Isolation and Characterization of a New *Arthrospira* Strain

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A filamentous microorganism, morphologically similar to the cyanobacterium *Arthrospira*, was isolated from Mangueira Lagoon in Brazil, from which *Arthrospira* has not previously been isolated. Random amplified polymorphic DNA (RAPD) comparison with the standard *Arthrospira platensis* strains LEB 52 and Paracas indicated that the organism isolated was an *Arthrospira* isolate, which we denominated strain LEB 18. The RAPD analysis showed conserved sequences which indicated that the three strains belonged to the same genus, and were all *Arthrospira* species, but there were sufficient differences between them suggesting that they were separate strains. The strain LEB 18 was cultivated in undiluted Zarrouk medium and in 60% and 20% (v/v) Zarrouk medium diluted with sterilized Mangueira Lagoon water (MLW) using illuminance rates of 32.5, 45.5 and 58.5 μmol m^{-2} s^{-1} according to a complete 3^2 factorial design with a triplicate central point. The strains LEB 52 and Paracas were cultived in the conditions central point. Our new isolate produced the highest specific growth rate (μ_{max} = 0.22 d^{-1}) in 60% Zarrouk medium diluted with MLW and illuminated with 58.5 μmol m^{-2} s^{-1} and the highest protein content (86.0% w/w).

Key words: Arthrospira, Brazil, Mangueira Lagoon

Introduction

The therapeutic importance of *Arthrospira* has been widely discussed. Schwartz *et al.* (1998) reported that treatment with *Spirulina* and *Dunaliella* produced a total regression of hamster squamous cancer cells in 30% of the animals treated. Mathew *et al.* (1995) reported that treatment with crude extracts of *Spirulina fusiformis* produced regression of small homogeneous oral cancer lesions. Mishima *et al.* (1998) demonstrated that a novel sulfated polysaccharide derived from *A. platensis*, calcium spirulan, inhibited human lung metastases by preventing the adhesion and migration of tumour cells. More recently, Hirahashi *et al.* (2002) reported that aqueous extracts of *A. platensis* inhibited the replication of HIV in human T-cells and promoted the activation of the immune system through increased interferon production.

Apart from its use for the production of bioactive molecules, *Arthrospira* has recently received increasing attention for its potential to sequester carbon. The ability of phototrophic algae and cyanobacteria to assimilate carbon could be one of the most efficient processes for carbon sequestering and allows carbon dioxide to be removed from the atmosphere without radical changes in the global energy matrix and thus leads to a reduction in global warming (Morais and Costa, 2007a).

Isolation of *Arthrospira* has been reported from many different sites, including Lake Chad in the Republic of Chad, Lake Paracas in Peru, Lake Texcoco in Mexico, Lakes Nakaru and Elementeita in Kenya, and Lakes Aranguadi and Kilotes in Ethiopia (Henrikson, 1994), but there have been no previous reports on the isolation of *Arthrospira* from Brazilian sites. However, some Brazilian coastal lakes are alkaline and rich in carbonates and bicarbonates and therefore seem to be suitable environments for *Arthrospira*. Mangueira Lagoon, an alkaline lagoon situated between the Atlantic Ocean and Mirim Lagoon in the southern Brazilian state of Rio Grande do Sul, extends from 32° 32′ 05″ S to 33° 31′ 57″ S and presents favourable conditions for the natural growth of *Arthrospira* (Costa *et al.*, 2004) but had not before been investigated for this organism. The study described in this paper reports the isolation and the evalua-

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tion of the kinetic characteristics of an *Arthrospira* strain from Mangueira Lagoon.

Material and Methods

Harvest of biological material

Water samples were collected at two sites (site 1: 33° 30′ 12″ S, 53° 08′ 58″ W; site 2: 32° 50′ 10″ S, 52° 39′ 36″ W) on Mangueira Lagoon. A sub-sample was taken from each water sample and concentrated by centrifugation at 7,000 × *g* for 15 min, the sediment being transferred to small test tubes and ELISA plates containing Zarrouk medium (Morais and Costa, 2007b) or 20% (v/v) Zarrouk medium diluted with sterilized Mangueira Lagoon water (MLW). Two different pH values (9.0 and 11.0) were tested and each run was triplicated. The samples were maintained at 30 °C in a growth chamber under a 12 h light/dark photoperiod and an incident light intensity of 32.5 μmol m^{-2} s^{-1} provided by 40 W daylight-type fluorescent tubes (Reichert *et al.*, 2006).

