Simultaneous Cultivation of *Spirulina platensis* and the Toxigenic Cyanobacteria *Microcystis aeruginosa*

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Mangueira Lagoon, located in the extreme south of Brazil, has water with physicochemical characteristics such as alkaline pH and carbonate levels propitious for the growth of the cyanobacterium *Spirulina platensis*. Previously published studies have shown that Mangueira Lagoon water supplemented with small quantities of carbon and nitrogen is suitable for *S. platensis* cultivation and can significantly reduce production costs. We studied mixed cultures of *Spirulina platensis* and the toxic cyanobacterium *Microcystis aeruginosa* using a 2^3 factorial design in which the three factors were the initial biomass concentration of *S. platensis* and *M. aeruginosa* and the type of culture medium (100% Zarrouk's medium or 80% Mangueira Lagoon water plus 20% Zarrouk's medium). The highest *S. platensis* maximum specific growth rate (μ_{max}) occurred in the culture with the highest *M. aeruginosa* biomass concentration and when undiluted culture medium was used ($\mu_{max} = 0.283\ d^{-1}$). The highest *M. aeruginosa* specific death rate (k) was obtained in the presence of *S. platensis* ($k = 0.555\ d^{-1}$) and was independent of the initial *M. aeruginosa* biomass concentration and culture medium, demonstrating that *S. platensis* cultures are not susceptible to contamination by *M. aeruginosa*. The culture medium had no significant influence ($p > 0.05$) on *S. platensis* μ_{max} values, indicating that production costs could be reduced by using a medium consisting of 80% Mangueira Lagoon water plus 20% Zarrouk's medium.

Key words: Cyanobacteria, *Microcystis aeruginosa*, *Spirulina platensis*

Introduction

The filamentous cyanobacterium *Spirulina platensis* is identified mainly by the helical arrangement of its multicellular cylindrical trichomes (Vonshak, 1997) which appear microscopically as greenish filaments up to 1 mm in length (Vymazal, 1995) and 1 to 12 μm in diameter with a free moving axis and no heterocysts (Richmond, 1990). This cyanobacterium inhabits soils, sands, swamps, alkaline lakes and brackish waters, marinas and docks where it uses photosynthesis to transform water and nutrients into biomass and oxygen.

Proteins make up 64 to 74% of the total biomass of *S. platensis* and, according to Vonshak *et al.* (1983), the lipid content varies from 6 to 13% and the carbohydrate content from 12 to 20%, which means that *Spirulina* biomass is highly nutritional and can be used as a food supplement for humans and animals and as a source of fine chemicals. Due to its ecological, nutritional and economic importance *Spirulina* has been the subject of intensive biotechnological studies. Reports showing that this cyanobacterium is an estuarine or marine species that can be produced on a commercial scale in temperate latitudes (Costa *et al.*, 2000) and several companies have industrialized *S. platensis* production because its cultivation requirements are simple, industrial residues can serve as feedstock and its growth rate is high. Clinical studies suggest that consumption of *S. platensis* has beneficial therapeutic effects on humans, including reduced serum cholesterol, hyperlipodemia and obesity, increased intestinal lactobacilli, improved immune response, protection against some cancers, and a reduction in the effects of exposure to radiation (Costa *et al.*, 2004).

The cosmopolitan freshwater colonial cyanobacteria *Microcystis aeruginosa* (Chroococcales, Chroococcaceae) principally inhabits temperate and tropical latitudes but has been reported from some subtropical areas. Cyanobacterial blooms of toxic *Microcystis* species pose serious ecological problems for animal and human health, *M. aeruginosa* is known to cause bad odors in lakes and

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reservoirs and to produce hepatotoxicity in animals and humans (Fleming *et al.*, 2002). Metabolically, *M. aeruginosa* is unable to fix atmospheric nitrogen and thus needs assimilable forms of nitrogen such as NH_4^+ or NO_3^- for growth (Yunes and Leon, 2001; Falconer, 1999; Falconer and Humpage, 1996; Bell and Codd, 1994; Carmichael, 1994; Gorham and Carmichael, 1980).

