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Pilot scale semicontinuous production of *Spirulina* biomass in southern Brazil

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ABSTRACT

We evaluated the feasibility of the pilot scale production of *Spirulina* strain LEB-18 in southern Brazil and assessed the quality of biomass produced in relation to its kinetics characteristic, nutritional value, heavy metal content and microbial content. The maximum mean biomass concentration was 1.24 g L^{-1} and the maximum productivity was 69.16 g m^{-2} d^{-1}. The biomass showed 84.0% digestibility, 86.0% (w/w) protein and 3.3% (w/w) lipid content. Analyses showed that the concentration (mg kg^{-1}) of heavy metals (As, 0.28 ± 0.01; Cd, <0.05; Hg, <0.01; and Pb, 0.17) and the microbial load (7.1×10^5 colony forming units per gram) were lower than the internationally accepted standards. These results show that pilot scale cultivation of *Spirulina* LEB-18 in southern Brazil is feasible and that the biomass produced is within the internationally recognized standards for use as a food additive for increasing the nutritional potential of conventional products.

1. Introduction

Increase in world population and the forecast of insufficient protein supply have spurred research into alternative food sources (Spolaore et al., 2006) and many studies have investigated the potential of microbial protein for use in human and animal foodstuffs. The cyanobacterium *Spirulina* (*Arthrospira*) contains about 60% to 70% protein and a nucleic acid and amino acid content similar to that recommended by the Food and Agriculture Organization (FAO) (Pelizer et al., 2003). This microalga also contains vitamins, including B12 and β-carotene, and minerals (Jiménez et al., 2003).

Pre-clinical and clinical studies suggest that the consumption of *Spirulina* biomass has therapeutic effects such as reducing blood cholesterol, protecting against some cancers, reducing drug and heavy metal nephrotoxicity, hyperlipidemia and obesity (Deshnium et al., 2000; Costa et al., 2004). Hirahashi et al. (2002) reported that aqueous extracts of *Spirulina platensis* partially inhibited the replication of HIV-1 in human cells.

Open raceway tanks are generally used for commercial *Spirulina* production but some companies use closed tubular bioreactors, cultivation in open raceway tanks is carried out in China, Israel and the USA (Jiménez et al., 2003). The largest *Spirulina* plant in the world is Earthrise Farms on a 440,000 m^2 site in the Californian desert near Calipatria City (California, USA), with 30 cultivation ponds of 5000 m^2 (total 150,000 m^2) each of which uses filtered mineral-rich Colorado River water supplemented with salts (Spolaore et al., 2006).

The large-scale production of *Spirulina* biomass depends on factors such as light, nutrients and temperature, all of which influence the quantity and composition of biomass produced. According to Vonshak (1997) nutrients, principally the carbon source, represent the largest cost in the production of *Spirulina* biomass, the main carbon source generally being sodium carbonate and bicarbonate.

Mangueira Lagoon is 92.0 km long × 7.6 km wide with a depth which varies from 1.2 m to 7.4 m and is an important hydrobiological resource located between the Atlantic Ocean and lake Mirim in the extreme south of the southernmost Brazilian state of Rio Grande do Sul. The pH and other physical–chemical characteristics of Mangueira Lagoon Water (MLW) are appropriate for the cultivation of *Spirulina* and contains carbon in the form of 0.126 g L^{-1} of HCO_3^- and nitrogen in the form of 0.132 of mg L^{-1} of NH^{4+}, 0.0195 mg L^{-1} of NO_2^{2-} and 1.040 mg L^{-1} of NO_3^- that can be used by *Spirulina* as nutrients (Costa et al., 2002).

The objective of the research published in this paper was to evaluate the feasibility of the pilot scale constant-volume semicontinuous production of biomass from *Spirulina* strain LEB-18 in the southern Brazil using MLW supplemented with mineral salts. We also assessed the quality of biomass produced with respect to kinetics of accumulation, nutritional characteristics, microbiological load and the presence of heavy metals.

2. Materials and methods

2.1. Strain selection and culture medium

We used the cyanobacterium *Spirulina* strain LEB-18 isolated from Mangueira Lagoon (Morais et al., 2008) and MLW supplemented with

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20% (v/v) Zarrouk medium (Costa et al., 2004), referred to in the text as MLW-S medium, for maintenance, inoculum and biomass production.