Each week samples were removed from the tubes and examined by bright-field microscopy using an Olympus CX31 microscope with a total magnification of ×400. Filaments presenting morphological characteristics similar to those of *Arthrospira* were isolated using an M-152 micromanipulator (Narishige, Japan) and inoculated into new tubes and ELISA plates of the same medium from which the filaments had been isolated.

Genetic analysis

The *Arthrospira* strains LEB 18, LEB 52 and Paracas were analyzed using the polymerase chain reaction random amplified polymorphic DNA (PCR-RAPD) method (Williams *et al.*, 1990) and the 10 arbitrary primers (Operon Technology, Alameda, CA, USA) shown in Table I.

Total DNA was extracted by the phenol/chloroform method (Sambrook *et al.*, 1989) and PCR-RAPD was carried out in a final volume of 20.0 μL containing 2.0 μL 10X PCR buffer, 0.8 μL cDNA, 0.4 μL dNTPs (2.5 mM), 0.8 μL primers (10 pM), 1.0 μL BSA (Bovine serum albumin, 10 mg mL^{-1}) and 0.2 μL (5 U μL^{-1}) of Platinum Taq DNA polymerase (Invitrogen, São Paulo, Brazil) using the following amplification protocol: 94 °C for 1 min, followed by 40 cycles of 94 °C for 30 s, 50 °C for 1 min, 72 °C for 1 min and 30 s, with a final 5 min extension at 72 °C.

The PCR-RAPD fragments were separated by electrophoresis on 1.5% (w/v) agarose gel. The banding patterns of the three *Arthrospira* strains were compared as presence (1) or absence (0) of bands. These banding patterns were used to construct a binary matrix, all the bands being considered irrespective of intensity. Genetic profiles were constructed for each strain using the MEGA 3 program version 3.0 (Kumar *et al.*, 2004) adapted to analyze RAPD data by changing the 0 and 1 values in the binary matrix to A and T, respectively. The *p* distance was calculated and used to compare the genetic profile for each strain (Paplomatas *et al.*, 2004).

Cultivation of Arthrospira strains

The standard *Arthrospira platensis* strains used for comparison with LEB 18 were Paracas (Reichert *et al.*, 2006) and LEB 52 (Costa *et al.*, 2006). An inoculum for all the *Arthrospira* strains was prepared and maintained in liquid Zarrouk medium (ZM). The cultures were inoculate in one-litre photobioreactors with 0.9 mL working volume and an initial *Arthrospira* biomass concentration of 0.15 g L^{-1}. The biomass concentration was determined by measuring the optical density at 670 nm in a model 700 Plus spectrophotometer (FEMTO, Brazil) by previously prepared calibration curves relating the optical density with dry weight of *Arthrospira* biomass (Costa *et al.*, 2006).

The bioreactors were maintained at 30 °C in a growth chamber under a 12 h light/dark photoperiod and an incident light intensity of 45.5 μmol m^{-2} s^{-1} for strains LEB 52 and Paracas, and 32.5, 45.5 and 58.5 μmol m^{-2} s^{-1} for strain LEB 18 provided by appropriate numbers of 40 W daylight-type fluorescent tubes (Reichert *et al.*, 2006). Strain LEB 18 was cultivated in undiluted ZM ($ZM_{100\%}$) and in 60% ZM ($ZM_{60\%}$) and 20% ZM ($ZM_{20\%}$) diluted (v/v) with sterilized Mangueira Lagoon water, while strains LEB 52 and Paracas were cultivated in $ZM_{60\%}$ only. Each bioreactor run lasted 38 d during which time the cultures were agitated and aerated with 20 L h^{-1} air supplied by diaphragm pumps. Samples were aseptically collected every 24 h and the biomass assessed as described above.

The biomass (*X*) values were used to calculate the following kinetic parameters: maximum specific growth rate (μ_{max}, d^{-1}) by exponential regression of the logarithmic phase of the biomass curve

(Bailey and Ollis, 1986); biomass doubling time (t_d, d) as $t_d = \ln 2/\mu_{max}$; biomass productivity (P, g L^{-1} d^{-1}) from the equation $P = (X_t - X_0) \cdot (t - t_0)^{-1}$, where X_0 is the biomass concentration at inoculation (t_0) and X_t is the biomass concentration (g L^{-1}) in t (d) after inoculation (Schmidell *et al.*, 2001); and maximum productivity (P_{max}, g L^{-1} d^{-1}) as the highest productivity for each strain.

