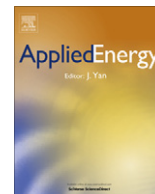2012

Arthrospira (Spirulina) platensis cultivation in tubular photobioreactor: Use of no-cost CO2 from ethanol fermentation

Arthrospira (Spirulina) platensis cultivation in tubular photobioreactor: Use of no-cost CO_2 from ethanol fermentation

L.S. Ferreira [a,b], M.S. Rodrigues [a,b], A. Converti [b], S. Sato [a], J.C.M. Carvalho [a,*]

[a] Department of Biochemical and Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, University of São Paulo, Av. Prof. Lineu Prestes, 580, Bloco 16, 05508-900 São Paulo-SP, Brazil
[b] Department of Chemical and Process Engineering "G.B. Bonino", University of Genoa, via Opera Pia 15, 16145 Genoa, Italy

ARTICLE INFO

ABSTRACT

The present study aimed at evaluating the production of Arthrospira platensis in tubular photobioreactor using CO_2 from ethanol fermentation. The results of these cultivations were compared to those obtained using CO_2 from cylinder at different protocols of simultaneous ammonium sulfate and sodium nitrate feeding. Maximum cell concentration (X_m), cell productivity (P_x), nitrogen-to-cell conversion factor ($Y_{X/N}$), and biomass composition (total lipids and proteins) were selected as responses and evaluated by analysis of variance. The source of CO_2 did not exert any significant statistical influence on these responses, which means that the flue gas from ethanol fermentation could successfully be used as a carbon source as well as to control the medium pH, thus contributing to reduce the greenhouse effect. The results taken as a whole demonstrated that the best combination of responses mean values (X_m = 4.543 g L^{-1}; P_x = 0.460 g L^{-1} d^{-1}; $Y_{X/N}$ = 15.6 g g^{-1}; total lipids = 8.39%; total proteins = 18.7%) was obtained using as nitrogen source a mixture of 25% $NaNO_3$ and 75% $(NH_4)_2SO_4$, both expressed as nitrogen.

1. Introduction

From the biotechnological viewpoint, Arthrospira (Spirulina) platensis is one of the most important cyanobacteria because it is able to produce inside its cells high concentrations of pigments (chlorophyll a and phycocyanin), fatty acids (i.e., γ-linolenic acid) and proteins [1,2]. The cultivation of this photosynthetic microorganism largely depends on the availability of light and nutrients, pH, cell density, temperature and eventual contamination by other microorganisms.

One of the main elements for A. platensis cultivation is nitrogen, which is supplied using either potassium [3] or sodium [4] nitrates as standard nitrogen sources. Nevertheless, urea and ammonium salts have been successfully used as alternative sources, and where chosen especially because of their low acquisition cost [5]. In addition, this cyanobacterium uptakes nitrogen directly in the form of ammonia [6] when either of these sources is present in the cultivation medium, whereas nitrates require an enzymatic reduction to nitrite and then ammonia, thus consuming energy [7]. When

added in alkaline media, ammonium salts are turned into ammonia, which is volatile and then toxic at high concentrations [8]. For these reasons, in order to reduce ammonia off-gassing, it would be preferable to cultivate A. platensis in closed photobioreactors [9,10] by the fed-batch process, in which small amount of this nitrogen source is daily added to cultivations [11]. The addition of only one kind of nitrogen source has been reported up to now by many authors [8–11]; however, Rodrigues et al. [5] have recently applied the combined use of two nitrogen sources, one nitrate and the other ammonia-based, to A. platensis cultivation in open tanks, thus ensuring better results in terms of maximum cell concentration and biomass quality when compared to cultivations carried out with only one nitrogen source.

Another important medium component is the carbon source, usually a mixture of carbonate and bicarbonate. High levels of these salts in a favorable ratio can in fact provide, at the same time, an optimum pH value (about 9.5) as well as a large availability of carbon source to A. platensis cultivations. During cultivation, photosynthetic cells preferably consume bicarbonate and release carbonate into the medium [12]; this bicarbonate/carbonate imbalance leads to a progressive pH increase that can successfully be avoided by a frequent CO_2 supply, enough to maintain this parameter within an optimal range [10,13]. On a bench-scale level, pH control is usually performed by the addition of pure CO_2 from cylinders, whereas on a large-scale one, the CO_2 contained in industrial gaseous effluents could be used. It is well known that

many industries eliminate this exhaust gases into the atmosphere being co-responsible for the greenhouse effect [14].

An alternative large source of CO_2 is the fermentation plants for bioethanol production, whose number is growing worldwide because ethanol is a renewable and less polluting fuel compared to oil [15]. According to *União da Agroindústria Canavieira de São Paulo* (UNICA) [16], the Brazilian ethanol production was of about 11.5 billion liters in 1990/1991 and 27.5 billion liters in 2008/2009. Taking into account that the production of ethanol and CO_2 is almost the same in terms of mass (0.96 g CO_2/g ethanol), the increase in ethanol production results in a worsening of the CO_2 emission situation. CO_2 fixation has been proposed as a method to remove this gas from the atmosphere, thereby contributing to the reduction of the greenhouse effect [17]. One of its possible applications could be in the production of photosynthetic microorganisms [13], which are able to capture light and fixate CO_2 much more efficiently than plants, and can be grown using a smaller cultivation areas when compared to crops [18]. Particularly, the state of São Paulo is the largest producer of ethanol in Brazil and its production achieved, in 2008/2009, about 17 billion liters [16], which corresponds to about 13 billion kg of CO_2. This huge amount of CO_2 could be profitably used to produce about 7 billion kg of biomass, which may be used to obtain energy through combustion [19], gasification [20] or processes involving anaerobic fermentation for the production of energetic gases such as biohydrogen and biomethane [21].

