Spirulina platensis Growth in Open Raceway Ponds Using Fresh Water Supplemented with Carbon, Nitrogen and Metal Ions

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To investigate the feasibility of using fresh water from Mangueira Lagoon (Rio Grande do Sul, Brazil) for biomass production in open raceway ponds (0.7 m long, 0.18 m wide, 0.075 m deep) we studied the influence of nutrient addition (carbon as sodium bicarbonate, nitrogen as urea, phosphate, sulfate, ferric iron, magnesium and potassium) on the growth rate of the cyanobacteria *Spirulina platensis* using a 2^2 factorial design. In unsupplemented lagoon water production of *S. platensis* was 0.78 ± 0.01 g/l (dry weight basis) while the addition of 2.88 g/l of sodium bicarbonate (without added urea, phosphate, sulfate or metal ions) resulted in 0.82 ± 0.01 g/l after 400 hours of culture. The further addition of phosphate and metal ions resulted in growth for up to 750 h and a final *S. platensis* biomass of 1.23 ± 0.04 to 1.34 ± 0.03 g/l.

Key words: Cyanobacteria, *Spirulina platensis*, Urea

Introduction

Biotechnological processes based on cyanobacteria have been receiving increasing interest due to their potential to produce a diverse range of chemicals and biologically active compounds, such as vitamins, carotenoid pigments, proteins, lipids and polysaccharides (Zhang *et al.*, 1999). For economic reasons, the culture system predominating in the large-scale commercial production of these types of organisms is the open-air system, closed systems being very expensive and often difficult to scale up (Borowitzka, 1999).

The cyanobacterium *Spirulina platensis* has been commercially exploited for the production of human food supplements, animal feed and pharmaceuticals because of its ability to produce large quantities of valuable products, such as phycocyanin (Estrada *et al.*, 2001; Miranda *et al.*, 1998; Sarada *et al.*, 1998) and ω-3 and ω-6 polyunsaturated fatty acids (Alonso and Maroto, 2000; Deshnium *et al.*, 2000; Marquez *et al.*, 1995; Quoc *et al.*, 1994; Cohen *et al.*, 1993).

Spirulina platensis is a multicellular, filamentous cyanobacterium, consisting of blue-green filaments of cylindrical cells (1 to 12 μm diameter) in unbranched helicoidal trichomes, the filaments being motile, gliding along their axis, and have no heterocysts (Richmond, 1990). *Spirulina* can colonize environments that are unsuitable for many other organisms, forming populations in freshwater and brackish lakes and some marine environments, mainly alkaline saline lakes (Belov and Giles, 1997; Richmond, 1990).

The large-scale production of *Spirulina* biomass depends on many factors, the most important of which are nutrient availability, temperature and light. These factors can influence the growth of *Spirulina* and the composition of the biomass produced by causing changes in metabolism, which considerably modify the time course of the accumulation of the main biomass components (Cornet *et al.*, 1992). Carbon is the principal nutrient required by *Spirulina*, and in alkaline lakes this organism is the dominant species because of the presence of high concentrations of sodium carbonate. Vonshak (1997) has shown that, after labor, the second major cost in *Spirulina* biomass production is the cost of nutrients, principally the carbon source.

Mangueira Lagoon is 92 km long by 7.6 km wide with a minimum depth of 1.2 m and a maximum depth of 7.4 m. It is located in the southern of Brazilian state of Rio Grande do Sul, between the At-

lantic Ocean and Mirim Lagoon and represents an important hydrobiology resource that is used principally as a source of water for paddy-rice. The pH 8.03 (Costa *et al.*, 2002) and other physicochemical characteristics of Mangueira Lagoon are ideal for the culture of *Spirulina* species.

