best conditions of that study, in terms of nitrogen costs, was selected to carry out the reference run of this study (run A).

To search conditions able to further improve biomass growth, additional runs were done changing medium composition and inoculum concentration with respect to run A. Thus, as shown in Table 1, the medium of Schlösser (1982) modified as described in
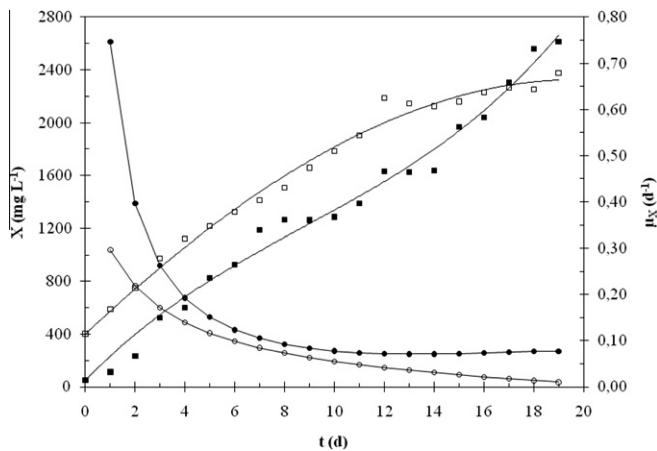
Fig. 1. Time behavior of the cell concentration (squares) and specific growth rate (circles) during *A. platensis* cultivations performed on modified Schlösser medium with an inoculum level of 50 mg L^{-1} (run B, ■ and ●) and 400 mg L^{-1} (run C, □ and ○).

Section 2.3 was used in run B, the inoculum concentration was increased from 50 to 400 mg L^{-1} in run C, and the pH was daily adjusted to 8.7 by addition of pure carbon dioxide in run D.

The use of the modified Schlösser (1982) medium resulted in a 24% increase in X_m when compared to the one of Paoletti et al. (1975). Although the nitrogen sources used in runs A and B were different [KNO$_3$ + NH$_4$Cl and NaNO$_3$ + (NH$_4$)$_2$SO$_4$, respectively], they were furnished in equimolar amounts to both cultures and detected in the media in almost the same concentrations, thus suggesting that such an increase should not be ascribed to these sources. Although both media are very similar and well suited for *A. platensis* cultivation, the medium of Schlösser (1982) has lower osmotic pressure than the one of Paoletti et al. (1975), as consequence of its lower salinity. The total monovalent cation concentration in medium of Paoletti et al. (1975) is in fact about 421 mM, whereas in the medium of Schlösser (1982) it is 28.3% lower (302 mM).

Contrary to expectations, the increase in the inoculum concentration from 50 (run B) to 400 mg L^{-1} (run C) did not promote any significant rise of X_m (Table 1); it accelerated, instead, the occurrence of pseudo-steady-state conditions from around the 12th day forward (Fig. 1), thereby highlighting that the amount of ammonium provided to the cultivation was insufficient to maintain exponential growth. This is confirmed in Fig. 1 by the values of μ_X, as well as in Table 1 by those of μ_m, that were significantly lower in runs started with 400 mg L^{-1} (runs C and D) than in those

started with 50 mg L^{-1} (runs A and B). Disappointedly, the control of pH through the daily addition of pure CO$_2$ did not lead to any statistically significant increase in X_m (runs C and D, Table 1) with respect to the 5% degree of confidence experimentally determined for the biomass concentration methodology. It is likely that, as for run C, the insufficient ammonium availability (long feeding time) hindered the cell growth during run D and masked the positive effect of pH control.

Moreover, according to previous tests (data not shown), it took around 9 h for pH to increase from 8.7 to 9.2 in the medium of Schlösser (1982). Since the addition of ammonium sulphate was done right after daily pH adjustment, cells in run D felt around 9 h of high ammonium availability per day, whereas starting pH in runs A, B and C was already higher than the pK_a of ammonia (9.25). The best ammonium uptake during run D was confirmed by the detectable presence of this ion in the medium only after the 8th day of cultivation, very late in comparison to all the other runs (2nd day).