In this work it was investigated the possibility of using CO_2 from ethanol fermentation and a mixture of two different nitrogen sources, namely $NaNO_3$ and $(NH_4)_2SO_4$, to cultivate *A. platensis* in a tubular photobioreactor. To this purpose, nitrate and ammonium salts were fed-batch added in three different ratios, and the cultivation pH was controlled by supplying either the flue gas from alcohol fermentation or pure CO_2 from cylinder. The results were evaluated in terms of maximum cell concentration (X_m), cell productivity (P_x), nitrogen-to-cell conversion factor ($Y_{X/N}$) and biomass composition (protein and lipid contents).

2. Material and methods

2.1. Arthrospira platensis cultivation

2.1.1. Microorganism, cultivation medium and inoculum preparation

The strain *Arthrospira platensis* UTEX 1926 was obtained from University of Texas Culture Collection. A standard medium [4] containing sodium nitrate as nitrogen source was used for maintenance and inoculum preparation, whereas runs carried out to investigate the effect of the simultaneous use of sodium nitrate and ammonium sulfate were done so with the same standard medium, but with different $NaNO_3/(NH_4)_2SO_4$ in different ratios.

The inoculum was grown in 500 mL Erlenmeyer flasks placed on rotary shaker at 100 min^{-1}, 29 ± 1 °C and using a photosynthetic photon flux density (PPFD) of 72 μmol photons $m^{-2} s^{-1}$. After 6–8 days of cultivation [22], the culture was filtered, washed with physiological saline solution to remove adsorbed nitrates, and resuspended in nitrogen-free cultivation medium. Starting cell concentration was set at 400 mg L^{-1} [10].

2.1.2. Photobioreactor, cultivation conditions and runs

The tubular photobioreactor (Fig. 1) used in this work was developed at the Fermentation Technology Laboratory of the Department of Biochemical and Pharmaceutical Technology of São Paulo University. It is made of a metal structure surrounded by 40 glass tubes with a 2% (1.15°) inclination, each of them having 0.01 m internal diameter [9], 0.0015 m wall thickness, and 0.5 m length. Light was provided by up to six 20 W-fluorescent lamps
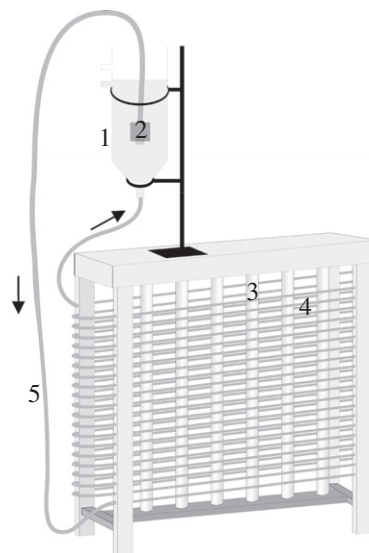

Fig. 1. Scheme of the photobioreactor. (1): Degasser; (2): motor driven pump; (3): 20 W Fluorescent lamps; (4): glass tubes; (5): external silicon tube.

located inside of the structure, illuminating around 57% of the total working volume. A cylinder glass flask placed on the top of the photobioreactor held part of the culture and worked as a degassing device. The culture flow was set at 0.2 m s^{-1}.

The culture pH was controlled at 9.5 ± 0.5 [20] by daily addition of flue gas from ethanol fermentation or pure CO_2 from cylinder, while temperature was maintained at 29 °C ± 1 °C [23] in a thermostatic chamber. The PPFD was set at 120 μmol photons $m^{-2} s^{-1}$ [24] and measured by an luminance meter, model LI-250A (LI-COR, Lincoln, NE, USA), at the beginning of each run and at nine different points of the photobioreactor, in order to ensure the selected average PPFD value. The working volume was 3.5 L and the cell circulation system used in this work was the motor driven-pumping [24].

The experimentation conditions of all runs performed in this work are listed in Table 1 together with the results. Runs C1 and C2, carried out with pure CO_2 (C), and runs E1 and E2, to which ethanol fermentation flue gas (E) was supplied, are standard runs taken as reference that were carried out using only nitrate as nitrogen source. Runs 1–6 and their respective repetitions were performed using mixtures of sodium nitrate and ammonium sulfate at different ratios (see later).

2.1.3. Nitrogen feeding protocols

A reference run was initially carried out at the selected PPFD (120 μmol photons $m^{-2} s^{-1}$) with the addition of sodium nitrate whenever it was necessary, so that its concentration in the medium never went below 1 g L^{-1} [25]. The resulting growth curve was used to calculate the amount of nitrogen that should be daily added, in the form of ammonium and nitrate salts mixture, to sustain the expected cell growth. Such calculation was based on the supposition that *A. platensis* nitrogen content is 7% of dry biomass, as previously reported [11].

According to the three selected protocols, all the nitrogen source necessary for cell growth was added in the following ratios, expressed as nitrogen percentages: (I) 25% $NaNO_3$ and 75% $(NH_4)_2SO_4$; (II) 50% $NaNO_3$ and 50% $(NH_4)_2SO_4$; (III) 75% $NaNO_3$ and 25% $(NH_4)_2SO_4$ (Table 2). The sodium nitrate was entirely added at the beginning of the runs, while the ammonium sulfate was pulse-fed every 12 h from the start up to the 8th day of cultivation.