Experimental design and statistical analysis

For the evaluation of strain LEB 18 we used a complete 3^2 factorial design (Table II) in which the variables were strain, culture media (X_2; $ZM_{100\%}$, $ZM_{20\%}$ and $ZM_{60\%}$) and illuminance (X_1 = 32.5, 45.5 and 58.5 μmol m^{-2} s^{-1}), with a triplicate central point ($ZM_{60\%}$, 45.5 μmol m^{-2} s^{-1}) for strain LEB 18. Strains LEB 52 and Paracas were maintained under the central point conditions only ($ZM_{60\%}$, 45.5 μmol m^{-2} s^{-1}). Growth curves were constructed from the biomass values for strain LEB 18, the kinetic and biomass data being subjected to analysis of variance (ANOVA) and the significance of the results being tested by the Tukey test at $p \leq 0.10$.

Physico-chemical characterization of Arthrospira biomass

Biomass of strains LEB 18, LEB 52 and Paracas were cultivated in $ZM_{60\%}$ at 45.5 μmol m^{-2} s^{-1} and a commercial powdered preparation of *Arthrospira maxima*. The biomass from strains LEB 18, LEB 52 and Paracas was separated from the culture medium by filtration, washed to remove the excess salts, dried to constant weight in an oven at 40 °C and milled. The commercial sample was untreated. Ash and micro-Kjeldahl protein (conversion factor 6.25) were assayed by standard methods (A.O.A.C., 1997). Lipids were determined by the method of Folch and Lees (1957), and digestible protein according to Furlong *et al.* (2000). All the analyses were triplicated.

Results and Discussion

Mangueira Lagoon water contained various photosynthetic microorganisms, but we were able to isolate filaments with the characteristic morphology of *Arthrospira*. The isolated strain was denominated *Arthrospira* LEB 18. Microscopy of strains LEB 18, LEB 52 and Paracas showed that they were similar greenish-blue, helical filaments, morphologically consistant with the genus *Arthrospira*.

It has been pointed out that the isolation of native strains of photosynthetic microorganisms is an option which eliminates the dependence on exotic strains, avoids problems regarding the import of biological material, reduces loss of viability during transport and eliminates the time needed for the microorganism to adapt to new cultivation conditions (Poli *et al.*, 2004). The use of native strains, better adapted to local conditions, can not only result in higher productivity and a better cost-benefit relationship but also decreases the risk of environmental impact.

Eight of the ten primers used in the RAPD analysis of uni-algal cultures of *Arthrospira* LEB 18 resulted in specific banding profiles which showed signs of polymorphism. The primers produced a total of 103 scorable bands, varying from 5 to 20 per primer with an average of approximately 13 (Table I). The genetic relationship among the *Arthrospira* strains are shown in the phenogram presented in Fig. 1, the genetic distances between the strains being 71.0% for LEB 18 and Paracas, 57.0% for LEB 18 and LEB 52, and 51.0% for LEB 52 and Paracas.

These results indicate that LEB 18 is more closely related to LEB 52 than to the Paracas strain, while LEB 52 is more closely related to the Paracas strain than to LEB 18. These results show that LEB 18 has a genetic profile which is different to the strains routinely used in our laboratory, discarding the possibility that strain LEB 18 was the result of laboratory or environmental contamination.

Table I. Oligonucleotide sequence used for random amplified polymorphic DNA (RAPD) analysis of the *Arthrospira* strains LEB 18, LEB 52 and Paracas.

