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Mixotrophic cultivation of microalga *Spirulina platensis* using molasses as organic substrate

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Abstract

Spirulina is a microalga rich in proteins, vitamins and polyunsaturated fatty acids. This microorganism grows photosynthetically but an organic substrate can stimulate its growth. Molasses is a by-product from sugar industry, containing more than 50% of sugar, and potentially useful as substrate to microalgae culture. On the other hand, light is needed to the photosynthetic fixation of CO_2. So, we determined the effects of molasses concentration and light levels on mixotrophic biomass production by *Spirulina platensis*. Molasses concentration was the main factor influencing maximum biomass concentration (X_{max}, g L^{-1}) and maximum specific growth rate (μ_{max}, d^{-1}), although light intensity also influenced both parameters after 11 days. X_{max} reached 2.94 g L^{-1} and μ_{max} 0.147 d^{-1} while the average maximum productivity (P_{max}, g L^{-1} d^{-1}) of 0.32 g L^{-1} d^{-1} occurred within the first few days and was not significantly affected either by the molasses concentration or light. Biomass production was stimulated by molasses, suggesting that this industrial by-product could be used as a low-cost supplement for the growth of *Spirulina platensis*.
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Keywords: Cyanobacterium; Light intensity; Mixotrophic cultivation; Molasses; *Spirulina platensis*

1. Introduction

The cultivation of microalgae is used for the production of valuable chemical compounds, including natural pigments, biofuels and dietary supplements. In spite of the progresses in cultivation techniques and the design of high efficiency photobioreactors, biomass productivity is still low and more research is needed to develop large-scale processes for the production of these microorganisms.

The cyanobacterium *Spirulina* contains up to 74% dry weight of proteins, along with high concentrations of minerals, pigments, unsaturated fatty-acids and vitamins (Cohen, 1997), because of which it is used as a dietary supplement for both animals and humans.

In *Spirulina* photosynthesis is the main carbon-fixation route, but during the light phase of cultivation *Spirulina* can combine autotrophic photosynthesis and heterotrophic assimilation of organic compounds in a process known as mixotrophy (Marquez et al., 1993; Villarejo et al., 1995; Chen et al., 1996). Photosynthetic fixation of inorganic carbon is influenced by light intensity while the heterotrophic assimilation of carbon is influenced by the availability of organic carbon (Zhang et al., 1999).

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Light plays an important role in the cultivation of photosynthetic microorganisms, with growth decreasing when illumination levels are too low (photo-limitation) or too high (photo-inhibition), but between these two extremes specific growth rate (μ, d^{-1}) becomes independent of light, in the light saturation range (Grima et al., 1996; Chojnacka and Noworyta, 2004). In mixotrophs the presence of an organic substrate means that cell growth is not strictly dependent on photosynthesis and hence light stops being an indispensable growth factor. *Spirulina* growth is stimulated during the light phase in media supplemented with glucose (Marquez et al., 1993; Chojnacka and Noworyta, 2004) and there is less biomass loss in the dark phase (Torzillo et al., 1991).

Brazil is the largest producer of sugarcane and produces large amounts of molasses (containing 50% w/w sugars) as a by-product. Although molasses has potential for use as substrate for the cultivation of microorganisms (Lee and Kim, 2001; Lazaridou et al., 2002; Jiménez et al., 2003), its use in the cultivation of photosynthetic microorganisms has not yet been reported.

The objective of this work was to determine the influence of light intensity and molasses concentration on the mixotrophic growth of *Spirulina platensis*.

2. Materials and methods

The *S. platensis* strain used was LEB-52 (Costa et al., 2004). The culture media was distilled water plus 20% (v/v) Zarrouk's medium (Zarrouk, 1966; Reinehr and Costa, 2006). Each component of the Zarrouk medium was sterilized separately by autoclaving at 121 °C for 15 min after which the cooled solutions were mixed and the medium supplemented with 0.25, 0.5 or 0.75 g L^{-1} of molasses obtained from the Brazilian sugar industry.