The aim of this work was to study the growth of *Spirulina platensis* in small open raceway ponds using fresh water from Mangueira Lagoon and to investigate the effect of adding of nutrients to the water in an attempt to prevent nutrient depletion and increase the biomass production of *Spirulina platensis.*

Materials and Methods

Microorganism and culture medium

The cyanobacterium used in this study was *Spirulina platensis* strain LEB-52 (Costa *et al.*, 2001). Zarrouk's medium (Zarrouk, 1966; Vonshak, 1982) being used to prepare and maintain the cultures. Water from Mangueira Lagoon was supplemented with nutrients, initial experiments being made with unsupplemented lagoon water and lagoon water supplemented with 2.88 g/l of sodium bicarbonate or 0.35 g/l urea or both. Further experiments were made using lagoon water (with and without added bicarbonate or urea) supplemented with various combinations of the nutrients contained in Zarrouk's medium (Table I).

Table I. Nutrient supplementation of lagoon water for experiments 1 to 8.

Nutrient	Supplementation
KH_2PO_4	0.5 g/l for experiments 5, 7
K_2SO_4	1.0 g/l for experiments 5, 7
$MgSO_4.7H_2O$	0.2 g/l for experiments 6, 8
$FeSO_4.7H_2O$	0.1 g/l for experiments 6, 8
$NaHCO_3$	2.88 g/l for experiments 2, 4, 7, 8
NH_2CONH_2 (urea)	0.35 g/l for experiments 3, 4, 7, 8
No addition	Experiment 1

Culture conditions

Experiments were carried out in a greenhouse in PVC open raceways (0.7 m long, 0.18 m wide, 0.075 m deep) containing 5 liters of *S. platensis* culture with an initial biomass concentration of 0.1 g/l (Costa *et al.*, 2000). The cultures were mixed using paddle wheels turning at 18 revs min^{-1} and illuminated with daylight-type fluorescent lights (Osram, 40 W, Brazil) at an intensity of 1,900 lux and a 12 h light/dark photoperiod at 30 °C (Sarada *et al.*, 1999; Zhang *et al.*, 1999).

Chemical and microbiological analysis

Samples were taken aseptically every 24 h, pH being measured potentiometrically and biomass determined by optical density at 670 nm using a VARIAN model CARY 100 spectrophotometer. Carbonate and bicarbonate concentrations were assessed weekly according to standard methods (AOAC, 1995). Each experiment was checked for bacterial contamination after 360 hours of cultivation using the total pour-plate method and plate count agar (ABNT, 1991), the results being reported as colony forming units per ml (CFU/ml).

Experimental design and statistical analysis

The study was separated into two stages, each containing four experiments. In the first stage we used a 2^2 factorial design to study the effects of the addition of sodium bicarbonate and urea to lagoon water on the growth rate and biomass concentration of *Spirulina platensis*, the resultant data being subjected to analysis of variance. In the second stage, the influence of adding various combinations of the components of Zarrouk's medium (Table I) was studied, this data being analyzed by the method described by Zar (1984). This technique detecting differences between regression lines using analysis of covariance through the statistical test of equation 1, where SSc = sum of common residual squares, SSp = sum of pooled residual squares and DFp = degrees of freedom of the pooled residual. When the hypothesis that all slopes were equal was rejected a multiple comparison test (Tukey test, 95% confidence interval) was used to determine which of the K population slopes differed from the others and thus detect differences between each pair of slopes.

$$F = \frac{\dfrac{[SSc - SSp]}{[K-1]}}{\dfrac{SSP}{DFp}} \qquad (1)$$

Results and Discussion

Microbiological analysis

The results of microbiological analysis showed that the levels of bacterial contamination in all experiments ($< 1.6 \times 10^4$ CFU/ml) were smaller than that previously described for dried *Spirulina.* It should also be remembered that these counts were taken before drying of the *Spirulina* culture and that the drying process would probably decreases the level of contamination. In large-scale culture, especially in open ponds, there is a greater susceptibility to bacterial contamination. Vonshak (1997) reported that the standard plate count limits for dried *Spirulina* are $< 0.1 \times 10^6$ CFU/g in France, $< 10 \times 10^6$ CFU/g in Sweden, $< 0.05 \times 10^6$ CFU/g in Japan and $< 1 \times 10^6$ CFU/g for *Spirulina* produced by Earthrise Farms (California, EUA) a large producer of products based on this organism.