Table 1

Runs and results of X_m (maximum cell concentration), P_x (cell productivity), $Y_{X/N}$ (nitrogen-to-cell conversion factor), lipid and protein contents for *Arthrospira platensis* cultivations in tubular photobioreactor.

Run[a]	CO$_2$ source	Nitrogen feeding protocols[b]	X_m (g L^{-1})	P_x (g L^{-1} d^{-1})	$Y_{X/N}$ (g g^{-1})	Lipids (%)	Proteins (%)
1	Ethanol fermentation	I	4.392	0.444	15.0	8.90	22.2
1$_r$	Ethanol fermentation	I	4.479	0.453	15.3	8.99	17.2
2	Ethanol fermentation	II	3.911	0.390	13.2	9.39	27.0
2$_r$	Ethanol fermentation	II	4.118	0.413	14.0	8.05	25.3
3	Ethanol fermentation	III	3.861	0.385	13.0	9.33	28.9
3$_r$	Ethanol fermentation	III	3.785	0.376	12.7	9.80	30.3
4	Cylinder	I	4.724	0.480	16.2	7.34	17.1
4$_r$	Cylinder	I	4.576	0.464	15.7	8.31	18.1
5	Cylinder	II	4.130	0.414	14.0	9.07	25.2
5$_r$	Cylinder	II	4.265	0.429	14.5	8.36	28.4
6	Cylinder	III	3.720	0.369	12.5	8.56	30.5
6$_r$	Cylinder	III	3.939	0.393	13.3	10.4	29.0
E	Ethanol fermentation	Only nitrate	4.041	0.405	5.35	9.79	28.2
E$_r$	Ethanol fermentation	Only nitrate	3.680	0.364	4.61	9.27	27.4
C	Cylinder	Only nitrate	4.038	0.404	4.73	8.60	29.3
C$_r$	Cylinder	Only nitrate	3.804	0.378	4.42	9.37	32.0

[a] r = Repetition.

[b] Nitrogen mixture of: protocol I = 25% NaNO$_3$ and 75% (NH$_4$)$_2$SO$_4$; protocol II = 50% NaNO$_3$ and 50% (NH$_4$)$_2$SO$_4$; protocol III = 75% NaNO$_3$ and 25% (NH$_4$)$_2$SO$_4$, expressed in terms of nitrogen.

Table 2

The daily addition of ammonium sulfate and sodium nitrate simultaneously according to the nitrogen feeding protocols: protocol I = 25% NaNO$_3$ and 75% (NH$_4$)$_2$SO$_4$; protocol II = 50% NaNO$_3$ and 50% (NH$_4$)$_2$SO$_4$, protocol III = 75% NaNO$_3$ and 25% (NH$_4$)$_2$SO$_4$, expressed in nitrogen.[a]

Time (days)	Cell concentration (mg L^{-1})	Nitrogen daily added (mM)	Nitrogen feeding protocol I (runs 1 and 4) (mM)		Nitrogen feeding protocol II (runs 2 and 5) (mM)		Nitrogen feeding protocol III (runs 3 and 6) (mM)	
			Nitrogen from NaNO$_3$	Nitrogen from (NH$_4$)$_2$SO$_4$	Nitrogen from NaNO$_3$	Nitrogen from (NH$_4$)$_2$SO$_4$	Nitrogen from NaNO$_3$	Nitrogen from (NH$_4$)$_2$SO$_4$
0	400	0.72	4.75	0.54	9.51	0.36	14.3	0.18
1	544	1.81	–	1.36	–	0.91	–	0.45
2	907	2.66	–	2.00	–	1.33	–	0.67
3	1439	2.82	–	2.12	–	1.41	–	0.71
4	2003	2.48	–	1.86	–	1.24	–	0.62
5	2500	2.71	–	2.04	–	1.36	–	0.68
6	3042	2.29	–	1.72	–	1.15	–	0.57
7	3501	1.93	–	1.45	–	0.97	–	0.48
8	3887	1.58	–	1.18	–	0.79	–	0.39
9	4202	–	–	–	–	–	–	–
10	4116	–	–	–	–	–	–	–

[a] The nitrogen from NaNO$_3$ was all added at the first cultivation day (zero day) and that from (NH$_4$)$_2$SO$_4$ was added each day at a different concentration, from the 1th until 8th day, according to the standard cultivation carried out using only nitrate as nitrogen source.

2.1.4. Analytical methods

Biomass concentration was determined by optical density measurements at 560 nm [26], using a calibration curve and a spectrophotometer, model 700 Plus (Femto, São Paulo, Brazil). In cell-free medium samples, the following analyses were performed: nitrate concentration according to Vogel [27], total ammonia concentration according to the phenol hypochlorite method [28], and total carbonate according to Pierce and Haenisch [29]. The pH was measured using a potentiometer, model 710-A (Orion, Beverly, MA, USA).

Finally, at the end of all runs, the cells were harvested by centrifugation, washed thrice with distilled water to remove salts and dried at about 55 °C for 12 h. The powdered samples were used in the analysis of total protein contents according to Kjeldhal method, and total lipid content by extraction with a chloroform-methanol solution 2:1 (v/v) [30].

2.1.5. Parameters calculation

The cell productivity (P_x), expressed in g L^{-1} d^{-1}, was calculated as the ratio between the total amount of cells produced per unit volume ($X_m - X_i$) and the cultivation time (T_c), according to the equation:

$$P_X = \frac{(X_m - X_i)}{T_c} \qquad (1)$$

The nitrogen-to-cell conversion factor ($Y_{X/N}$) was calculated according to the following equation:

$$Y_{X/N} = \frac{(X_m - X_i)V}{N_t} \qquad (2)$$

where X_m and X_i are the maximum and starting cell concentrations (g L^{-1}), V (L) is the photobioreactor volume and N_t is the total nitrogen amount added.