Conclusively, the increase in the inoculum level could be successful in cultivations of *A. platensis* only alongside with ammonium feeding time decrease, and the pH control could have the potential of increasing X_m, just as well as μ_X, as long as ammonium supply were adequate to support cell growth.

3.2. Influence of the ammonium feeding time and pH control on growth

As it can be observed in Table 1, the reduction of (NH$_4$)$_2$SO$_4$ feeding time from 17 to 15 and to 13 days was enough to reach in runs 1 and 2 (X_i = 400 mg L^{-1}) the same X_m value as in run B (X_i = 50 mg L^{-1}). Since runs 1 and 2 lasted less than run B, their P_X values were also higher. These results indicate that the regulation of the ammonium feeding time was indeed essential to support the increase in the inoculum concentration.

According to the statistical analysis (Table 2), the ammonium feeding time exerted significant influence especially on X_m (p = 0.003) and $Y_{X/N}$ (p = 0.005), indicating that T = 13 and 15 were both adequate to *A. platensis* growth and ammonium uptake. A feeding time of 13 days did in fact lead to the highest μ_m values (0.76–0.77 d^{-1}, Table 1), hence confirming its adequacy for this cyanobacterium cultivation under the conditions studied.

Moreover, despite the sufficient nitrogen source addition, runs 3–5 exhibited low values of both X_m (about 1800–2000 mg L^{-1}) and $Y_{X/N}$ (about 5.9–6.5 mg mg^{-1}), but relatively high values of P_X (127–147 mg L^{-1} d^{-1}), because of short feeding time (7–11 days). However, since ammonia was detectable in run 5 only from the 4th day forward, but never at inhibitory levels, other environmental factors were likely responsible for this growth

Table 2
Mean values of the results of *A. platensis* cultivation (cultivation time, T_C; maximum cell concentration, X_m; cell productivity, P_X; maximum specific growth rate, μ_m; yield of biomass on nitrogen, $Y_{X/N}$) and biomass contents (chlorophyll, CLH; protein, PTN; lipid, LIP; carbohydrate, CBH; ashes, A) obtained by the two-way analysis of variance (ANOVA).

	X_m (mg L^{-1})	P_X (mg L^{-1} d^{-1})	μ_m (d^{-1})	$Y_{X/N}$ (mg mg^{-1})	CLH (mg g^{-1})	PTN (%)	LIP (%)	CBH (%)	A (%)
Ammonium feeding time (d)									
7	1959[a]	148[a]	0.53[a]	6.69[a]	4.6[a]	34[a]	15[a]	45[a]	5[a]
9	2149[a]	159[a]	0.52[a]	6.84[a]	4.6[a]	31[a]	18[a]	43[a]	7[a]
11	2218[a]	152[a]	0.66[b]	7.58[a]	4.7[a]	27[a]	26[b]	37[a]	8[a]
13	2774[b]	170[a]	0.77[c]	9.48[b]	4.8[a]	26[a]	28[b]	40[a]	6[a]
15	2798[b]	150[a]	0.60[a,b]	9.54[b]	4.9[a]	24[a]	25[a,b]	42[a]	8[a]
p^*	0.003	0.332	0.004	0.005	0.807	0.257	0.010	0.650	0.169
pH control (free or CO$_2$ addition)									
Free	2197[d]	143[d]	0.61[d]	7.31[d]	5.0[d]	29[d]	22[d]	42[d]	7[d]
CO$_2$	2563[e]	169[e]	0.63[d]	8.75[e]	4.5[d]	28[d]	23[d]	41[d]	7[d]
p^*	0.004	0.014	0.004	0.307	0.050	0.608	0.554	0.910	0.587

Different lower-case letters indicate statistical difference in each column, for each experimental condition studied.
* p-values (descriptive level) obtained by the two-way analysis of variance (ANOVA), being the results considered statistically significant when $p < 0.05$ (confidence level > 95%).

limitation or inhibition. Taking into account also the low μ_m values of runs 4 and 5 (0.48–0.50 d^{-1}), one can conclude that $T \leqslant 11$ days are unsuitable to A. platensis cultivation with 7.50 mM (NH$_4$)$_2$SO$_4$.

