Production of biomass and nutraceutical compounds by *Spirulina platensis* under different temperature and nitrogen regimes

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Abstract

The cyanobacterium *Spirulina platensis* has been used by humans because of its nutritional and possibly medicinal effects. Our study evaluated the influence of temperature and nitrogen concentration in the medium on the production of biomass by this cyanobacterium and the biomass composition in protein, lipid and phenolic compounds. We found that at 35 °C there was a negative effect on biomass production but a positive effect on the production of protein, lipids and phenolics, the highest levels of these compounds being obtained in Zarrouk’s medium containing 1.875 or 2.500 g l⁻¹ sodium nitrate. Higher biomass densities and productivity were obtained at 30 °C than at 35 °C, but nitrogen concentration appeared to have no effect on the amount of protein, lipid or phenolics, indicating that at 30 °C the concentration of sodium nitrate in Zarrouk’s medium (2.50 g l⁻¹) can be reduced without loss of productivity, an important cost-saving factor in large-scale cultivation.

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*Keywords:* Nitrogen; Nutraceuticals; *Spirulina platensis*; Temperature

1. Introduction

*Spirulina platensis* is a planktonic photosynthetic filamentous cyanobacterium that forms massive populations in tropical and subtropical bodies of water which have high levels of carbonate and bicarbonate and alkaline pH values of up to 11. This cyanobacterium is recognizable by the main morphological feature of the genus, i.e. the arrangement of multicellular cylindrical trichomes in an open left-hand helix along the entire length of the filaments (Vonshak, 1997).

For centuries, native peoples have harvested *S. platensis* from Chad Lake in Africa and Texcoco Lake in Mexico for use as a source of food (Vonshak, 1997), a fact which means that *Spirulina* deserves special attention both as a source of single cell protein (SPC) (Anupama, 2000) and because of its nutraceutical properties. The chemical composition of *Spirulina* indicates that it has high nutritional value due to its content of a wide range of essential nutrients, such as provitamins, minerals, proteins and polyunsaturated fatty acids such as gamma-linolenic acid (Miranda et al., 1998). More recently, *Spirulina* has been studied because of its therapeutic properties (Belay et al., 1993) and the presence of antioxidant compounds (Estrada et al., 2001; Miranda et al., 1998) such as phenolics. The occurrence of phenolic compounds in plants is well documented and these compounds are known to possess antioxidant activity in biological systems but the antioxidant characteristics of algae and cyanobacteria are less well documented, although decreased cholesterol levels have been reported in hypercholesterolemic patients fed *Spirulina* (Ramamoorthy and Premakumari, 1996) and the antioxidant activity of phycobiliproteins extracted from *S. platensis* has also been demonstrated (Estrada et al., 2001).
The influence of growth conditions on the chemical composition of *Spirulina* has been studied by many researchers with the purpose of optimizing the production of economically and nutritionally interesting compounds, especially gamma-linolenic acid (GLA) and phycocyanin (*Tanticharoen et al.,* 1994; *Cohen et al.,* 1993). Although the use of *Spirulina* for the production of phycocyanin for use as a natural pigment seems commercially efficient, the production of GLA from this cyanobacterium presents higher costs when compared to production using other sources (*Cohen et al.,* 1993). The extraction of nutritionally active compounds in pure form is expensive, but the direct consumption of *Spirulina* as a nutritious food is a viable alternative. Growth conditions optimized for biomass production and productivity are usually used in the commercial production of *Spirulina* without considering chemical composition, but higher concentrations of potentially useful compounds such as polyunsaturated fatty acids, proteins and phenolics can be obtained by manipulating growth conditions.

The objective of the work presented in this paper was to evaluate the influence of nitrogen concentration and temperature on the protein, lipid and phenolic compound content, maximum specific growth rate and productivity of *S. platensis*.