Growth curves and statistical analysis

The water from Mangueira Lagoon already contained levels of carbon (0.126 HCO_3^{-2} g/l) and nitrogen (0.132 mg/l NH_4^+, 0.0195 mg/l NO_2^-, 1.040 mg/l NO_3^- – Costa *et al.*, 2002). We chose urea (CH_4N_2O) as supplementary nitrogen source due to the fact that it is cheaper than other nitrogen sources (e. g. potassium nitrate, sodium nitrate or ammonium chloride) and contains two nitrogen atoms (46% nitrogen) whereas nitrates have only one (14–16% nitrogen) (Faintuch *et al.*, 1992). Urea is a good nitrogen source for *Spirulina*, producing no ill effects at pH 8.4 as long as its concentration is kept below 1.5 g/l (Richmond, 1990). The maximum levels of urea and sodium bicarbonate were based on a previous study (in Erlenmeyer flasks) on the growth of *Spirulina platensis* using water from Mangueira Lagoon (Costa *et al.*, 2002).

Table II shows the results of maximum biomass concentration (X_{max}), maximum specific growth rate (μ_{max}) and doubling time (t_d), being the specific growth rate and doubling time calculated during the exponential growth phase.

In Table II, experiment 1 represents the control with no added nutrients while experiment 3 used lagoon water supplemented with 0.35 g/l urea. It can be seen that the these experiments presented similar values of maximum biomass concentration (0.78 g/l) for X_{max}, although for experiment 1 μ_{max} is 0.1394/day while for experiment 3 it is 0.1573/day (Table II), which is significantly different at $p = 0.0002$ and it appears that the addition of urea at this concentration was beneficial in promoting biomass production by *S. platensis.* This agrees with the results of Richmond (1990), who reported that *Spirulina* produced 0.32 g/l of biomass with 0.06 g/l of urea, 1.16 g/l with 0.3 g/l of urea and 0.51 g/l with 1.2 g/l of urea.

In experiment 2 only sodium bicarbonate was added, but the biomass production (X_{max}) was higher than in the other experiments, being 0.82 g/l compared with 0.78 and 0.79 g/l up to 400 h of culture. The maximum specific growth rate of experiment 2 (0.1357/day) was significantly ($p = 0.0002$) smaller than the μ_{max} of experiment 1 (0.1394/day). According to Vonshak *et al.* (1982), higher specific growth rates are to be expected in systems that are primarily light-limited. However, the relative effect of decreasing the population density, and thus increasing the availability of light to each cell, is more significant at higher temperatures, although this didn't occur in our experiments, be-

Table II. Growth parameters *Spirulina platensis* in lagoon water supplemented and unsupplemented with sodium bicarbonate ($NaHCO_3$) and urea.

Run	$NaHCO_3$ (g/l)	Urea (g/l)	X_{max}[a]	μ_{max}[b]	t_d[c]	Δt[d]	r^2[e]
1	0.0	0.0	0.78 ±0.01	0.1394 ± 0.0001	4.97	48–360	0.967
2	2.88	0.0	0.82 ±0.01	0.1357 ± 0.0001	5.11	48–408	0.965
3	0.0	0.35	0.78 ±0.01	0.1573 ± 0.0002	4.41	96–360	0.990
4	2.88	0.35	0.79 ±0.01	0.0735 ± 0.0001	9.43	144–384	0.958

[a] Maximum concentration of *Spirulina platensis* (mean ± standard deviation, g/l, dry weight basis); [b] Maximum specific growth rate (mean ± standard deviation , 1/day); [c] doubling time (1/day); [d] Length of exponential growth phase (hours); [e] correlation coefficient.