2.2. Production site, pilot plant and biomass production system

The pilot plant for production of *Spirulina* LEB-18 was located near the shore of Mangueira Lagoon (33° 30′ 13″ S; 53° 08′ 59″ W) and consisted of raceway tanks of different dimensions and volumes depending on their purpose. We used one inoculum tank (4.0 m long × 1.0 m wide × 0.50 m high) with a surface area of 3.87 m^2 and a working volume of 1000 L and three production tanks (13.0 m long × 3.0 m wide × 0.50 m high), each with a surface area of 37.10 m^2 and a working volume of 10,000 L. All the tanks were lined with glass fiber and covered by a greenhouse structure constructed from transparent polyethylene film. All tank cultures were agitated by one paddle wheels rotating at 18 rpm 24 h per day. The runs were carried out from 12th July 2005 to 2nd August 2006 (387 days) when the water temperature ranged from 4 °C to 44 °C. The volume of culture media was maintained by the periodic addition of MLW to compensate for evaporation, about 12 L d^{-1}, for tank of 10,000 L, being added over the course of the experiment.

Stock cultures were produced in using MLW-S in conical flasks in which the volume of media did not exceed 20% of the volume of the flask. The cultures were agitated and aerated with air at a rate of 0.1 volume of air per volume of media per minute (v v^{-1} m^{-1}) and the cultures were maintained at 30 °C at an illuminance of 2500 lx supplied by 40 W daylight-type fluorescent lamps and under a 12 h light/dark photoperiod for acclimatization (Costa et al., 2006). The biomass concentration was estimated spectrophotometrically as described below.

First-stage inoculum for the bioprocess was produced using MLW-S in conical flasks in which the volume of media did not exceed 20% of the volume of the flask, volumes being scaled up from 20.0 mL to 20.0 L. These cultures were inoculated with 10% v/v of stock culture and maintained under the same conditions as used for the stock cultures. The biomass concentration was estimated spectrophotometrically as described below.

Second-stage inoculum was produced by adding 20.0 L of flask inoculum and scaling up by doubling the culture volume each 3 days by adding MLW supplemented with 20% (v/v) Zarrouk medium (MLW-S) until it reached the working volume of 1000 L and a biomass concentration of 1.00 g L^{-1}. For production, the culture was removed from the inoculum tank and added to each of the production tanks containing sufficient MLW-S to produce initial working volume of 10,000 L, and after 3 days the culture volume reached 10,000 L and 0.5 g L^{-1} by adding MLW-S. The cultures were maintained under natural light in the tank cultures for up to 387 days. The biomass concentration was estimated directly as dry biomass as described below. The system is summarized in Fig. 1.

2.3. Biomass measurement

Spectrophotometric measurements of biomass concentration were made in all tanks every 24 h by measuring the optical density (OD) at 670 nm using a 700-Plus spectrophotometer (Femto, Brazil), a calibration curve being used to relate optical density to *Spirulina* LEB-18 biomass dry mass (Morais and Costa, 2007). The pH of the cultures was also determined every 24 h using a Q400H digital pH meter (Quimis, Brazil) and the daily temperature range inside the greenhouse was measured using a maximum and minimum thermometer.

Each 72 h, 25% of the biomass produced in the pilot scale was removed and the biomass was separated by filtration of the sample through a 200 μm filter, the filter cake being compressed and extruded using a hydraulic press. At this stage the biomass contained about 76% (w/w) water and was dried at 50 °C for 5 h in a tray-dryer, and then vacuum-packed and stored until needed for testing. To conserve nutrients in the culture media we returned the filtrate to the tank from which it had been removed. The filtrate not returned to the tank was added to the tanks containing fish *Odonthestes* sp.