### 2. Methods

#### 2.1. Microorganism and culture medium

The cyanobacterium *S. platensis* strain LEB-52 (*Costa et al.,* 2002, 2003) was used in this study. To prepare and maintain the inoculum we used Zarrouk’s medium, a standard synthetic medium containing 2.50 g l\(^{-1}\) sodium nitrate as nitrogen source (*Zarrouk, 1966*), this medium also being used to study the batch growth of *S. platensis* but in this case the concentration of sodium nitrate was modified as explained below in section *Experimental design*. All the reagents used were of analytical grade, obtained from the Merck Chemical Co. (Darmstadt, Germany) or the Synth Chemical Co. (São Paulo, Brazil).

### 2.2. Cultivation

*S. platensis* was cultivated in 20 l photo-bioreactors with an initial volume of 141 l and an initial biomass concentration of 0.15 g l\(^{-1}\). Two glass tubes were passed through the photo-bioreactor’s stopper, one for sample collection and the other for continuous aeration. The cultures were mixed and aerated using filtered air at a flux of 1701 h\(^{-1}\) supplied by diaphragm pumps. The cultures were illuminated with daylight-type 40 W fluorescent lights (Osram, Brazil) which provided an irradiance of 31.35 \(\mu\text{mol photons m}^{-2}\text{s}^{-1}\) (*Costa et al.,* 2000). Cultures were maintained in a greenhouse under a 12 h light/12 h dark photoperiod at 30 °C and 35 °C.

To monitor biomass changes in cultures, samples were taken every 24 h using aseptic technique. At the end of each batch run, replicate cultures of each experiment were pooled, filtered, washed with distilled water to remove soluble salts, centrifuged at 15,000 rpm, lyophilized and stored at −20 °C for protein, lipid and phenolic compound determinations.

### 2.3. Experimental design

For this study we used a multilevel factorial design (MFD) in which the concentration of sodium nitrate in Zarrouk’s medium was set at four different levels (0.625, 1.250, 1.875 and 2.500 g l\(^{-1}\)) and the temperature at 30 and 35 °C (Table 1), all experiments being triplicated. Analysis of variance (ANOVA) was used to compare the data and calculate *p*-values.

#### 2.4. Analytical methods

Biomass concentration (g l\(^{-1}\)) was calculated by measuring optical density at 670 nm to produce a standard curve relating dry weight of *S. platensis* biomass (g l\(^{-1}\)) to optical density, this standard curve was subsequently used to calculate the biomass of individual samples based on their optical density. The calculated biomass (the average of three experiments) were used to construct growth curves from which we obtained maximum specific growth rates (\(\mu_{\text{max}}\)).

### Table 1

Cultivation of *Spirulina platensis* in media with different sodium nitrate concentrations and at different temperatures