Although the commercial cultivation of cyanobacteria is basically quite a simple industrial process the fact that the growth medium for these organisms is complex makes industrial scale production difficult and costly and has led to the search for new, lower cost, nutrient sources. Previous studies (Costa *et al.*, 2003, 2002) have demonstrated that water from Mangueira Lagoon (33°30′S; 53°08′W), situated in the southern Brazilian state of Rio Grande do Sul between Mirim Lagoon and the Atlantic Ocean, has favorable physico-chemical characteristics for the cultivation of *S. platensis* in that it has the alkaline pH and high carbonate and bicarbonate content necessary for the growth of this cyanobacterium.

One problem that may arise with the use of natural unprocessed lake water for the cultivation of *S. platensis* is that cultures could be subject to contamination with toxigenic *M. aeruginosa.* The aim of the work described in this paper was to evaluate the growth of *M. aeruginosa* in artificially-contaminated *S. platensis* cultures and to ascertain whether or not the growth of toxigenic algae could pose a problem for the cultivation of *S. platensis.*

Material and Methods

Microorganisms and culture medium

The cyanobacteria *Spirulina platensis* strain LEB-52 (Costa *et al.*, 2000) and *Microcystis aeruginosa* strain RST 9501 (Yunes and Leon, 2001) were used in this study. The toxic *Microcystis aeruginosa* strain RST 9501 was supplied by the "Unidade de Pesquisa em Cianobactéria" of Fundação Universidade Federal do Rio Grande. Its toxicity and toxin content have been reported by Yunes *et al.* (1996) and Matthiensen *et al.* (2000). For maintenance and inoculum preparation we used Zarrouk's medium (Zarrouk, 1966) for *S. platensis* and BGN/2 medium (Rippka *et al.*, 1979) for *M. aeruginosa.* The production runs were carried out using undiluted Zarrouk's medium (medium A), Mangueira Lagoon water supplemented with 20% (v/v) Zarrouk's medium (medium B) and Mangueira Lagoon water supplemented with 60% (v/v) Zarrouk's medium (medium C). Mangueira Lagoon is situated at 33°30′S; 53°08′W in the southern Brazilian state of Rio Grande do Sul between Mirim Lagoon and the Atlantic Ocean. All reagents were at least of analytical quality.

Cultivation

The cyanobacteria were cultivated in 0.25 L closed bioreactors using a 0.2 L working-volume and aerated at a rate of 20 L h^{-1} using diaphragm pumps. Light was provided using 20 W daylight-type fluorescent lamps (General Electric) at an illuminance of 1200 Lux and a 12 h:12 h light:dark photoperiod (Tanticharoen *et al.*, 1994). The bioreactors were maintained at a constant 30 °C in a growth chamber (Sarada *et al.*, 1999; Zhang *et al.*, 1999) and the total growth time was 623 h.

The bioreactor experiments were carried out, in duplicate, as separate runs according to a complete 2^3 factorial design where the initial biomass was 0 or 0.1 g L^{-1} for *S. platensis* and 0.01 or 0.03 g L^{-1} for *M. aeruginosa* (Table I). Two additional monoculture *S. platensis* standard runs were carried out with an initial biomass concentration of 0.1 g L^{-1}, one employing medium A and the other medium B, along with two mixed-species standard runs in medium C using 0.1 g L^{-1} initial *S. platensis* biomass concentration and a *M. aeruginosa* initial biomass concentration of 0.2 g L^{-1}.

Table I. Initial and final *S. platensis* and *M. aeruginosa* biomass values during growth for 623 h in undiluted Zarrouk's medium (medium A) and in Mangueira Lagoon water supplemented with 20% (v/v) Zarrouk's medium (medium B). Each run was duplicated and the final biomass values represent the arithmetic mean of the two runs.

Run	Initial biomass (g L^{-1})	
	S. platensis	*M. aeruginosa*
Medium A		
1	0.00	0.01
2	0.10	0.01
3	0.00	0.03
4	0.10	0.03
Medium B		
5	0.00	0.01
6	0.10	0.01
7	0.00	0.03
8	0.10	0.03

In pre-run trials samples were collected, aseptically, every 48 h and the total number of *S. platensis* filaments or *M. aeruginosa* cells calculated using a S50 Sedgewick-Rafter cell and phase-contrast microscopy at 100x magnification and the data used to construct calibration curves (data not shown) relating dry biomass weight to cell or filament numbers for *M. aeruginosa* or *S. platensis*, respectively. For each experimental run samples were collected every 48 h and the dry biomass values used to calculate the biomass doubling time (t_d) and maximum specific growth rate (μ_{max}) for *S. platensis* and the death time (D_t = time needed for the *M. aeruginosa* biomass to drop by 50%) and specific death rate (k) for *M. aeruginosa*, μ_{max} and k are calculated by exponential regression of the ascending and descending logarithmic sections of the growth curves. Death rate was calculated as $t_d = \ln 2/\mu_{max}$ and death time as $D_t = \ln 2/k$.