Primer data		Number of bands	
Primer	Sequence (5′–3′)	Simple	Polymorphic
AB3	TGGCGCACAC	11	9
AB4	GGCACGCGTT	12	4
AB5	CCCGAAGCGA	0	0
AB6	GTGGCTTGGA	5	1
AB7	GTAAACCGCC	15	6
AB9	GGGCGACTAC	20	8
AB11	GTGCGCAATG	0	0
AB14	AAGTGCGACC	15	4
AB17	TCGCATCCAG	11	5
AB18	CTGGCGTGTC	14	7

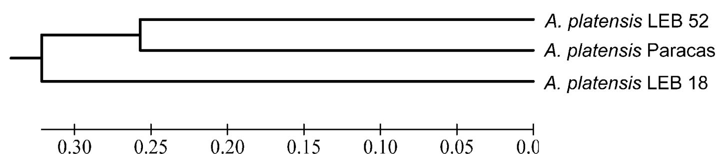

Fig. 1. Phenogram, generated by the MEGA 3 software, showing the genetic relationship between the *Arthrospira* strains LEB 18, LEB 52 and Paracas based on the polymorphic profile produced by 8 arbitrary derived RAPD primers. The numbers indicate the *p* distance calculated and used to compare the genetic profile for each strain.

The kinetic data for the *Arthrospira* strains tested are shown in Table II. When *A. platensis* strains LEB 52 and Paracas were cultivated at the LEB 18 central point values of the 3^2 factorial design (*i.e.* $ZM_{60\%}$ and 45.5 μmol m^{-2} s^{-1}) strain LEB 52 gave μ_{max} = 0.08 d^{-1} and the Paracas strain μ_{max} = 0.07 d^{-1}, while strain LEB 18 reached an average of μ_{max} = 0.13 d^{-1} under the same conditions. The highest μ_{max} values for strain LEB 18 were 0.22 d^{-1} in run 6 and 0.18 d^{-1} in run 4 while the lowest μ_{max} values were 0.07 d^{-1} in run 7 and 0.09 d^{-1} in run 9, indicating that strain LEB 18 grew better in diluted than in undiluted Zarrouk media (Table II). The highest μ_{max} value was 0.22 d^{-1} for strain LEB 18, which was higher than the 0.11 d^{-1} obtained for *A. platensis* in semi-continuous cultivation by Reichert *et al.* (2006). Thus we found that the highest μ_{max} value was obtained with diluted ZM (LEB 18, run 6) while the lowest values occurred with undiluted ZM (LEB 18 in run 6 and LEB 52).

These μ_{max} values were reflected in the biomass doubling time (t_d), which was only 5.38 d for strain LEB 18 (average for the three central points) but 8.66 d for strain LEB 52 and 9.90 d for the Paracas strain. These results show that, when cultivated under the same conditions, strain LEB 18 duplicates its biomass about 2 d faster than the other *Arthrospira* strains studied. Strain LEB 18 produced the largest biomass values (X_{max}), 2.36 g L^{-1} in run 6 (58.5 μmol m^{-2} s^{-1}, $ZM_{60\%}$) and 2.22 g L^{-1} in run 10 (45.5 μmol m^{-2} s^{-1}, $ZM_{60\%}$). The X_{max} value for run 6 was the highest one (p = 0.0095) compared with the X_{max} values for the other runs. As with the μ_{max} values, the highest X_{max} values were obtained in the runs with $ZM_{60\%}$.

Table II. Growth conditions and kinetic data for *Arthrospira* strains growing in different contents of Zarrouk medium (ZM, %) diluted with sterilized Mangueira Lagoon water (MLW) at different illumination rates (*I*).

Run	Factorial coding*		Physical variable		Kinetic data			
	X_1	X_2	I [μmol m^{-2} s^{-1}]	ZM**	μ_{max} [d^{-1}]	t_d [d]	X_{max} [g L^{-1}]	P_{max} [g L^{-1} d^{-1}]
LEB 18								
1	−1	−1	32.5	20	0.12[a]	5.78[a]	1.56[a]	0.08[a]
2	0	−1	45.5	20	0.11[ab]	6.30[ab]	1.66[b]	0.15[ab]
3	+1	−1	58.5	20	0.14[c]	4.95[ac]	1.82[c]	0.10[bc]
4	−1	0	32.5	60	0.18[d]	3.85[d]	1.93[d]	0.12[abcd]
5[†]	0	0	45.5	60	0.15[cde]	4.62[ace]	1.51[a]	0.09[abcde]
6	+1	0	58.5	60	0.22	3.15[d]	2.36	0.10[bcdef]
7	−1	+1	32.5	100	0.07[f]	9.90[f]	1.30	0.09[abcdefg]
8	0	+1	45.5	100	0.11[abg]	6.30[abg]	1.76[bcg]	0.12[h]
9	+1	+1	58.5	100	0.09[abfgh]	7.70	2.16[e]	0.18
10[†]	0	0	45.5	60	0.12[abcgi]	5.78[abcegh]	2.22[e]	0.14[bcdefgi]
11[†]	0	0	45.5	60	0.13[acegi]	5.33[acehi]	1.89[cd]	0.11[cfhij]
12[†]	0	0	45.5	60	0.12[abcgi]	5.03[abceghi]	2.02[f]	0.10[bcdeifgjk]
LEB 52	0	0	45.5	60	0.08[bfghj]	8.66	1.77[bcg]	0.07[a]
Paracas	0	0	45.5	60	0.07[fhj]	9.90[f]	2.20[ef]	0.11[cdefhijk]