<table>
<thead>
<tr>
<th>Run</th>
<th>NaNO(_3) (g l(^{-1}))</th>
<th>(\mu_{\text{max}}) (day(^{-1}))</th>
<th>(\Delta t) (h)</th>
<th>(P_{450}) (mg l(^{-1}) day(^{-1}))</th>
<th>Protein (%)</th>
<th>Lipid (%)</th>
<th>Phenolics (mg g(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(T = 30 ^\circ \text{C})</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.625</td>
<td>0.073 ± 0.002</td>
<td>24–468</td>
<td>34.0 ± 0.1</td>
<td>59.76 ± 2.07</td>
<td>6.73 ± 0.40</td>
<td>3.09 ± 0.26</td>
</tr>
<tr>
<td>2</td>
<td>1.250</td>
<td>0.073 ± 0.002</td>
<td>24–468</td>
<td>30.0 ± 1.0</td>
<td>57.36 ± 1.13</td>
<td>6.69 ± 0.27</td>
<td>3.66 ± 0.27</td>
</tr>
<tr>
<td>3</td>
<td>1.875</td>
<td>0.073 ± 0.001</td>
<td>48–612</td>
<td>34.0 ± 3.7</td>
<td>60.82 ± 1.88</td>
<td>7.61 ± 0.43</td>
<td>3.27 ± 0.39</td>
</tr>
<tr>
<td>4</td>
<td>2.500</td>
<td>0.074 ± 0.005</td>
<td>24–468</td>
<td>30.2 ± 0.7</td>
<td>57.61 ± 1.16</td>
<td>8.16 ± 0.23</td>
<td>3.78 ± 0.30</td>
</tr>
<tr>
<td>(T = 35 ^\circ \text{C})</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.625</td>
<td>0.048 ± 0.001</td>
<td>96–588</td>
<td>23.9 ± 3.9</td>
<td>58.92 ± 0.96</td>
<td>7.49 ± 1.10</td>
<td>2.46 ± 0.22</td>
</tr>
<tr>
<td>6</td>
<td>1.250</td>
<td>0.050 ± 0.006</td>
<td>72–588</td>
<td>24.8 ± 4.0</td>
<td>56.73 ± 0.79</td>
<td>7.95 ± 1.42</td>
<td>2.42 ± 0.21</td>
</tr>
<tr>
<td>7</td>
<td>1.875</td>
<td>0.054 ± 0.002</td>
<td>24–564</td>
<td>26.4 ± 3.9</td>
<td>70.15 ± 0.82</td>
<td>10.37 ± 0.63</td>
<td>4.99 ± 0.37</td>
</tr>
<tr>
<td>8</td>
<td>2.500</td>
<td>0.054 ± 0.003</td>
<td>24–468</td>
<td>24.8 ± 4.0</td>
<td>65.47 ± 2.19</td>
<td>10.03 ± 0.63</td>
<td>4.92 ± 0.29</td>
</tr>
</tbody>
</table>

Except for \(\Delta t\), all values show means ± standard deviation. 

\(\mu_{\text{max}}\) = maximum specific growth rate; \(\Delta t\) = start–end of the exponential growth phase; \(P_{450}\) = productivity at 450 h.
from the log phase of the growth curves by exponential regression. Productivities were calculated from the equation \( P = (X_i - X_0) / t_i \), where \( P \) = productivity (mg l\(^{-1}\) day\(^{-1}\)), \( X_0 \) = initial biomass density (mg l\(^{-1}\)), \( X_i \) = biomass density at time \( i \) (mg l\(^{-1}\)) and \( t_i \) = time interval (h) between \( X_0 \) and \( X_i \).

Protein was determined by the micro-Kjeldahl method according to AOAC standard methods (AOAC, 1995) in which protein is assumed to contain 16% nitrogen. Four replicates were analyzed for each lyophilized biomass sample.

Lipid content was evaluated using Folch’s method (Folch and Lees, 1957) by extracting lipids in a 2:1 chloroform/methanol mixture and determining lipid content gravimetrically. Three replicates were used for each lyophilized biomass sample.

Phenolic compounds were determined by extraction with methanol, followed by partitioning with hexane and precipitation of non-phenolics with Ba(OH)\(_2\) and ZnSO\(_4\). Total phenolics were determined spectrophotometrically by the Folin–Ciocalteau method using tyrosine as standard (Singleton and Rossi, 1965). Five replicates were used for each lyophilized biomass sample.

3. Results and discussion

The growth curves for 30°C are shown in Fig. 1a while Fig. 1b shows the curves for 35°C. Because the S. platensis cells had previously been adapted to the medium there was no lag phase. The biomass concentrations achieved in the 30°C runs (0.82–0.92 g l\(^{-1}\)) were higher than in the 35°C runs (0.59–0.65 g l\(^{-1}\)). It has been shown by previous workers (Danesi et al., 2001; Vonshak, 1997) that the optimal growth temperature for S. platensis is between 30 and 35°C, with 40°C definitely being deleterious to this cyanobacterium. In respect to increase in biomass, the best responses were obtained at 30°C, which agrees with the studies by Danesi et al. (2001).