Results and Discussion

The t_d and μ_{max} values for *Spirulina platensis* and k and D_t for *Microcystis aeruginosa* are shown in Table II. The mean μ_{max} value for *S. platensis* was (0.245 ± 0.029) d^{-1} but in mixed cultures μ_{max} varied from (0.283 ± 0.030) d^{-1} in medium A inoculated with 0.03 g L^{-1} of *M. aeruginosa* to (0.214 ± 0.007) d^{-1} in medium B inoculated with 0.01 g L^{-1} of *M. aeruginosa*, while the lowest *S. platensis* t_d values occurred in runs 4 and 8 in which the initial *M. aeruginosa* biomass concentration was highest (0.03 g L^{-1}) (Table II).

The *S. platensis* monoculture standard runs with medium A gave μ_{max} = 0.153 d^{-1} and 0.173 d^{-1} for medium B, these values were lower than the lowest μ_{max} values occurring in the mixed cultures (Table II). It thus seems that the presence of *M. aeruginosa* did not reduce the *S. platensis* μ_{max} value but, on the contrary, in some cases increased it.

Alkaline medium with pH 8.5 to 11 is normally used to cultivate *Spirulina* species and this generally prevents the growth of algae, bacteria, fungi and yeasts, additional bacteriostatic or bactericidal protection is supplied by compounds such as sterols which represent up to 1.5% w/w of the non-polar lipid fraction of *Spirulina* species (Parada *et al.*, 1998).

The highest *M. aeruginosa* specific death rate was k = (0.555 ± 0.014) d^{-1} for run 2, *S. platensis* was present in run. Taken in conjunction with the results described above, the data suggests that not only *S. platensis* is not susceptible to contamination with *M. aeruginosa* but that *S. platensis* can inhibit the growth of *M. aeruginosa* during the first few hours of cultivation. The D_t value represents the time taken for the biomass concentration to drop by 50%, and it can be seen from Table II that there are large differences between the D_t values for *M. aeruginosa* grown in monoculture or in the presence of *S. platensis*, with the mean *M. aeruginosa* monoculture D_t value being (3.40 ± 0.24) d as compared to (2.04 ± 0.21) d when grown in mixed culture with *S. platensis.*

Our data supports the work of Goodman *et al.* (2000) who analyzed 62 products enriched with *S. platensis* but found no evidence of microcystin (the toxin produced by *Microcystis aeruginosa*), although this toxin has been found in products enriched with other cyanobacteria and/or microalgae.

The growth curves for the *S. platensis* and *M. aeruginosa* monocultures growing in medium B (Mangueira Lagoon water supplemented with 20% Zarrouk's medium) are shown in Fig. 1, from which it can be seen that the *M. aeruginosa* biomass remained constant during the first 100 h of

	S. platensis		*M. aeruginosa*	
Run	t_d [d]	μ_{max} [d^{-1}]	D_t [d]	k [d^{-1}]
1	–	–	3.25 ± 0.42	0.202 ± 0.045
2	3.22 ± 0.11	0.214 ± 0.007	1.31 ± 0.04	0.525 ± 0.017
3	–	–	3.62 ± 0.24	0.191 ± 0.012
4	2.79 ± 0.14	0.248 ± 0.012	3.03 ± 0.56	0.312 ± 0.015
5	–	–	4.32 ± 0.26	0.160 ± 0.009
6	2.94 ± 0.04	0.235 ± 0.002	2.61 ± 0.24	0.265 ± 0.024
7	–	–	2.30 ± 0.07	0.299 ± 0.010
8	2.46 ± 0.26	0.283 ± 0.030	1.24 ± 0.03	0.555 ± 0.014

Table II. Biomass doubling times (t_d) and maximum specific growth rates (μ_{max}) for *S. platensis* and death times (D_t) and specific death rates (k) for *M. aeruginosa* in a 2^3 factorial design experiment. Values shown are means ± standard deviation.