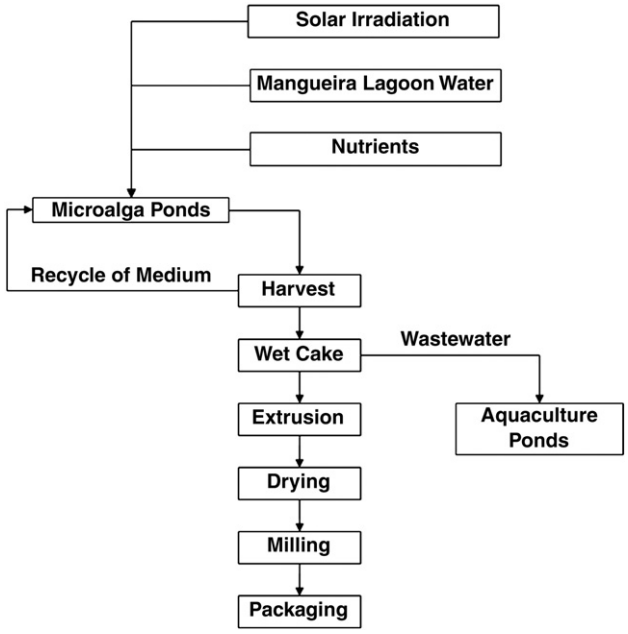

Fig. 1. Flow diagram for the pilot scale production of biomass from *Spirulina* strain LEB-18 in southern Brazil.

2.4. Bacteriological and chemical analysis of the biomass

The biomass was checked for bacterial contamination using the total pour-plate method and plate count agar (AOAC, 1997), the results were being reported as colony forming units per g (CFU g^{-1}). The caloric value of the dried microalgal biomass was determined using an adiabatic bomb calorimeter series 1300 plain type (Parr Instrument Co., USA). Arsenic (Mello et al., 1999), cadmium and lead (Horwitz, 2000) and mercury (Yallouz et al., 2001) were determined by flame absorption spectrometry using a Varian VGA 96 by direct comparison with standards of known concentration (Sigma). All determinations on dry biomass were carried out in duplicate or triplicate and all percentages are weight for weight (w/w) or simple percentages unless otherwise stated.

Total protein, moisture and ash were determined by standard methods (AOAC, 1997). Nitrogen content was measured by the micro-Kjeldahl process using a 6.25 factor to convert total nitrogen to protein content. Digestibility was determined by the method of Furlong et al. (2000). Total and free amino acids were assayed using the automatic amino acid analysis method of Alonso and Hirs (1968) and the automatic recording apparatus for use in amino acid chromatography described by Spackman et al. (1958). Total amino acids were obtained by analysis after hydrolysis of 3.0 mg to 5.0 mg for 22 h at 110 °C in 0.50 mL of constantly boiling HCl containing 0.01% (w/v) phenol in an evacuated and sealed tube. Tryptophan was determined by amino acid analysis after hydrolysis of 3.0 mg to 5.0 mg of protein or hydrolyzed with lithium hydroxide (Lucas and Sotelo, 1980).

Lipids were extracted with 2:1 (v/v) chloroform/methanol, purified with 0.9% (w/v) NaCl and a 2:1 (v/v) methanol/water mixture according to Folch and Lees (1957) and transferred to a rotary evaporator and the solvent was removed at approximately 37 °C. The lipid content was determined gravimetrically (Folch and Lees, 1957). Fatty acids were transmethylated by treatment with hexane boron trifluoride (Metcalfe and Schimitz, 1966). The fatty acid methyl esters (FAME) were analyzed in a Varian-3400CX gas chromatograph

(Varian, EUA) equipped with a flame ionization detector (FID) and a 0.32 mm diameter 30 m fused silica capillary column (Varian, EUA) rising from 100 °C to 230 °C at 8 °C min^{-1} with an injector temperature of 250 °C and a detector temperature of 280 °C. Hydrogen was used as the carrier gas at a flow rate of 0.5 mL min^{-1}. Identification of fatty acids was made by comparing the relative retention times of fame peaks of the samples with those of Sigma (USA) standards. The peak areas were determined using the Varian Star Chromatography workstation software to normalize the percentage areas of the total fatty acids.