2.2. Saccharomyces cerevisiae cultivation

2.2.1. Microorganism, inoculum preparation and cultivation

The inoculum for alcoholic fermentation was prepared using 1.1 kg of commercial pressed yeast (*S. cerevisiae*) homogenized in 6 L of distilled water, and the mash was prepared by diluting blackstrap molasses in distilled water until a concentration of about 220 g L^{-1} of total reducing sugars (TRS). 500 U L^{-1} of penicillin [31] and 0.5 g L^{-1} of urea were added to the mash. Its pH was adjusted at 4.5–5.0 using sulfuric acid or sodium hydroxide as needed [32].

2.2.2. Alcoholic fermentation bioreactor and conditions

The fermentation runs were carried out in a New Brunswick fermenter at 32 ± 2 °C. The impeller speed was 200 min^{-1}, no air was supplied, and the medium pH was not controlled [32]. After the inoculum addition, the impeller speed and temperature were adjusted, and the mash was constantly fed into the reactor at 2.8 L h^{-1} during the first 5 h, when the total volume reached 20 L. The fermentations were kept running for four more hours, until ethanol concentration was constant. Samples were withdrawn at the times 1, 3, 5, 7, 8 and 9 h. Nine fed-batch ethanol fermentations were done to supply the necessary carbon dioxide to *A. platensis* cultivations.

2.2.3. Analytical methods

Biomass concentration was determined by filtering 5 mL samples of the broth through a 1.2 μm Millipore® membrane, after washing with 50 mL of distilled water; they were then dried at about 100 °C for 3 h and weighed until to constant weight. Additional 12 mL samples were centrifuged at 3000 rpm for 15 min, and the supernatant was used to analyze sugars and ethanol by Somogyi [33] and the dichromate [34] methods, respectively.

2.3. Statistical analysis

Comparison of results was made by the analysis of variance (ANOVA – program MINITAB 16), specifically one-way ANOVA for ethanol fermentation and two-way ANOVA for *A. platensis* cultivations. The statistical significance was evaluated by estimation of the descriptive level (p), being the results considered statistically significant when $p < 0.05$ (confidence level of 95%).

3. Results and discussion

3.1. Effect of CO_2 feeding

The average results of fed-batch ethanol fermentations performed to supply CO_2 to the *A. platensis* cultivation system are

presented in Fig. 2. During feeding time (first 5 h), the total reducing sugars (TRS) increased up to about 35 g L^{-1}. From the fifth hour, residual TRS concentration of about 5 g L^{-1} was observed, which is common in fermentations carried out using sugar cane molasses as carbon source [32] and, probably, corresponds to non-sugars substances determined as reducing sugars by Somogyi method. The cell concentration decreased from 56.2 to 30.9 g L^{-1} as a consequence of medium addition, while ethanol concentration increased up to 68.8 g L^{-1} at the 7th hour of the run, after the end of mash addition. It is important to notice that all nine ethanol fermentation results were considered statistically equal ($p = 0.998$–1.000) when analyzed by one-way ANOVA.

The highest ethanol mean production obtained in this work (68.8 g L^{-1}) was higher than that obtained by Converti et al. [35] (58.6 g L^{-1}), who also cultivated commercial yeast, using a fed-batch process for the ethanol production, but adding clarified blackstrap molasses as substrate, using a working volume of 10 L and the filling up time of 7 h. Taking into account that ethanol and CO_2 are produced in equimolar amounts, such a high value of ethanol production pointed out an equally high CO_2 release that was expected to be able to sustain the fed-batch growth of *A. platensis* in the photobioreactor.

The CO_2, either pure from cylinder or contained in the ethanol fermentation flue gas, was bubbled into the photobioreactor tubes to control the pH at an optimum value (9.5) as well as to act as a carbon source. As already highlighted by Binaghi et al. [12], bicarbonate is the prevalent species of the $H_2CO_3/HCO_3^-/CO_3^=$ system at pH 9.6, while at pH 11.3 it is practically absent. This being so, the optimum pH for *A. platensis* growth is around 9.5, because this microorganism consumes preferentially bicarbonate, two moles of which, once transported actively into the cells, are converted into $CO_3^=$ and CO_2, the latter being used in the photosynthesis and the former released into the medium.