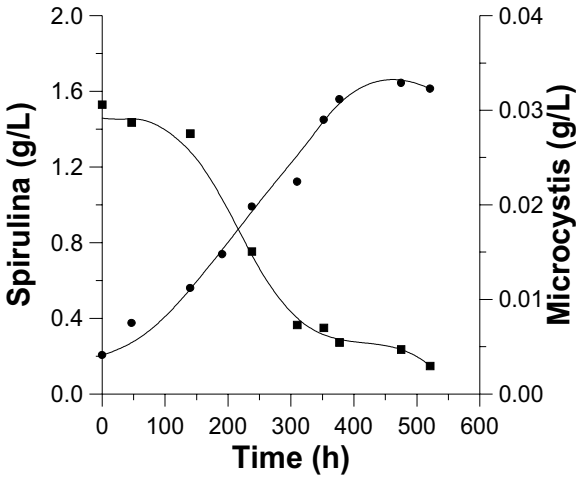

Fig. 1. Growth curves (biomass versus time) of pure cultures of *S. platensis* (●) and *M. aeruginosa* (■) grown separately in 20% Zarrouk's medium plus 80% Mangueira Lagoon water. Initial biomass was 0.1 g L^{-1} for *S. platensis* and 0.03 g L^{-1} for *M. aeruginosa.*

cultivation but by 300 h had declined to almost zero and remained at this level until the end of the experiment.

When growing together both *S. platensis* and *M. aeruginosa* showed similar tendencies in runs 2 and 4 in that the growth of *S. platensis* seemed to

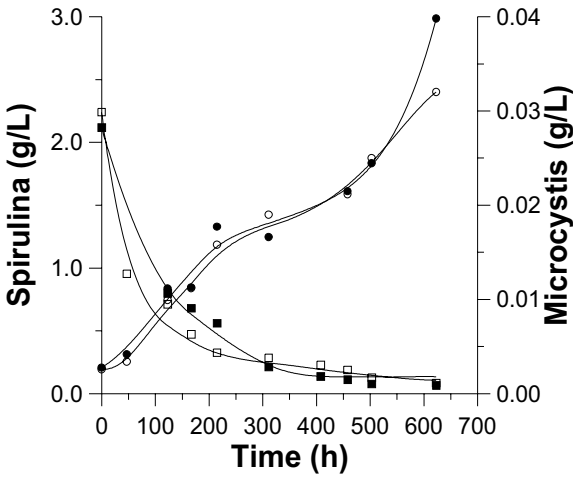

Fig. 2. Growth curves (biomass versus time) for *S. platensis* and *M. aeruginosa* growing simultaneously in 20% Zarrouk's medium plus 80% Mangueira Lagoon water. Results for run 2 with an initial biomass of 0.1 g L^{-1} for *S. platensis* (●) and 0.01 g L^{-1} for *M. aeruginosa* (■) and run 4 with an initial biomass of 0.1 g L^{-1} for *S. platensis* (○) and 0.03 g L^{-1} for *M. aeruginosa* (□).

Table III. Maximum specific growth rate (μ_{max}, d^{-1}) effects, errors and significance values (p) obtained in a complete 2^2 factorial analysis experiment with *S. platensis.*

Factor	Effect [d^{-1}]	Error	p
X_1	0.040	0.023	0.145
X_2	0.027	0.023	0.294
X_1*X_2	0.007	0.023	0.787

X_1, initial *M. aeruginosa* biomass concentration; X_2, culture medium.

be unaffected by the initial biomass concentration of *M. aeruginosa* (Fig. 2). The curves show that shortly after inoculation there was a sharp decrease in *M. aeruginosa* biomass which reached close to zero at 300 h and remained at this level until the end of the experiments, while for *S. platensis* a small lag phase lasting until about 40 h after inoculation followed by exponential growth. Comparing the behavior of *M. aeruginosa* in monoculture (Fig. 1) and mixed culture (Fig. 2) it can be seen that this toxigenic cyanobacterium shows a lag phase in monoculture but not when cultivated simultaneously with *S. platensis.*

The *S. platensis* μ_{max} values obtained in the runs involving this cyanobacteria were analyzed as a 2^2 factorial experiment, the results showing that neither the biomass concentration of *M. aeruginosa* nor the type of medium (*i.e.* medium A or B, medium C being excluded from this analysis) had any statistically significant ($p > 0.05$) effect on the *S. platensis* μ_{max} values (Table III).