2.5. Kinetic parameters

The biomass concentration values in the production tanks were used to calculate the maximum biomass dry weight (X_{max}, g L^{-1}) and the maximum biomass productivity (P_{max}, g m^{-2} d^{-1}) was calculated as $P_{max} = (X_t - X_o) \cdot (t - t_o)^{-1}$, where X_o is the initial biomass concentration (g L^{-1}) at time t_o (d) and X_t is the biomass concentration (g L^{-1}) at any time t (d) subsequent to t_o (d) (Schmidell et al., 2001). The mean productivity (P, g m^{-2} d^{-1}) was calculated as the mean of the productivities values (Bailey and Ollis, 1986).

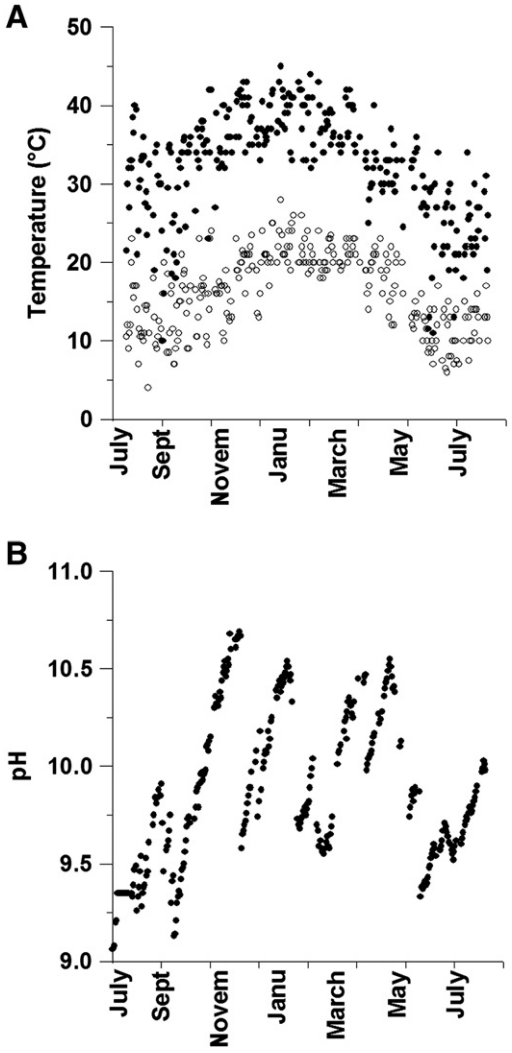

Fig. 2. Maximum (●) and minimum (○) daily temperature (A) and pH (B) values of the cultures from July 12th 2005 to August 2nd 2006 during the cultivation of *Spirulina* strain LEB-18.

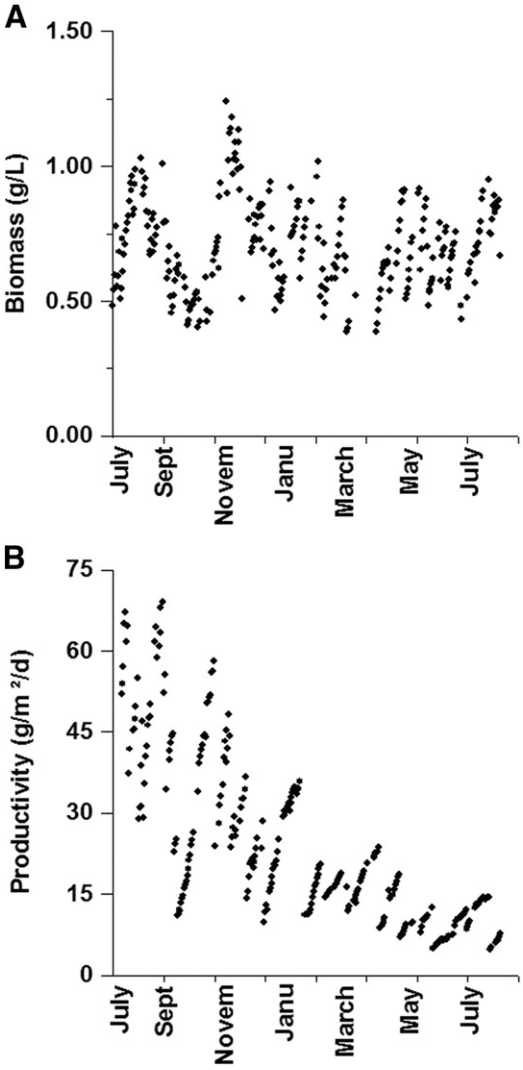

Fig. 3. Biomass concentration (A) and productivity (B) during the period of operation from 12th July 2005 to 2nd August 2006 *Spirulina* strain LEB-18.