Cultivation was carried out in sterilized photobioreactors consisting of 2 L Erlenmeyer flasks equipped with a device for aseptic removal of samples. The cultures were incubated at 30 °C and agitated using filter-sterilized air provided by diaphragm pumps. Illumination was provided by 40 W daylight-type fluorescent lamps to give light intensities (I) of 32.5, 45.5 or 58.5 μmol m^{-2} s^{-1} with a photoperiod of 12 h light/dark (Costa et al., 2000).

Each discontinuous culture was inoculated with an initial *S. platensis* biomass concentration (X_0, g L^{-1} dry weight) of 0.15 g L^{-1} (Colla et al., 2004) previously adapted to molasses and hence incubated for up to 25 days. The runs were planned using a complete factorial 3^2 design and central point duplication in respect of molasses concentration and light intensity (Table 1).

The *S. platensis* biomass concentration was determined daily by measuring the optical density (λ_{max}=670 nm) of samples and comparing these values with previously prepared standard calibration curves of optical density versus *S. platensis* biomass dry weight (Costa et al., 2002). The pH of the cultures was measured every three days using a Quimis Q.400H digital pH meter. At the end of each run (25 days) the maximum *S. platensis* biomass concentration (X_{max}, g L^{-1}) was recorded and the maximum productivity (P_{max}, g L^{-1} d^{-1}) calculated from the equation $P=(X_t-X_0)/(t_x-t_0)$, where X_t is biomass concentration (g L^{-1}) at time t_x (days) and X_0

Table 1
Experimental variables and growth parameters for *Spirulina platensis* cultivated at various light intensities in mineral media supplemented with molasses

Run	Variables		Growth parameters[1]				
	Light intensity (μmol m^{-2} s^{-1})	Molasses (g L^{-1})	$X_{max\ 1}$ (g L^{-1})	$X_{max\ 2}$ (g L^{-1})	P_{max} (g L^{-1} d^{-1})	$\mu_{max\ 1}$ (d^{-1})	$\mu_{max\ 2}$ (d^{-1})
1	32.5	0.25	1.03	1.57	0.26	0.093	0.089
2	45.5	0.25	0.86	1.41	0.18	0.067	0.147
3	58.5	0.25	1.04	1.91	0.20	0.089	0.054
4	32.5	0.50	1.07	2.09	0.46	0.056	0.057
5	45.5	0.50	0.96	2.32	0.34	0.058	0.042
6	58.5	0.50	1.17	2.16	0.24	0.035	0.023
7	32.5	0.75	1.21	2.03	0.56	0.042	0.039
8	45.5	0.75	1.14	2.94	0.29	0.063	0.064
9	58.5	0.75	1.55	2.80	0.44	0.069	0.048
10	45.5	0.50	0.96	2.05	0.13	0.070	0.062
11	45.5	0.50	1.13	2.21	0.42	0.055	0.042

[1] $X_{max\ 1}$=maximum biomass concentration (X_{max}, g L^{-1}) at 11 days (t_{11}); $X_{max\ 2}=X_{max}$ after t_{11}; $\mu_{max\ 1}$=maximum specific growth rate (μ_{max}, d^{-1}) at t_{11}; $\mu_{max\ 2}=\mu_{max}$ after t_{11}; P_{max}=maximum biomass productivity (g L^{-1} d^{-1}).

the initial biomass concentration (g L^{-1}) at t_0 (day) (Schmidell et al., 2001). The maximum specific growth rate (μ_{max}, d^{-1}) was calculated by exponential regression of the logarithmic growth phase (Bailey and Ollis, 1986). The results were submitted to Analysis of Variance (ANOVA) at the 90% confidence interval ($p<0.1$).

3. Results and discussion

The variables for all runs are shown in Table 1. Runs 3, 4, 6, 7 and 8 showed two growth trends, one up to 11 days (t_{11}) and one after t_{11} (Fig. 1a, b), enabling two values to be calculated for the maximum *S. platensis* biomass concentration (X_{max}, g L^{-1}), i.e. X_{max} at t_{11} ($X_{max\ 1}$) and X_{max} after t_{11} ($X_{max\ 2}$). The same was true for the maximum specific growth rate (μ_{max}, d^{-1}), i.e. μ_{max} at t_{11} ($\mu_{max\ 1}$) and μ_{max} after t_{11} ($\mu_{max\ 2}$).