Our results indicate that Mangueira Lagoon water supplemented with 20% Zarrouk's medium (medium B) has suitable physico-chemical characteristics (*e.g.* alkalinity, carbonate content and micronutrient level) for the growth of *S. platensis*, supporting the view (Costa *et al.*, 2003, 2002) that the addition of small quantities of carbon and nitrogen sources to Mangueira Lagoon water is enough to ensure that this water can be used for the cultivation of *S. platensis* and can thus significantly reduce production costs for this cyanobacterium.

The data in Table IV was produced using not only the results shown in Table II but also the data from the two standard central point values with *M. aeruginosa* (0.02 g L^{-1}) and *S. platensis* (0.1 g L^{-1}) in mixed-culture runs using medium C. This analysis shows that the presence of *S. platensis* had a significant effect ($p = 0.010$) in increasing the

Table IV. Specific death rate (k, d^{-1}) effects, errors and significance values (p) obtained in a complete 2^3 factorial analysis experiment with *M. aeruginosa* in simultaneous culture with *S. platensis.*

Factor	Effect [d^{-1}]	Error	p
X_1	0.153	0.048	0.010*
X_2	0.024	0.048	0.638
X_3	– 0.137	0.048	0.022*
X_1*X_2	0.007	0.048	0.881
X_1*X_3	0.080	0.048	0.136
X_2*X_3	– 0.121	0.048	0.036*

X_1, initial *S. platensis* biomass concentration; X_2, initial *M. aeruginosa* biomass concentration; X_3, culture medium.
* Statistically significant at $p \geq 0.05$.

specific death rate (k) of *M. aeruginosa.* The interaction among initial *M. aeruginosa* biomass concentration and medium composition had a significant effect (p = 0.036) in decreasing the specific death rate (k) of *M. aeruginosa* and the Mangueira Lagoon water supplemented with 20% (v/v) Zarrouk's medium had a significant effect (p = 0.022) in increasing the specific death rate (k) of *M. aeruginosa* (Table IV). Our results show that *S. platensis* accelerated the decline in *M. aeruginosa* biomass not only in medium B but also in medium C.

Conclusions

Neither cultures of *Spirulina platensis* were not susceptible to contamination by *Microcystis aeruginosa* nor did the initial concentration of this toxigenic microalgae have any significant negative effect on the maximum specific growth rate (μ_{max}) of *S. platensis*, although it is possible that the μ_{max} value for *S. platensis* increased in mixed cultures with *M. aeruginosa.* The presence of *S. platensis*, type of culture medium and the interaction between the initial biomass concentration of *M. aeruginosa* and culture medium all had a significant influence in the specific death rate (k) of *M. aeruginosa.* There was no significant difference between the different culture media [undiluted Zarrouk's medium or Mangueira Lagoon water supplemented with 60% or 20% (v/v) of Zarrouk's medium], indicating that undiluted Zarrouk's medium could be replaced by Mangueira Lagoon water supplemented with 20% (v/v) of Zarrouk's medium thereby considerably reducing *S. platensis* production costs.