Fig. 3 illustrates the daily pH variation and cell growth of duplicate runs carried out using only nitrate as nitrogen source, taken as examples, and using either pure CO_2 from cylinder (C) or the ethanol fermentation flue gas as a CO_2 source (E). This figure clearly shows that, as expected, the pH increased as the cells multiplied, and that this increment was higher when the cells were in the exp-phase, which is consistent with the well-known quicker cell growth in this phase. The total carbonate concentration (about 9.0 g L^{-1}) kept almost constant during all the runs, thus suggesting that the CO_2 addition was able to replace the consumed carbon source, avoiding, at the same time, any accumulation in the medium. Taking as an example the run 1 (Table 1), whose P_X value was 0.444 g L^{-1} d^{-1} and considering that the dry cell weight is constituted by about 50% of carbon [36], we can estimate a carbon
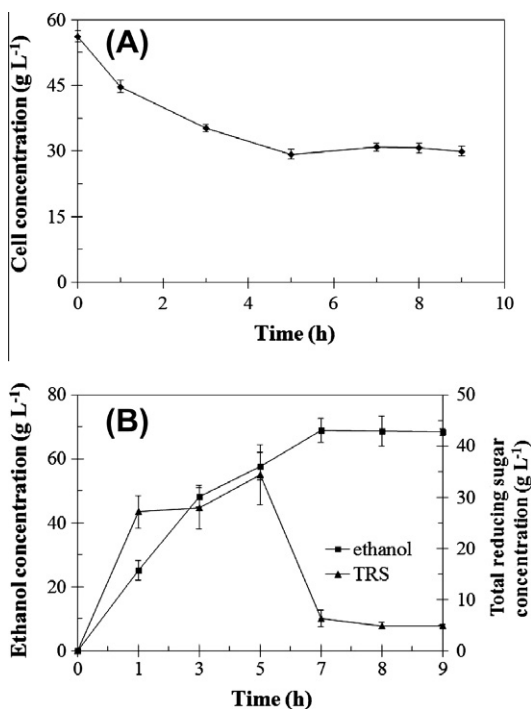
Fig. 2. Cell (A), ethanol and total reducing sugars (B) concentrations as a function of time with the standard deviation for the nine ethanol fermentations carried out in this work.
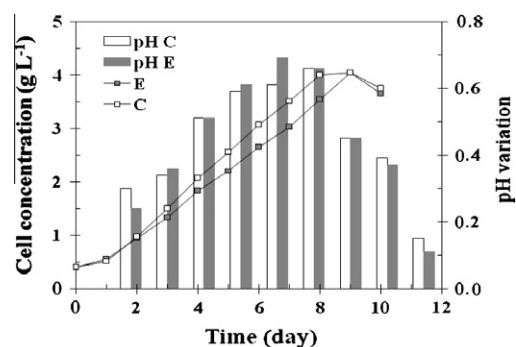
Fig. 3. Maximum cell concentration (X_m) and pH as a function of time (days) for the cultivations carried out using only NaNO₃ as nitrogen source, and flue gas from ethanol fermentation (run E: gray squares and bars) and pure CO_2 from cylinder (run C: empty squares and bars).

Table 3
Influence of the CO_2 from cylinder and ethanol fermentation on mean values of X_m (maximum cell concentration), P_x (cell productivity), $Y_{X/N}$ (nitrogen-to-cell conversion factor), lipid and protein contents and respectively p values obtained from *A. platensis* cultivations.[b]

CO_2	X_m (g L^{-1})	P_x (g L^{-1} d^{-1})	$Y_{X/N}$ (g g^{-1})	Lipid (%)	Protein (%)
Ethanol fermentation	4.033^A	0.404^A	11.6^A	9.19^A	25.8^A
Cylinder	4.150^A	0.416^A	11.9^A	8.75^A	26.2^A
P[a]	0.144	0.154	0.212	0.242	0.676

[a] According to ANOVA, the results were considered statistically significant when $p < 0.05$ (confidence level of 95%).
[b] Different capital letters were used for means significantly different, for each response analyzed, according to ANOVA.

Table 4
Influence of the three nitrogen feeding protocols: protocol I = 25% $NaNO_3$ and 75% $(NH_4)_2SO_4$; protocol II = 50% $NaNO_3$ and 50% $(NH_4)_2SO_4$; protocol III = 75% $NaNO_3$ and 25% $(NH_4)_2SO_4$, on response's mean values of X_m (maximum cell concentration), P_x (cell productivity), $Y_{X/N}$ (nitrogen-to-cell conversion factor), lipid and protein contents and respectively p values obtained from *A. platensis* cultivations.[b]

Nitrogen feeding protocol	X_m (g L^{-1})	P_x (g L^{-1} d^{-1})	$Y_{X/N}$ (g g^{-1})	Lipid (%)	Protein (%)
I	4.543^A	0.460^A	15.6^A	8.39^A	18.7^A
II	4.106^B	0.412^B	13.9^B	8.72^A	26.5^B
III	3.826^B	0.381^B	12.9^C	9.52^A	29.7^B
Only nitrate	3.891^B	0.388^B	4.78^D	9.26^A	29.2^B
p[a]	0.000	0.000	0.000	0.166	0.000

[a] According to ANOVA, the results were considered statistically significant when $p < 0.05$ (confidence level of 95%).
[b] Different capital letters were used for means significantly different, for each response analyzed, according to ANOVA.

dioxide necessity of 2.849 g/day for the 3.5 L of culture. Taking into account that cells achieved their maximum concentration within 9 days, the total amount of CO_2 required for their growth was about 26 g, which accounts for approximately 84% of the total carbon dioxide present in the initial Schlösser medium (30.8 g), either in the form of bicarbonate or carbonate. Previous study demonstrated that, if the pH is not regulated, cells stop growing [13], thus justifying the significant daily CO_2 addition required in this work to this purpose. It is worth mentioning that pH correction with CO_2 is preferable to the one with inorganic acid, because it allows preventing a reduction of total carbon along cultivations, which otherwise would lead to carbon shortage [23]. Moreover, bicarbonate and carbonate present in the starting medium at the beginning of runs are not totally consumed by the microorganisms until the end of the cultivations; but, if this medium is reused there is no need to add carbon source, which will result in cost savings to the process.