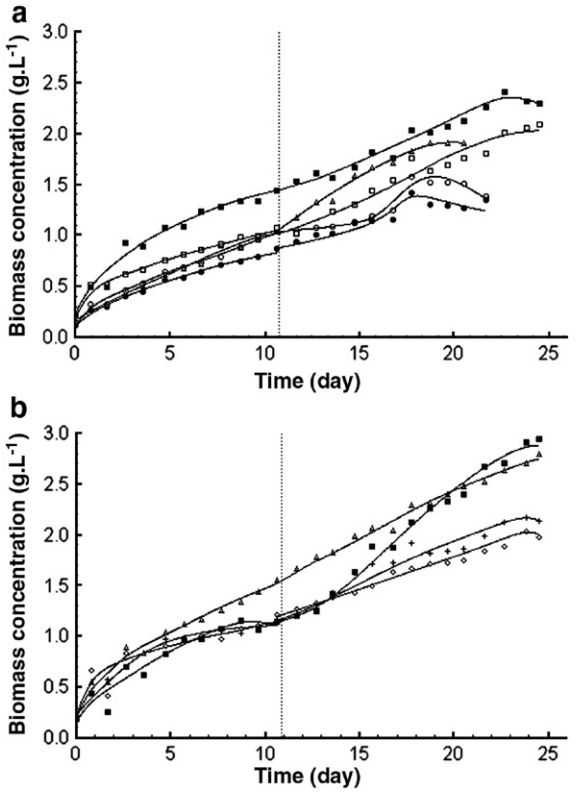

Fig. 1. Biomass production by *Spirulina platensis* cultivated at various light intensities (I, μmol m^{-2} s^{-1}) in mineral media supplemented with different molasses concentrations (S_0, g L^{-1}). Fig. 1a: run 1 (○) I=32.5, S_0=0.25; run 2 (●) I=45.5, S_0=0.25; run 3 (△) I=58.5, S_0=0.25; run 4 (□) I=32.5, S_0=0.5; run 5 (■) I=45.5, S_0=0.5. Fig. 1b: run 6 (+) I=58.5, S_0=0.5; run 7 (◇) I=32.5, S_0=0.75; run 8 (■) I=45.5, S_0=0.75; run 9 (△) I=58.5, S_0=0.75. The vertical dotted line delimits two different growth stages, i.e. up to 11 days (t_{11}, left of dotted line) and after 11 days (right of dotted line).

The highest $X_{max\ 1}$ values occurred in runs 6, 7 and 9 (Fig. 1b) with the highest initial substrate (molasses) concentration (S_0, g L^{-1}) whereas the highest $X_{max\ 2}$ values occurred in the runs with the highest light intensity and S_0 values (run 5, Fig. 1a; runs 8 and 9, Fig. 1b). These results suggest mixotrophy, with predominantly heterotrophic growth dependent on substrate concentration before t_{11} and mainly autotrophic growth dependent on light intensity after t_{11}. These results are in agreement with those of Martínez et al. (1997) who also found two growth trends during mixotrophic cultivation of *Chlorella*.

Although heterotrophic growth and photosynthesis has been reported to occur simultaneously and independently in mixotrophic *Spirulina* cultures (Marquez et al., 1993), the presence of organic carbon can alter both the photosynthetic and heterotrophic metabolism of *Chlorella* (Villarejo et al., 1995) and decreases production of photosynthetic pigments as compared with the amounts present in the absence of organic carbon source (Ogbonna and Tanaka, 1998). Moreover cultivation conditions can favor autotrophy or heterotrophy metabolism and when this occurs the limiting factor for the unfavorable metabolic process (light in the case of photosynthesis or organic carbon for heterotrophic growth) can inhibit growth (Zhang et al., 1999).