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Bell S. G. and Codd G. A. (1994), Cyanobacterial toxins and human health. Med. Microbial. **5**, 256–264.
Carmichael W. W. (1994), The toxins of cyanobacteria. Sci. Am. **270**, 64–70.
Costa J. A. V., Linde G. A., Atala D. I. P., Mibieli G. M., and Krüger R. T. (2000), Modelling of growth conditions for cyanobacterium *Spirulina platensis* in microcosms. World J. Microb. Biot. **16**, 15–18.
Costa J. A. V., Colla L. M., Duarte P., Kabke K., and Weber A. (2002), Modelling of *Spirulina platensis* growth in fresh water using response surface methodology. World J. Microb. Biot. **7**, 603–607.
Costa J. A. V., Colla L. M., and Duarte P. (2003), *Spirulina platensis* growth in open raceway ponds using fresh water supplemented with carbon, nitrogen and metal ion. Z. Naturforsch. **58c**, 76–80.
Costa J. A. V., Colla L. M., and Filho P. F. D. (2004), Improving *Spirulina platensis* biomass yield using a fed-batch process. Bioresource Technol. **92**, 237–241.
Falconer I. R. (1999), An overview of problems caused by toxic blue-green algae (cyanobacteria) in drinking and recreation water. Environ. Toxicol. **14**, 5–12.
Falconer I. R. and Humpage A. R. (1996), Potential impact on human health of toxic cyanobacteria. Phycologia **35**, 6–11.
Fleming L. E., Rivero C., Burns J., Williams C., Bean J., Shea K. A., and Stinn J. (2002), Blue green algal (cyanobacterial) toxins, surface drinking water, and liver cancer in Florida. Harmful Algae **1**, 157–168.
Goodman T. K., Lawrence J. F., and Morrisey M. (2000), Risk Assessment of microcystins in blue-green algal health food products. Mycotoxins and phycotoxins in perspective at the turn of the millennium. Proceedings of the Xth International IUPAC symposium on Mycotoxins and Phycotoxins. Guarujá (Brazil), pp. 549–556.
Gorham P. R. and Carmichael W. W. (1980), Hazards of freshwater blue-green (cyanobacteria). In: Algae and Human Affairs (Lembi C. A., ed.). Cambridge University Press, Cambridge, England.
Matthiensen A., Beattie K. A., Yunes J. S., Kaya K., and Cood G. A. (2000), [D-leu] microcystin-LR, from the cyanobacterium *Microcystis* RST 9501 and from a *Microcystis* bloom in the Patos Lagoon Estuary, Brazil. Phytochemistry **55**, 383–387.

Parada J. I., Caire G. Z., Mulé M. C. Z., and Cano M. M. S. (1998), Lactic acid bacteria growth promoters from *Spirulina platensis.* Int. J. Food Microbiol. **45**, 225–228.

Richmond A. (1990), Handbook of Microalgal Mass Culture. CRC Press, Boca Raton, USA.

Rippka J. E., Derruelles J., Waterbury J. B., Herdeman M., and Satiner R. Y. (1979), Generic assignments, stain histories, and properties of pure culture of cyanobacterial. J. Gen. Appl. Microbiol. **111**, 1–6.

Sarada R., Pillai G., and Ravishankar G. A. (1999), Phycocyanin from *Spirulina* sp.: influence of processing of biomass on phycocyanin yield, analysis of efficacy of extraction methods and stability studies on phycocyanin. Process Biochem. **34**, 795–801.

Tanticharoen M., Reungjitchachawali M., Boonag B., Vonktaveesuk P., Vonshak A., and Cohen Z. (1994), Optimization of γ-linolenic acid (GLA) production in *Spirulina platensis.* J. Appl. Phycol. **6**, 295–300.

Vonshak A. (1997), *Spirulina platensis* (Arthrospira): Physiology, Cell-biology and Biotechnology. Taylor & Francis, London, UK.

Vonshak A., Boussiba S., Abeliovich A., and Richmond A. (1983), Production of *Spirulina* biomass: maintenance of monoalgal culture outdoors. Biotechnol. Bioeng. **25**, 341–349.

Vymazal J. (1995), Algae and Element Cycling in Wetlands. Ed. Lewis Publishers, Boca Raton, Florida, USA.

Yunes J. S. and Leon L. (2001), First report of a microcystin-containing bloom of the cyanobacterium *Microcystis aeruginosa* in the La Plata River, South America. Environ. Toxicol. **16**, 110–112.

Yunes J. S., Salomon P. S., Matthiensen A., Beattie K. A., Ragget S. L., and Codd G. A. (1996), Toxic blooms of cyanobacteria in the Patos Lagoon Estuary, southern Brazil. J. Aquatic. Ecosyst. Health. **5**, 223–229.

Zarrouk C. (1966), Contribution à l'étude d'une cyanophycée. Influence de divers facteurs physiques et chimiques sur la croissance et la photosynthèse de *Spirulina maxima*. Ph. D. Thesis, Université de Paris.

Zhang X. W., Zhang Y. M., and Chen F. (1999), Application of mathematical models to the determination optimal glucose concentration and light intensity for mixotrophic culture of *Spirulina platensis.* Process Biochem. **34**, 477–481.