cause the temperature remained constant at 30 °C. When both sodium bicarbonate and urea were added (experiment 4) the maximum specific growth rate was lowest at 0.0735/day. Highest biomass production (X_{max}) was obtained without addition of urea but with 2.88 g/l of sodium bicarbonate, whereas the best μ_{max} value was obtained with no addition of sodium bicarbonate but 0.35 g/l of urea. When sodium bicarbonate was not added but the concentration of urea increased from 0 to 0.35 g/l there was little change in X_{max} and μ_{max} but the addition of 2.88 g/l of sodium bicarbonate and an increase in urea concentration from 0 to 0.35 g/l resulted in a decrease in X_{max} and $\mu_{max.}$ The high values obtained with the addition of sodium bicarbonate without urea may be the result of an increase in pH (alkalinity) due to the formation of a $CO_2/H_2CO_3/HCO_3^-/CO_3^{-2}$ system, which is a very useful buffer system for maintained the alkaline pH which is important for the optimum growth of *Spirulina* and which helps to prevent carbon depletion (Vonshak, 1997; Richmond, 1990).

Figure 1 shows the results for experiments 5 to 8 in which lagoon water was supplemented with sulfate, phosphate, ferric iron, magnesium and potassium as well as carbon and nitrogen. The growth curves obtained presented the same behavior, and showed no lag phase. The ZAR method showed that the best biomass values occurred in experiment 8 when both bicarbonate and urea were added, the largest slope value being 0.155 ($p < 0.05$). The second best result occurring in experiment 7, where the slope value was 0.144 ($p < 0.05$). These results show that the addition of other nutrients in addition to sodium bicarbonate results in *S. platensis* growing for 750 hours with biomass concentrations reaching from 1.23 to 1.33 g/l, indicating the need for additional of sources of potassium, sulfate, phosphate and metal ions.

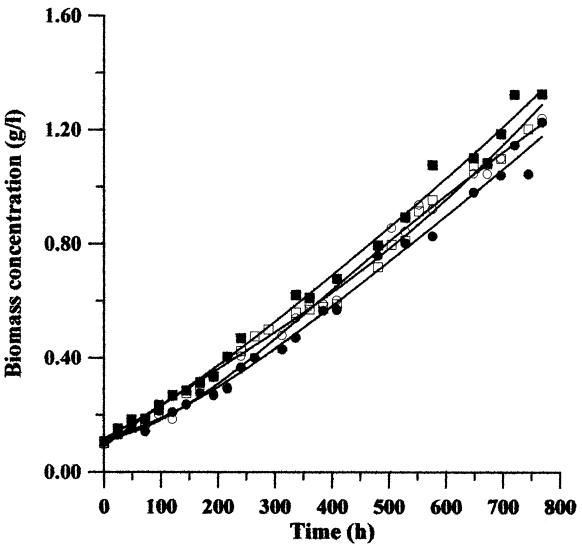

Fig. 1. Growth (g/l) of *Spirulina platensis* in unsupplemented lagoon water and lagoon water supplemented with sodium bicarbonate, urea, phosphate and metal ions. Supplement key: ● = 0.5 g/l potassium dihydrogen phosphate and 1.0 g/l potassium sulfate (experiment 5), ○ = 0.2 g/l magnesium sulfate and 0.01 g/l ferric sulfate (experiment 6), ■ = 0.5 g/l potassium dihydrogen phosphate, 1.0 g/l potassium sulfate, 2.88 g/l $NaHCO_3$ and 0.35 g/l urea (experiment 7), □ = 0.2 g/l magnesium sulfate, 0.01 g/l ferric sulfate, 2.88 g/l $NaHCO_3$ and 0.35 g/l urea (experiment 8). Maximum biomass concentrations: experiment 5 (1.23 ± 0.04 g/l), experiment 6 (1.24 ± 0.03 g/l), experiment 7 (1.33 ± 0.03 g/l) and experiment 8 (1.31 ± 0.06 g/l).

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