3. Results and discussion

The daily temperatures, pH, biomass concentration and productivity during the cultivation of *Spirulina* strain LEB-18 in the production tanks are presented in Figs. 2 and 3.

The maximum air temperature in the greenhouse covering the tanks was 44 °C in December 2005 and the minimum temperature was 4 °C in August 2005 (Fig. 2A). As strain LEB-18 was originally isolated from Mangueira Lagoon it was, presumably, adapted to the environmental conditions in the area such that cultivation under the prevailing environmental conditions was not impeded by temperature variations (Fig. 3A).

The optimum temperature range for the growth of *S. platensis* is variable and strains can differ both in their optimal growth temperature and their extreme temperature ranges (Vonshak, 1997). For example, Richmond (1990) reported that during outdoor cultivation the minimal temperature for the growth of *S. platensis* was around 18 °C and that cultures deteriorated quickly when the maximum day-time temperature was below 12 °C. However, other lower temperature limits can exist for different *Spirulina* species or strains, with Jiménez et al. (2003) having reported that cultures of *Spirulina* grew in the Spanish city of Malaga at temperatures ranging from 9 °C to 28 °C, a less extreme temperature range than the 4 °C to 44 °C seen in our pilot plant experiments in southern Brazil.

The pH of the medium in the production tanks was pH 9.0 to pH 10.7, but fell during some periods (Fig. 2B). According to Vonshak (1997) the pH optimum for *Spirulina* ranges from pH 9.5 to pH 10.5, with a reduction in cell numbers occurring at pH 8.0 and below. Jiménez et al. (2003) report that pH 9.5 and above is ideal for pilot scale cultivation of *Spirulina*, with their *Spirulina* cultures in Malaga having pH values of pH 9.0 to 10.9, similar to the pH 9.0 to pH 10.7 seen in our production tanks in southern Brazil.

In the production tanks strain LEB-18 presented a maximum biomass (X_{max}) of 1.24 g L^{-1} a maximum productivity (P_{max}) of 69.16 g m^{-2} d^{-1} and mean biomass productivity (P) of 21.59 g m^{-2} d^{-1}, although the productivity decreased with time (Figs. 3A and 3B). Semicontinuous cultures with recycled medium allowed strain LEB-18 to use the medium more efficiently than if they had been grown in discontinuous or batch culture but because no fresh media was added there was a gradual depletion of nutrients, and hence productivity, with time, possibly due in part to the reduction in osmotic pressure caused by the decrease in nutrients which had been converted to biomass (Carrión et al., 2001).

According to Travieso et al. (2001) the ideal biomass concentration for the maximum productivity of *Spirulina* is 0.5 g L^{-1} to 0.7 g L^{-1}, and in our study the biomass concentration was 0.5 g L^{-1} to 1.24 g L^{-1} (Fig. 3A). Vonshak (1997) reported that photoinhibition in *Spirulina* cultures is possible to occur with a biomass concentration below 0.5 g L^{-1} due to the excess incident light and that this can result in physiological and metabolic processes, such as the accumulation of H_2O_2, which decrease or inhibit the use of light by *Spirulina*. However, Chojnacka and Noworyta (2004) have pointed out that at biomass concentrations above 1.0 g L^{-1} the large number of cells can block light penetration into the cultures and result in photolimitation. Thus at low luminosity incidences CO_2 fixation rates are negative due to cell respiration, interfering with photosynthesis, CO_2 biofixation rates and cell multiplication. The levels of light causing photoinhibition or photolimitation depends on the genera, species and strain of the photosynthetic microorganism concerned, with discontinuous cultivations being more susceptible to photolimitation caused by high cell numbers which decrease available light due to shading effects. However, due to the regular removal of 25% of the biomass in the tank, photolimitation does not generally occur in constant-volume semicontinuous cultures such as the pilot process used by us.