Values with the same letters of the same parameters indicate that the values did not differ by the Tuckey test at $p \leq 0.10$.

* X_1, illuminance; X_2, culture medium.

** Percentage ZM (v/v).

† These are the central point replicates for strain LEB 18.

The highest biomass productivity (P_{max} = 0.18 g L^{-1} d^{-1}, $p < 0.0001$) of all the strains occurred with strain LEB 18 in run 9 ($ZM_{100\%}$, 58.5 μmol m^{-2} s^{-1}); this strain also produced the highest average P_{max} value (0.11 g L^{-1} d^{-1}) at the central point conditions ($ZM_{60\%}$, 45.5 μmol m^{-2} s^{-1}).

The highest μ_{max} values for LEB 18 occurred in 40% to 60% ZM diluted with MLW at illumination levels above 52 μmol m^{-2} s^{-1}. The lowest LEB 18 μ_{max} values occurred in undiluted Zarrouk media at all illumination levels and in $ZM_{20\%}$ at intermediary illuminance levels between 39 μmol m^{-2} s^{-1} and 45.5 μmol m^{-2} s^{-1}.

Although the cultivation of *Arthrospira* is a simple industrial process, the preparation of Zarrouk and other complex media is difficult and expensive and makes the production of *Arthrospira* unduly difficult. An attractive alternative for reducing production costs is the partial or total substitution of Zarrouk medium by natural alkaline waters such as MLW which contains carbonates and micronutrients and has appropriate physico-chemical characteristics for the growth of *Arthrospira* (Costa *et al.*, 2002).

During the semi-continuous cultivation of *A. platensis* in open bioreactors the highest P_{max} value was 0.043 g L^{-1} d^{-1} in water supplemented with 20% ZM, the productivity in undiluted ZM being 0.038 g L^{-1} d^{-1} (Reichert *et al.*, 2006). These results, in conjunction with our data, suggest that ZM contains more nutrients than is needed for the optimum growth of *Arthrospira*. Costa *et al.* (2004) reported that although *Arthrospira* can be cultivated in MLW it has to be supplemented with carbon and nitrogen because the unsupplemented water supports the growth of *Arthrospira* for only 12 d. Our current results show that growth can extend for at least 38 d (the maximum time of each run) and that the best results were obtained in MLW supplemented with 60% Zarrouk medium, which supplied sufficient carbon and nitrogen to extend each run to over one month.

Linear analysis was carried out for the 3^2 complete factorial analysis with a triple central point for strain LEB 18 using culture media and illuminance as factors and μ_{max} as effect (Table III). The results showed that although μ_{max} increased while illuminance increased this was not statistically significant ($p > 0.0890$), indicating that the lowest illuminance (32.5 μmol m^{-2} s^{-1}) could be used for cultivation of *Arthrospira* and this not only reduce costs compared to higher light intensity but also makes economically the provision of a constant light intensity unavailable under natural conditions. For the culture medium factor we found that the μ_{max} value decreased as the concentration of ZM increased, indicating that diluted ZM accelerates the growth as well as lowers the costs of the final media (Table III).

Table III. Factors (culture media and illuminance), effect (maximum specific growth rate μ_{max}, d^{-1}) and significance (p) obtained with the 3^2 complete factorial analysis with a triple central point for strain LEB 18.

Factor	Effect (μ_{max} [d^{-1}])	Error	p
Mean	0.13	>0.00	>0.0000
X_1(L)	0.03	0.02	0.2313
X_1(Q)	−0.02	0.01	0.2342
X_2(L)	−0.03	0.02	0.1721
X_2(Q)	0.07	0.01	0.0240*
X_1(L) · X_2(L)	>0.00	0.02	0.9635
X_1(L) · X_2(Q)	>0.00	0.02	0.7129
X_1(Q) · X_2(L)	>0.00	0.02	0.3340
X_1(Q) · X_2(Q)	−0.04	0.01	0.0619*

X_1, illuminance; X_2, culture media; L, linear term; Q, quadratic term.
* Statistically significant by the Tukey test at $p \leq 0.10$.