Run 3 had the lowest molasses concentration (S_0=0.25 g L^{-1}) but a highest light intensity (I=58.5 μmol m^{-2} s^{-1}) and produced one of the lowest biomass concentrations ($X_{max\ 1}$=1.04 g L^{-1}) before t_{11}, although after t_{11} biomass production increased and reached levels just below those found in cultures with the highest initial molasses concentration (S_0=0.75 g L^{-1}).

In run 9 the highest light intensity (I=58.5 μmol m^{-2} s^{-1}) and the highest initial substrate concentration (S_0=0.75 g L^{-1}) appear to have produced a balance between photosynthesis and heterotrophy because this run showed continuous growth and not the two growth trends seen in the other runs.

In run 8 (S_0=0.75 g L^{-1}, I=45.5 μmol m^{-2} s^{-1}) *S. platensis* biomass concentration ($X_{max\ 2}$) was 2.94 g L^{-1}. This was the highest of all the runs and also higher than that encountered by Marquez et al. (1993) during autotrophic (X_{max}=1.77 g L^{-1}) and mixotrophic growth (X_{max}=2.52 g L^{-1}) of *S. platensis* with glucose, however these authors used undiluted Zarrouk medium while we used only 20% (v/v) Zarrouk medium.

Molasses concentration was the factor that most influenced X_{max} before t_{11}, the average $X_{max\ 1}$ value being 0.32 g L^{-1} larger (p=0.056) in runs with higher levels of molasses as compared with lower levels of molasses, independent (p=0.202) of the light level. The

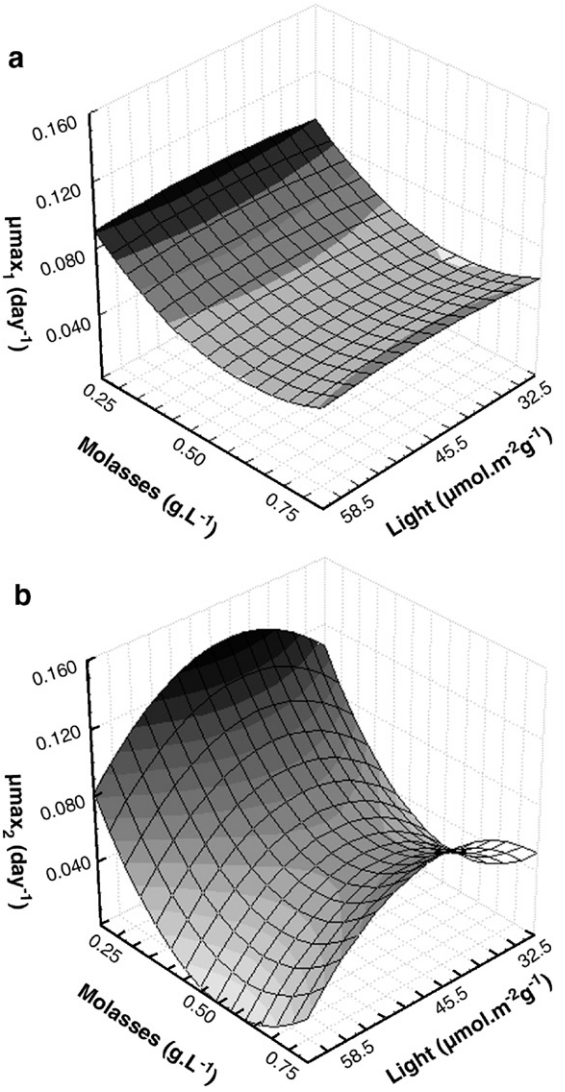

Fig. 2. Maximum specific growth rate of *Spirulina platensis* cultivated at various light intensities in mineral media supplemented with different concentrations of molasses. Fig. 2a shows the maximum specific growth rate (μ_{max}, d^{-1}) at 11 days ($\mu_{max\ 1}$, left of dotted line in Fig. 1) while Fig. 2b shows μ_{max} after 11 days ($\mu_{max\ 2}$, right of dotted line in Fig. 1).

same relationship was found by García et al. (2005), when studying the mixotrophic growth of the microalga *Phaeodactylum tricornutum* growing on glucose. X_{max} after t_{11} was influenced by both molasses concentration ($p=0.013$) and light intensity ($p=0.071$), with higher average X_{max} values occurring at the higher levels of both variables. Chen and Zhang (1997) report 10.24 g L^{-1} of *S. platensis* in fed batch mixotroph culture where light is increased progressively with increasing biomass.