The dry biomass of *Spirulina* strain LEB-18 consisted of 86.0% ± 1.3% protein, higher than the 61.0% protein reported by Grinstead et al. (2000) for *S. platensis* or the 57.1% reported by Yanagi et al. (1995) for *Chlorella* strain HA-1 cultivated with 10.0% (v/v) of CO_2. The quality of the proteins is determined by their amino acid content and digestibility and strain LEB-18 presented 84.0% ± 0.1% digestibility, agreeing with Richmond (1990) who obtained about 83.0% of digestibility for *Spirulina*. Vonshak (1997) has pointed out that *Spirulina* has a layer of soft mucopolysaccharide instead of a cellulose cell wall, facilitating cell lysis and release of proteins.

Table 1
Amino acid content (%, w/w) of *Spirulina* strain LEB-18 compared with a *Spirulina platensis* strain (Grinstead et al., 2000), *Chlorella* sp. strain HA-1 (Yanagi et al., 1995), *Nannochloropsis* sp. strain MFD-2 (James et al., 1989) and the FAO/WHO standard for essential amino acids for children aged 2 years to 5 years.

Amino acid	LEB-18	*S. platensis*	HA-1	MFD-2	FAO/WHO
Glut. acid	10.70	–	5.78	5.17	–
Aspart. acid	9.20	–	4.66	3.85	–
Leucine	8.02	5.10	4.71	2.98	6.60
Alanine	6.51	–	4.55	2.42	–
Phenylalan.	5.75	2.60	2.69	2.03	–
Glycine	5.17	–	3.35	1.96	–
Arginine	4.94	3.90	3.06	2.41	–
Threonine	4.87	2.80	2.46	1.72	3.40
Valine	4.61	3.60	3.03	1.51	3.50
Isoleucine	4.36	3.20	2.01	1.16	2.80
Serine	4.31	–	1.04	1.69	–
Proline	4.04	–	2.52	2.74	–
Tyrosine	3.20	2.40	2.40	1.37	–
Lysine	2.95	2.70	3.98	2.29	5.80
Histidine	2.72	1.00	2.18	0.75	1.90
Tryptophan	2.53	0.60	1.04	–	1.10
Methionine	1.64	1.40	1.19	0.21	–
Cystine	0.47	0.70	0.65	1.67	–

Table 2
Heavy metal concentration (mg kg^{-1}) in *Spirulina* biomass from different sources and the maximum levels (mg kg^{-1}) permitted by the Brazilian National Health Surveillance Agency (Agência Nacional de Vigilância Sanitária, ANVISA).

	Heavy metal concentration (mg kg^{-1}) in *Spirulina* biomass			Maximum levels (mg kg^{-1})
Heavy metal	LEB-18	Richmond (1990)	Earthrise[a]	ANVISA (1965)
Arsenic	0.28[b]	1.1	<1.0	1.0
Cadmium	<0.05	<0.1	<0.05	1.0
Lead	0.17	0.4	0.8	0.17
Mercury	<0.01	0.24	<0.01	<0.01

[a] According to Vonshak (1997).
[b] This value ± 0.01 mg kg^{-1}.

The essential amino acids isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine were found in the biomass of LEB-18 (Table 1), this *Spirulina* strain presenting a better amino acid content than the *S. platensis* described by Grinstead et al. (2000), *Chlorella* sp. strain HA-1 (Yanagi et al., 1995) or *Nannochloropsis* strain MFD-2 (James et al., 1989), especially with respect to glutamic acid which constituted 10.70% of the dry biomass of LEB-18 (Table 1). Strain LEB-18 generally presented larger amounts of essential amino acids than the theoretical quantities recommended in dietary protein for children aged 2 years to 5 years (FAO, 1990), the exception being lysine which accounted for 2.95% of the dry biomass of LEB-18 (Table 1) as against the 5.8% recommended in the dietary protein of children by FAO (1990).