Evaluating the quadratic terms, we found that the maximum growth occurred at 32.5 and 58.5 μmol m^{-2} s^{-1}, while for culture media the best growth occurred at the central point values ($ZM_{60\%}$). These results were statistically significant at (p = 0.0910) indicating that the effects of illuminance and culture medium need to be studied further.

The protein, lipid, and ash content and the digestibility (%) of the *Arthrospira* strains LEB 18, LEB 52 and Paracas and *A. maxima* are shown in Table IV as means ± standard deviation. The most abundant component was protein, the highest

Table IV. Protein, lipid, and ash content and protein digestibility (%) for the *Arthrospira* strains LEB 18, LEB 52 and Paracas. The values represent means ± standard deviation.

Strain	Protein	Lipid	Ash	Protein digestibility
LEB 18	86.0 ± 1.3[a]	3.3 ± 1.9[a]	6.7 ± 0.1[a]	84.0 ± 0.1
LEB 52	82.5 ± 0.2[a]	3.3 ± 1.1[a]	6.7 ± 0.1[a]	86.5 ± 0.2
Paracas	73.7 ± 1.0[b]	7.9 ± 1.1[b]	7.0 ± 0.6[b]	80.2 ± 0.3
A. maxima	73.6 ± 0.1[b]	6.9 ± 1.4[b]	7.1 ± 0.1[b]	78.4 ± 0.2

Values with the same letters of the same parameters indicate that the values did not differ by the Tuckey test at $p \leq 0.10$.

value occurring in strain LEB 18 (86.0%). Pelizer *et al.* (2003) investigated the effects of inoculum on the cultivation of *Arthrospira platensis* and found that the highest protein content was 61% when growing at 30 °C with an illuminance of 45.5 μmol m^{-2} s^{-1}. Our *Arthrospira* results were also higher than those found for *Chaetoceros* sp. (64.1%), *Rhodomonas* sp. (58.8%) and *Cryptomonas* sp. (49.5%) growing at 30 °C (Renaud *et al.*, 2002). In cultivations of *Arthrospira maxima* growing in water supplemented with KNO_3 (Macedo and Alegre, 2001) the maximum protein value was about 72.0%, lower than for any of the strains shown in Table IV.

It is known that 64.0% to 74.0% by dry weight of *Arthrospira* is protein, and these values may be up to 15.0% higher when samples are collected at the start of the photoperiod (Henrikson, 1994). This content is higher than in the best non-microbial commercial sources of proteins, such as soya (35.0%), powdered milk (35.0%), fish (15.0–25.0%), eggs (12.0%), cereals (8.0–14.0%) and fresh whole milk (3.0%) (Henrikson, 1994). Large-scale production of *Arthrospira* protein could be a lucrative alternative to conventional agro-industrial processes.

Table IV shows that the protein digestibility values for strains LEB 18, LEB 52 and Paracas agree with the 80.0% to 90.0% reported by Henrikson (1994), although the value for *A. maxima* was close to the lower limit of (78.4 ± 0.2)% (Henrikson, 1994). Unlike other microorganisms used as protein sources, the cyanobacterium *Arthrospira* contains no cellulose in its cell wall but is instead covered with a relatively fragile mucopolysaccharide which, in part, explains the high digestibility of its proteins (Costa *et al.*, 2002), which is a considerable advantage from a processing point of view and results in the retention of high concentrations of vitamins and polyunsaturated fatty acids.

The lipid content of strains LEB 18 and LEB 52 was lower, and more similar to each other, than in the Paracas strain and *A. maxima* (Table IV). Fuentes *et al.* (2000) cultivated the microalga *Porphyridium cruentum* supplemented with carbon dioxide and obtained a lipid content of 7.5%. In our study the ash content varied from 6.7% to about 7.1%, comparable with the 7.0% reported for *A. maxima* growing at 25 °C in water supplemented with 2.5 g L^{-1} of KNO_3 (Macedo and Alegre, 2001).

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