According to Cachot et al. [37] the carbon dioxide from ethanol fermentation can carry relatively low levels of volatile organic compounds (VOC), as propanol, isobutanol, acetaldehyde and acetic acid. Matsudo et al. [13] who worked with the same cyanobacterium in continuous process, detected, by gas-chromatographic analysis, CO_2 as the main volatile compound released from ethanol fermentation by *Saccharomyces cerevisae*, together with small amount of ethanol and traces of acetaldehyde. Thus, in the present work, it is possible that the microorganism assimilated ethanol as a carbon source, but, as suggested by the statistical analysis, if so, it occurred to so low extent that was not sufficient to significantly increase the maximum cell concentration when compared to the runs carried out with CO_2 from cylinder (Table 3). This result suggests the use of ethanol fermentation flue gases as no-cost carbon source for the cultivation of photosynthetic microorganisms as a means to mitigate the greenhouse effect.

3.2. Effect of nitrogen feeding

Nitrogen is the second most important element in biomass composition and, in the case of commercial cyanobacteria cultivations, it is usually added in the form of nitrate salts. This choice is dictated by their easy handling and ability to guarantee high maximum cell concentrations; however, these nitrogen sources are in general expensive when compared to ammonium compounds. These last ones, in solution, release ammonium ions that, under alkaline conditions, become ammonia; therefore, they are toxic at high concentrations [8] and, for this reason, it is recommended that only the necessary amount to sustain cell growth should be daily supplied to cultivations by the so-called fed-batch process [11] rather than to be entirely added at the beginning of a batch run. In this work, the simultaneous addition of two nitrogen sources was suggested by the promising results reported by Rodri-

gues et al. [5] in open photobioreactor and using another kind of nitrogen feeding protocol.

The effect of the nitrogen feeding protocol was analyzed in terms of maximum cell concentration (X_m), cell productivity (P_x), nitrogen-to-cell conversion factor ($Y_{X/N}$), and lipid and protein contents (Table 1), which were selected as responses. As it can be seen in Table 4, the lipid content was the only response that was not statistically influenced by the nitrogen feeding protocol.

Cell growth was statistically higher when the protocol I [25% $NaNO_3$ and 75% $(NH4)_2SO_4$] was applied, which can be explained by the fact that photosynthetic microorganisms consume energy to reduce nitrate to nitrite and then to ammonia, by the enzymes nitrate and nitrite reductases [38], respectively, before uptaking this nitrogen source in the form of ammonia. Therefore, the high X_m values can be ascribed to the higher $(NH_4)_2SO_4$ amount of protocol I. It is clear why these microorganisms do prefer ammonium salts as nitrogen sources, when available at non-toxic levels [6]. As expected by the lower content of ammonium and higher content of nitrate, the feeding protocols II and III and the standard runs (containing only nitrate) resulted in lower X_m values compared to protocol I (Table 4), though statistically equivalent among them.

According to Ohmori et al. [39], who cultivated *Anabaena cylindrica*, when ammonia is available in the medium, this cyanobacterium almost does not utilize nitrate as nitrogen source until practically all the ammonia has been consumed. However, in the present work, the nitrogen consumption profile did not reflect a so strict selectivity, in that the nitrate and ammonia concentrations decreased at the same time. On the basis of this comparison, one can suspect that some induction mechanism could have been involved in the assimilation of these nitrogen sources by the above microorganism. Fig. 4 shows an example of ammonia and nitrate consumption (run 2), in which ammonia was almost entirely uptaken throughout the cultivation, while nitrate was slowly consumed until the end of cultivation. This simultaneous consumption could have been due to the optimum nitrogen uptake conditions ensured by the feeding protocols, i.e., the amount of nitrogen added through the fed-batch process was exactly the one necessary for the cell growth. So, although both nitrogen sources were simultaneously consumed, ammonium uptake took place at higher rate, as suggested by the higher slope of its curve depicted in Fig. 4.

As far as cell productivity is concerned, since all the runs carried out with both nitrogen sources were done following a pre-fixed nitrogen addition, the cultivation time was the same in all the runs, which resulted in a P_x pattern qualitatively resembling that of X_m. This being so, the lowest mean P_x value (0.381 g L^{-1} d^{-1}) was obtained applying the feeding protocol III, followed by protocols II (0.412 g L^{-1} d^{-1}) and I (0.460 g L^{-1} d^{-1}). This last value was higher than that obtained by Rodrigues et al. [5] for the same strain in
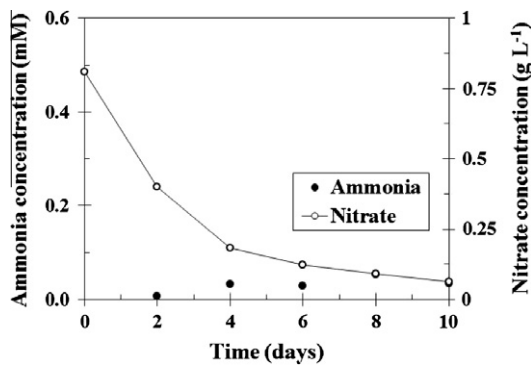
Fig. 4. Concentrations of nitrate (g L^{-1}) = $\bigcirc$ and ammonia (mM) = $\bullet$ as a function of time (days) for the run 2 carried out using the nitrogen feeding protocol II = 50% $NaNO_3$ and 50% $(NH_4)_2SO_4$.

open tank, and this result confirms the better performance of tubular closed photobioreactors.

Because of the same reason, i.e. a pre-fixed nitrogen addition based on the actual needs of the microorganism, the higher the cell concentration (protocol I), the higher $Y_{X/N}$ (Table 4), which provides a further confirmation of the preference of the cyanobacterium for ammonium salts as nitrogen source [6]. These $Y_{X/N}$ values are satisfactory and similar to those obtained by Ferreira et al. [11] using a different nitrogen feeding protocol for the same microorganism. These results taken as a whole suggest that the use of suited protocols for nitrogen supply is able to ensure high $Y_{X/N}$ values and, consequently, a reduction of costs.