Strain LEB-18 contained 6.7 ± 0.1% ash, 5.3 ± 0.26% moisture and 3.3 ± 0.19% lipids. Pelizer et al. (2003) cultivated *Spirulina* at 30 °C in raceway tanks using an initial *Spirulina* biomass concentration of 0.5 g L^{-1} and obtained 5.3% moisture but 15.6% lipids. It has been reported that lipid content increased when several strains of microalgae were grown under conditions of nitrogen limitation, with lipid content of *Chlorella protothecoides* increasing from 11.0% when growing without nitrogen limitation to 23.0% under nitrogen limitation and the caloric power increasing from 19.0% to 24.0% under the same conditions (Illman et al., 2000).

The fatty acid profile showed 46.4% palmitic acid (C16:0), 32.2% oleic acid (C18:1), 7.6% behenic acid (C22:0), 6.3% elaidic acid (C18:1) and 1.9% myristoleic acid (C24:1), comparing favorably with the fatty acid profile of the *Spirulina* cultivated by Romano et al. (2000) which showed 44.4% palmitic acid, 15.2% elaidic acid and 3.9% oleic acid. Olguín et al. (2001) obtained 43.9% of palmitic acid and 16.8% of oleic acid. Palmitic acid is an important source of energy in infant feeding because maternal milk contains 20.0 to 30.0% of palmitic acid (Willis et al., 1998). Colla et al. (2004) cultivated *Spirulina* at different temperatures and under different nitrogen concentrations and also found high levels (45.0%) of palmitic acid. The predominance of palmitic acid reported by several authors in the biomass of photosynthetic microorganisms is probably due the fact that the synthesis of fatty acids generally begins with saturated fatty acids (Lehninger, 2004).

Carbohydrate, protein and lipid content influence the caloric value of a food, with the high protein and relatively low lipid content of LEB-18 possessing the relatively low caloric value of 16.9 kJ g^{-1} compared to the 18.0 kJ g^{-1} to 21.0 kJ g^{-1} reported for microalgae (Scragg et al., 2001).

Large scale cultivations in open tanks are susceptible to contamination by unwanted microalgae or bacteria. In our study, microbiological analysis of the dry LEB-18 biomass showed 7.1×10^5 CFU g^{-1}. This was lower than the values reported by Vonshak (1997) for *Spirulina* cultures in France (<0.1×10^6 CFU g^{-1}), Japan (<0.05×10^6) and California

(1.0×10^6 CFU g^{-1}, Earthrise Farms), probably due to alkaline pH values (pH 9.0 to pH 10.7) prevailing in our tanks.

Analyses showed that the concentration of heavy metals (Table 2) in the *Spirulina* LEB-18 biomass was lower than the values reported by Richmond (1990) for *Spirulina* sp. biomass and Vonshak (1997) for Earthrise *Spirulina* sp. biomass and were within the maximum limits as stated by the Brazilian National Health Surveillance Agency (Agência Nacional de Vigilância Sanitária (Anvisa), Decree N° 55.871, 26 March 1965).

4. Conclusions

The pilot scale cultivation of *Spirulina* strain LEB-18 in south of Brazil lasted for 387 days and indicates that this strain is resistant to fluctuations in environmental conditions such as temperature, which varied between 4 °C and 44 °C. The pH was maintained between 9.0 and 10.7, indicating that supplementing Mangueira Lagoon Water with 20% (v/v) Zarrouk medium does not harm the cultivation of this strain and results in reduced cultivation costs.

The maximum LEB-18 biomass concentration (X_{max}) was 1.24 g L^{-1} and the maximum productivity (P_{max}) was 69.16 g m^{-2} d^{-1}, while the mean productivity (P) was 21.59 g m^{-2} d^{-1}. Strain LEB-18 had a digestibility of 84.0% and contained 86.0% protein, the most abundant amino acid being glutamic acid (10.70%). The lipid content was 3.3%, with a predominance of palmitic acid (46.4%). Heavy metal content (As, 0.28 ± 0.01; Cd, <0.05; Hg, <0.01; and Pb, 0.17) and microbiological load (7.1×10^5 CFU g^{-1}) were within Brazilian and international standards. These results show that *Spirulina* strain LEB-18 biomass produced in southern Brazil on a pilot scale can be used as a nutritional supplement or a food source without causing risks to health linked to the parameters studied.

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