It is known that A. platensis uptakes exclusively bicarbonate and that no significant growth is observed when only carbonate is available to cells; in aqueous solution both ions coexist in chemical equilibrium, and bicarbonate prevails at pH < 10.2 (Binaghi et al., 2003). As explained by Miller and Colman (1980), bicarbonate is actively transported to the inside of cells and converted into carbon dioxide and carbonate, the former being incorporated as carbon source for growth and the latter released into the medium, leading to pH increase. A. platensis was shown to reach its optimum growth at pH values around 9.0 (Ogbonda et al., 2007) because of the bicarbonate preponderance in the HCO$_3^-$/CO$_3$ = equilibrium under these conditions. Runs 6–10 had pH controlled in the range 8.7–9.7 with an average daily value of 9.1, which means that A. platensis was maintained at around its optimum pH for the largest duration of the cultivations, contrary to runs 1–5, carried out without any pH control, where pH reached 10.7.

Table 1 shows that the daily pH adjustment by addition of CO$_2$ resulted in a X_m increase under all conditions studied. Such a X_m increase led to consequent increases in P_X and $Y_{X/N}$; therefore, these three parameters were the ones statistically influenced by pH control, with $p = 0.004$, 0.014 and 0.005, respectively (Table 2). Runs 7–9 and 6–7 did in fact exhibit the highest values of P_X (172–179 mg L^{-1} d^{-1}) and $Y_{X/N}$ (9.95–10.2 mg mg^{-1}), respectively. The ammonium level in the medium was detectable only after the last (NH$_4$)$_2$SO$_4$ addition in runs 6 and 7 ($T = 15$ and 13 days, respectively), whereas it occurred on the 8th day of cultivation in most runs carried out without pH control. These results indicate that pH adjustment is of paramount importance to ammonium uptake, especially at the beginning of A. platensis cultivation.

An ammonium feeding time of 13 days either with or without pH control (runs 2 and 7) ensured the highest values of μ_m (0.76–0.77 d^{-1}). These values are higher than that reported in the literature for fed-batch cultivation of A. platensis in open tanks with, for example, urea as nitrogen source (0.63 d^{-1}) (Sánchez-Luna et al., 2007), and then confirm the advantage of using the mixture of nitrogen sources proposed in this study. Although the pH control did not exert any statistically significant influence on μ_m (Table 2), Fig. 2 shows its positive effect on both the specific growth rate and the cell growth along runs 2 and 7 taken as examples, in that it decelerated μ_X decrease, especially after the 10th day of cultivation.

On the basis of these results taken as a whole, $T = 13$ days with pH control (run 7) was considered to be the best condition for A. platensis cultivation in open tanks in this work.

3.3. Influence of the ammonium feeding time and pH control on biomass composition

Piorreck et al. (1984) suggested that chlorophyll content of A. platensis biomass depends on medium composition. Since nitrogen is a fundamental element in the chlorophyll molecule, it could be influenced by the kind and amount of the nitrogen source. In addition, Hendry (1996) reported that, at high cell concentrations, A. platensis increases the production of chlorophyll in order to improve its photon capture efficiency, to compensate the low light intensity received by the inner cells, i.e. the shadowing effect (Richmond and Qiang, 1997).