The mean values of lipid content of cells obtained in this work (Table 4) are in accordance to the range (6–13%) found in literature for cyanobacteria [1]; but, the kind of nitrogen feeding protocol did not exert any statistically significant effect ($p = 0.166$) on such a response. On contrary, it did so on the protein content of cells, in that the runs carried out according to protocol I resulted in cells with protein content statistically lower (18.7%) than those obtained through protocol II and III (26.5% and 29.7%, respectively) (Table 4). Such a stimulation of protein synthesis by nitrate was already observed Ferreira et al. [11] for the same system using a different nitrogen feeding protocol.

It should be noticed that we can exclude any phosphorus limitation under the conditions adopted in this work. In fact, the N/P ratio in the nutrients solution added during the cultivation (2.99) was by far lower than that of dry *A. platensis* biomass (12.8), which can estimated from the elemental composition reported for this microorganism by Cornet et al. [36] ($CH_{1.650}$ $O_{0.531}$ $N_{0.170}$ $S_{0.007}$ $P_{0.006}$). Finally these results suggest that, under bioenergetic stress conditions, i.e. nitrate excess and ammonium shortage, the microorganism prefers to accumulate the nitrogen source in the form of proteins rather than to grow.

4. Conclusions

The aim of this study was to evaluate the influence of three different ratios of nitrate and ammonium salts as nitrogen sources and two different sources of CO_2 (pure from cylinder or flue gas from ethanol fermentation) on the maximum cell concentration (X_m), cell productivity (P_x), nitrogen-to-cell conversion factor ($Y_{X/N}$), and biomass composition (protein and lipid contents).

The kind of CO_2 source did not exert any statistical influence on *A. platensis* growth, from which we can suggest the possibility of using the flue gas from ethanol fermentation as an alternative and hugely available no-cost carbon source for cyanobacteria cultivations. On the other hand, the selected responses were statisti-

cally influenced by the nitrogen feeding protocol. The best set of results was obtained when using the nitrogen mixture with the lowest nitrate level (protocol I) ($X_m = 4.543$ g L^{-1}; $P_x = 0.460$ g L^{-1} d^{-1}; $Y_{X/N} = 15.6$ g g^{-1}; total lipids = 8.39%), likely due to the less bioenergetics requirements under these conditions. On the other hand, the highest content of proteins (26.5% and 29.7%) obtained under these conditions suggests a preference of the microorganism to accumulate the nitrogen source in the form of proteins rather than to grow.

References

[1] Cohen Z. The chemicals of *Spirulina*. In: Vonshak A, editor. *Spirulina platensis (Arthrospira)*: physiology, cell-biology and biotechnology. London: Taylor and Francis; 1997. p. 175–204.
[2] Pulz O, Gross W. Valuable products from biotechnology of microalgae. Appl Microbiol Biotechnol 2004;65:635–48.
[3] Paoletti C, Pushparaj B, Tomaselli Feroci L. Ricerche sulla nutrizione minerale di *Spirulina platensis*. In: Atti del XVII Congresso Nazionale della Società Italiana di Microbiologia. Padua, Italy: Italian Society of Microbiology; 1975. p. 833–9.
[4] Schlösser UG. Sammlung von Algenkulturen. Ber Dtsch Bot Ges 1982;95:181–276.
[5] Rodrigues MS, Ferreira LS, Converti A, Sato S, Carvalho JCM. Fed-batch cultivation of *Arthrospira (Spirulina) platensis*: potassium nitrate and ammonium chloride as simultaneous nitrogen sources. Bioresource Technol 2010;101:4491–8.
[6] Boussiba S. Ammonia uptake in the alkalophilic cyanobacterium *Spirulina platensis*. Plant Cell Physiol 1989;30:303–8.
[7] Markou G, Georgakakis D. Cultivation of filamentous cyanobacteria (blue–green algae) in agro-industrial wastes and wastewaters: A review. Appl Energ 2011;88:3389–401.
[8] Carvalho JCM, Francisco FR, Almeida KA, Sato S, Converti A. Cultivation of *Arthrospira (Spirulina) platensis* by fed-batch addition of ammonium chloride at exponentially-increasing feeding rate. J Phycol 2004;40:589–97.
[9] Carlozzi P, Pinzani E. Growth characteristics of *Arthrospira platensis* cultured inside a new closed-coil photobioreactor incorporating a mandrel to control culture temperature. Biotechnol Bioeng 2005;90:675–84.
[10] Soletto D, Binaghi L, Ferrari L, Lodi A, Carvalho JCM, Zilli M, et al. Effects of carbon dioxide feeding rate and light intensity on the fed-batch pulse-feeding cultivation of *Spirulina platensis* in helical photobioreactor. Biochem Eng J 2008;39:369–75.
[11] Ferreira LS, Rodrigues MS, Converti A, Sato S, Carvalho JCM. A new approach to ammonium sulfate feeding for fed-batch *Arthrospira (Spirulina) platensis* cultivation in tubular photobioreactor. Biotechnol Progr 2010;26:1271–7.
[12] Binaghi L, Del Borghi A, Lodi A, Converti A, Del Borghi M. Batch and fed-batch uptake of carbon dioxide by *Spirulina platensis*. Process Biochem 2003;38:1341–6.
[13] Matsudo MC, Bezerra RP, Converti A, Sato S, Carvalho JCM. CO$_2$ from alcoholic fermentation for continuous cultivation of *Arthrospira (Spirulina) platensis* in tubular photobioreactor using urea as nitrogen source. Biotechnol Progr 2011;27:650–6.
[14] Ramanathan V, Feng Y. Air pollution, greenhouse gases and climate change: global and regional perspectives. Atmos Environ 2009;43:37–50.
[15] Oliveira M, Vasconcelos Y. Revolução no canavial. Revista Pesquisa FAPESP 2006;122:1–6.
[16] União da Agroindústria Canavieira de São Paulo. Desenvolvimento ecologicamente correto. www.portalunica.com.br/dadosCotacao/estatistica [Accessed 10.08.2011].
[17] Maeda K, Owada M, Kimura N, Omata K, Karube J. CO$_2$ fixation from the flue gás on coal-fired thermal power plant by microalgae. Energy Convers Manage 1995;36:717–20.
[18] Janssen M, Tramper J, Mur LR, Wijffels RH. Enclosed outdoor photobioreactors: light regime, photosynthetic efficiency, scale-up, and future prospects. Biotechnol Bioeng 2002;81:193–210.
[19] Chen C, Ma X, Liu K. Thermogravimetric analysis of microalgae combustion under different oxygen supply concentrations. Appl Energ 2011;88:3189–96.
[20] Venkitasamy C, Hendry D, Wilkinson N, Fernando L, Jacoby WA. Investigation of thermochemical conversion of biomass in supercritical water using a batch reactor. Fuel 2011;90:2662–70.
[21] Cheng J, Zhang M, Song W, Xia A, Zhou J, Cen K. Cogeneration of hydrogen and methane from *Arthrospira maxima* biomass with bacteria domestication and enzimatic hydrolysis. Int J Hydrogen Energy 2011;36:1474–81.
[22] Pelizer LH, Danesi EDG, Rangel CO, Sassano CEN, Carvalho JCM, Sato S, et al. Influence of inoculum age and concentration in *Spirulina platensis* cultivation. J Food Eng 2003;56:371–5.