The fact that the highest biomass values occurred at 30°C may have been due to the fact that the partial pressure of CO\(_2\) in the medium is higher at 30°C than at 35°C, leading to a higher concentration of bicarbonate and consequently an increased rate of photosynthesis. Another factor that should be considered is that at higher temperatures (i.e. 35°C) there is an increase in dark cycle respiratory activity in which the cells use reserve material (e.g. carbohydrates) for respiration and a concomitant decrease in cell weight (Vonshak et al., 1982). According to Torzillo and Bernardini (1991), for outdoor cultures of Spirulina up to 34% of the biomass produced during the daylight period may be lost through respiration at night.

Values for maximum specific growth rate (\( \mu_{\text{max}} \)), duration of the exponential phase (\( \Delta t \)), productivity at 450 h (\( P_{450} \)), and protein, lipid and phenolic compound content are shown in Table 1.

In general the biomass and \( \mu_{\text{max}} \) values were higher at 30°C (0.073–0.074 day\(^{-1}\)) than they were at 35°C (0.048–0.054 day\(^{-1}\)). In Spirulina, the lower the population density, the higher the specific growth rate. This is to be expected for a system that is primarily light-limited because reducing the population density increases the availability of light to each cell. However, the effect of decreasing the population density in a light-limited system is most pronounced at high temperatures and much less so at low temperatures (Vonshak et al., 1982). This explains why the highest \( \mu_{\text{max}} \) values were observed at 30°C and not at 35°C. The \( \mu_{\text{max}} \) values at 30°C appear not to have been affected by sodium nitrate concentration. The highest productivity (\( P_{450} = 30–34\) mg l\(^{-1}\) day\(^{-1}\)) was obtained at 30°C, and it thus appears that the concentration of sodium nitrate in Zarrouk’s medium (2.500 g l\(^{-1}\)) could be reduced to 0.625 g l\(^{-1}\) without loss of productivity, decrease production costs in large-scale cultivation.

The \( p \)-values (Table 2) show that the concentration of sodium nitrate in Zarrouk’s medium had a significant effect on the production of protein, lipid and phenolics, while cultivation temperature had a significant effect on all variables. There were significant interactions between protein, lipid and phenolics contents at the 99% confidence interval,
indicating that the interaction between these variables should be investigated in more detail.

The lower sodium nitrate concentrations (0.625 and 1.250 g l\(^{-1}\)) gave lower values for cellular proteins and lipids (Fig. 2a and b). Nitrogen is required for synthesis of the amino acids, which make up proteins and other cellular components such as phycocyanin. However, at 30 °C, nitrogen uptake seems to be limited because the experiments with higher concentrations of sodium nitrate (1.875 and 2.500 g l\(^{-1}\)) showed no increase in the level of protein, while at 35 °C an increase was observed (Runs 7 and 8 in Table 1). With regard to lipids, higher concentrations of sodium nitrate resulted in an increase in lipids, similar to that which was obtained by Manabe et al. (1992), who demonstrated higher total lipid in *Spirulina* grown in media containing up to 25 mM of ammonium chloride. However, Piorreck et al. (1984) found that the concentration of nitrogen had little influence on the total lipid and fatty acid composition of some cyanobacteria, the influence of nitrogen being more marked in eukaryotic algae. Piorreck et al. (1984) also found that for cyanobacteria, total lipid content remained constant at all nitrogen concentrations studied (0.001–0.1% of potassium nitrate), with only a slight increase occurring at the highest nitrogen concentration tested (0.1%). Olguín et al. (2001) observed a higher content of total lipids in *Spirulina* growing in Zarrouk’s medium as compared to *Spirulina* cultivated under conditions of nitrogen starvation. In our experiments, higher protein and lipid content may have been associated with nitrogen uptake because Runs 7 and 8 presented lower biomass densities which would have meant that more light was available to individual cells, light being the only limiting factor in self-shading cells at high densities (Qiang et al., 1996). Because there was less biomass in the 35 °C experiments more light may have been available to individual cells and light ceased to be a limiting factor, allowing better use of available nitrogen and, consequently, higher production of protein and lipids at the higher nitrogen content of Runs 7 and 8. According to Walach et al. (1987), higher quantities of carbohydrates are synthesized when nitrogen availability is decreased but carbon availability is constant. These two factors together may explain the higher production of protein, lipids and phenolics in Runs 7 and 8.