However, although the nitrogen availability to cells was varied in this study, the chlorophyll content only varied in the range 4.1–5.3 mg g^{-1} (Table 1), and it was not statistically influenced by T (Table 2). Such low chlorophyll contents can be ascribed to the fact that the light intensity was not a matter, and cell concentration was not so high in this study. In addition, since the nitrogen fraction addressed to chlorophyll production is very low compared to proteins and other biopolymers, the amount of nitrogen supplied under all the conditions tested in this study was never likely to be a limiting factor for the synthesis of this component.

It is a well-known fact that protein and lipid contents of A. platensis biomass vary mainly according to nitrogen supply. Rodrigues et al. (2010) observed that, when two different nitrogen sources are simultaneously supplied to cultivations, the ratio between them, as well as the total amount of nitrogen, has a direct influence on the lipid content. This is evident also in this work, where the lipid content of biomass progressively decreased from 25–30% to 14–16% with decreasing the feeding time from 15 to 7 d ($p = 0.010$), with no clear influence of the pH control (Table 2). As expected, an opposite behavior was observed for the protein content (Table 1), even if the statistical analysis did not indicate any influence of T and pH control on protein content (Table 2). This fact occurred likely because in the runs with pH control (runs 6–10), the ammonia lost by off-gassing was minimized, and the microorganism met high nitrogen availability, thus leading to similar results. In fact, when comparing only runs carried out without pH control, the p value obtained was only 0.060 (data not shown), indicating that the lower the value of T, the higher the protein content.

As it can be seen in Table 2, neither the feeding time ($p = 0.650$) nor the pH control ($p = 0.910$) exerted any significant effect on the carbohydrate content, which varied from 35% to 50%, not far from 40%, which is the value reported as a standard for biomass of a great number of cyanobacteria and microalgae (Vargas et al., 1998). The ash content varied from 5.31% to 9.48% (Table 1) and, once again, no influence by the experimental conditions was detected (Table 2).

4. Conclusions

A. platensis cultivations were carried out at 156 μmol photons m^{-2} s^{-1} in open tanks on Schlösser medium modified using a mixture of sodium nitrate (5.90 mM) and ammonium sulphate (7.50 mM) as nitrogen source and an inoculum of 400 mg L^{-1}, either without pH control or adjusting it at 8.70 by CO$_2$ addition. The fed-batch exponential supply of ammonium sulphate at a feeding time of 13 d under pH control allowed obtaining the best results in terms of growth parameters: $X_m = 2911$ mg L^{-1}, $P_X = 179$ mg L^{-1} d^{-1} and $\mu_m = 0.77$ d^{-1}. Under these conditions,
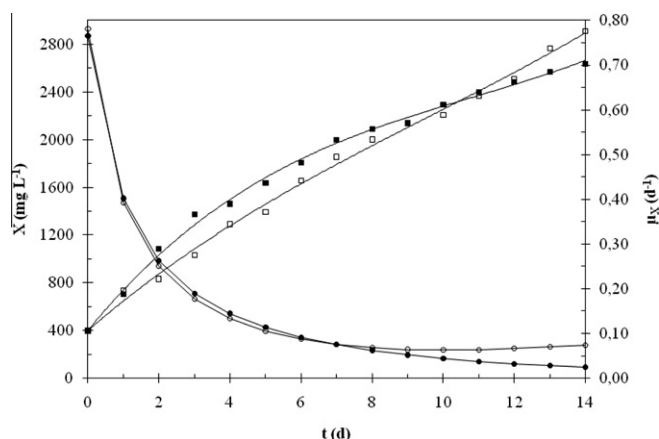

Fig. 2. Time behavior of the cell concentration (squares) and specific growth rate (circles) during A. platensis cultivations in which 7.50 mM (NH$_4$)$_2$SO$_4$ was fed throughout 13 d, with pH control (run 7, □ and ○) and without pH control (run 2, ■ and ●).

protein, lipid and carbohydrate contents were 28%, 30% and 35% respectively.

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