[23] Sánchez-Luna LD, Bezerra RP, Matsudo MC, Sato S, Converti A, Carvalho JCM. Influence of pH, temperature and urea molar flowrate on *Arthrospira platensis* fed-batch cultivation. A kinetic and thermodynamic approach. Biotechnol Bioeng 2007;96:702–11.

[24] Ferreira LS, Rodrigues MS, Converti A, Sato S, Carvalho JCM. Kinetic and growth parameters of *Arthrospira (Spirulina) platensis* cultivated in tubular photobioreactor under different cell circulation systems, Biotechnol Bioeng, in press.

[25] Faintuch BL. Análise comparativa da produção de biomassa a partir de três cianobactérias empregando distintas fontes nitrogenadas. Master Thesis, University of São Paulo, Brazil; 1989.

[26] Leduy A, Therien N. An improved method for optical density measurement of the semicroscopic blue algae *Spirulina maxima*. Biotechnol Bioeng 1977;19:1219–24.

[27] Vogel AI. Análise Química Quantitativa. Rio de Janeiro: Livros Técnicos e Científicos Ltda; 1992.

[28] Solorzano L. Determination of ammonia in natural waters by the phenol hypochlorite method. Limnol Oceanogr 1969;14:799–801.

[29] Pierce WC, Haenisch EL. Quantitative analysis. 3th ed. New York: John Willey & Sons; 1948.

[30] Olguín EJ, Galicia S, Angulo-Guerrero O, Hernández E. The effect of low light flux and nitrogen deficiency on the chemical composition of *Spirulina sp.* growth on pig waste. Bioresource Technol 2001;77:19–24.

[31] Aquarone E. Penicillin and tetracycline as contamination control agents in alcoholic fermentation of sugar cane molasses. Appl Microbiol 1960;8:263–8.

[32] Carvalho JCM, Aquarone E, Sato S, Brazzach ML, Almeida KA, Borzani W. Influence of exponentially decreasing feeding rates on fed-batch ethanol fermentation of sugar cane blackstrap molasses. Biotechnol Lett 1990;12:777–82.

[33] Somogyi M. Notes on sugar determination. J Biol Chem 1952;195:19–23.

[34] Joslyn MA. Methods in food analysis. 2th ed. New York: Academic Press; 1970.

[35] Converti A, Arni S, Sato S, Carvalho JCM, Aquarone E. Simplified modeling of fed-batch alcoholic fermentation of sugarcane blackstrap molasses. Biotechnol Bioeng 2003;84:88–95.

[36] Cornet JF, Dussap CG, Cluzel P, Dubertret GA. Structured model for simulation of cultures of the cyanobacterium *Spirulina platensis* in photobioreactors: II. Identification of kinetic parameters under light and mineral limitations. Biotechnol Bioeng 1992;40:826–34.

[37] Cachot T, Müller M, Pons M. Kinetics of volatile metabolites during alcoholic fermentation of cane molasses by *Sacchamoryces cerevisiae*. Appl Microbiol Biotechnol 1991;35:450–4.

[38] Hattori A, Myers J. Reduction of nitrate and nitrite by subcellular preparations of *Anabaena cylindrica* II. Reduction of nitrate to nitrite. Plant Cell Physiol 1967;8:327–37.

[39] Ohmori M, Ohmori K, Strotmann H. Inhibition of nitrate uptake by ammonia in a blue–green alga *Anabaena cylindrica*. Arch Microbiol 1977;114:225–9.