At 35 °C higher nitrogen concentration (Runs 7 and 8) promoted higher levels of phenolics (Fig. 2c). According to Miranda et al. (1998), the main phenolic compounds found in *Spirulina* are salicylic, *trans*-cinnamic, synaptic, chlorogenic, quinic and caffeic acids. However, the metabolic pathways for the formation of these compounds in cyanobacteria and their importance are still unknown. For green plants, Rechner et al. (2001) reported that the amino acid phenylalanine could be converted by an ammonia lyase to *trans*-cinnamic acid, which is then converted to cuminic acid that in turn is converted to caffeic acid, these compounds being converted through several chemical reactions to flavonoids, natural pigments with antioxidant activity. According to Duval and Shetty (2001), biosynthetic path-

### Table 2

<table>
<thead>
<tr>
<th>Factors</th>
<th>( p )-value</th>
<th>Productivity</th>
<th>Protein</th>
<th>Lipid</th>
<th>Phenolics</th>
</tr>
</thead>
<tbody>
<tr>
<td>( X_1 )</td>
<td>0.1788</td>
<td>0.3889</td>
<td>&lt;0.0001*</td>
<td>&lt;0.0001*</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>( X_2 )</td>
<td>&lt;0.0001*</td>
<td>&lt;0.0001*</td>
<td>0.0002*</td>
<td>0.0004*</td>
<td></td>
</tr>
<tr>
<td>( X_1 \cdot X_2)</td>
<td>0.3637</td>
<td>0.5180</td>
<td>&lt;0.0001*</td>
<td>0.0039*</td>
<td>&lt;0.0001*</td>
</tr>
</tbody>
</table>

* Significant at the 99% confidence interval. \( X_1 \) = nitrogen concentration; \( X_2 \) = temperature; \( X_1 \cdot X_2 \) = interaction of factors.

![Fig. 2](image-url)
ways that lead to the formation of flavonols and phenylpropanols are related to the pentose-phosphate (Calvin) cycle, and the amounts synthesized are characteristic of each organism. These same pathways can lead to the formation of such compounds in cyanobacteria because the Calvin cycle is part of the photosynthetic carbon fixing mechanism of these organisms (Fay, 1993).

Although the mechanisms used by cyanobacteria to synthesize phenolic compounds and their metabolic functions in cells are still unknown, we found that at 35 °C there was a significant increase in the quantity of phenolic compounds synthesized by the cells, and this increase was also related to an increase in other cellular components such as proteins and lipids. In spite of the fact that biomass production at 30 °C presented better results in terms of productivity and number of cells, cultivation at 35 °C presented advantages related to the quantities of useful compounds such as proteins, lipids and, especially, phenolics.

4. Conclusions

In this paper we have demonstrated that temperature has an important influence on the production of biomass, proteins, lipids and phenolics by S. platensis (35 °C having a negative effect on biomass production but a positive effect on the production of protein, lipids and phenolics, the highest levels of these compounds being obtained at 35 °C and 1.875 g l⁻¹ sodium nitrate or 2.500 g l⁻¹ sodium nitrate). Therefore, cultivation at 35 °C can be exploited when the purpose is to produce S. platensis with nutritional characteristics. Highest biomass density and productivity were obtained at 30 °C, although nitrogen concentration appeared to have no effect on the quantity of protein, lipid or phenolics produced, which indicates that at this temperature the concentration of sodium nitrate in Zarrouk’s medium (2.50 g l⁻¹) can be reduced to 0.625 g l⁻¹ without loss of biomass productivity.

References