In all runs the maximum productivity (P_{max}, g L^{-1} d^{-1}) values (Table 1) occurred up to 3 days (t_3). After attaining P_{max} productivity decreased until t_{11}, after which productivity remained more or less constant at 0.05 to 0.13 g L^{-1} d^{-1}. According to ANOVA P_{max} was not significantly ($p>0.1$) influenced by the different levels of the tested variables or the interaction among variables.

The molasses concentration (X_s) caused a decrease in μ_{max} as X_s increased from 0.25 to 0.5 g L^{-1} and a subsequent increase in μ_{max} as X_s increased from 0.5 to 0.75 g L^{-1} (Fig. 2a, b). Before t_{11} $\mu_{max\ 1}$ was influenced by molasses concentration but independent of light intensity ($p=0.927$), indicating that light saturation had been reached (Fig. 2a). However, after t_{11} $\mu_{max\ 2}$ was influenced not only by molasses concentration but also by light intensity (Fig. 2b). When increasing molasses concentration increases X_{max} and reduces μ_{max}, this would mean that a longer growth period would be required to get to the high biomass.

In *Spirulina* cultivation pH is one of the most important factors and should be maintained above 9.5 in order to avoid contamination by microalgae (Belay, 1997). pH 9.5 to 10.5 is considered ideal for *Spirulina* cultivation (Richmond and Grobbelaar, 1986). In our study even though we used diluted Zarrouk medium the pH remained between 9.5 and 10.5.

Before t_{11} runs 1, 2 and 3 (all with $X_s=0.25$ g L^{-1}) showed an increase and then a fall in pH. The decrease in pH before t_{11} may have been due to the release of carbon dioxide (CO_2) caused by the heterotrophic component of mixotrophic metabolism.

In all the runs there was a tendency for the pH to increase and we found that where light was highest ($I=58.5$ μmol m^{-2} s^{-1}; runs 3, 6 and 9) and photosynthesis could proceed at a higher rate pH was higher than in the runs for which the light intensity was lower. These results are in agreement with those of Hase et al. (2000) who reported an increase of pH in *Chlorella* cultures during the light phase when photosynthesis was occurring and a decrease in pH during the dark period when photosynthesis ceased and only heterotrophic growth occurred.

4. Conclusions

The mixotrophic cultivation of *Spirulina platensis* using molasses as organic substrate resulted in two growth trends in some conditions, with the transition point occurring about 11 days after inoculation. Before 11 days growth present evidences of essentially heterotrophic. After 11 days growth was also influenced by light intensity and showed increasing pH values

typical of predominantly autotrophic growth and photosynthesis.

Molasses concentration was the main factor influencing the growth of *Spirulina*. Increased molasses concentration favored biomass production ($X_{max}=2.94$ g L^{-1} for $X_s=0.75$ g L^{-1} and $I=45.5$ μmol m^{-2} s^{-1}) but was unfavorable for the specific growth rate ($\mu_{max}=0.093$ d^{-1} for $X_s=0.25$ g L^{-1} and $I=32.5$ μmol m^{-2} s^{-1}). After 11 days light intensity started to influence growth parameters but showed smaller effects than molasses concentration. The variables studied did not influence the maximum productivity ($P_{max}=0.32$ g L^{-1} d^{-1}).

These results highlight the potential of molasses as an organic substrate for the mixotrophic cultivation of *Spirulina platensis* and open up the possibility of using low cost agricultural by-product for the growth of this microorganism.

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