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Kinetics and Bioenergetics of *Spirulina platensis* Cultivation by Fed-Batch Addition of Urea as Nitrogen Source

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Abstract

The cyanobacterium *Spirulina platensis* was cultivated in bench-scale miniponds on bicarbonate/carbonate solutions using urea as nitrogen source. To minimize limitation and inhibition phenomena, urea was supplied semicontinuously using exponentially increasing feeding rates. The average growth rates obtained alternately varying the total mass of urea added per unit reactor volume ($275 < m_T < 725$ mg/L) and the total feeding time ($9 < t_T < 15$ d) clearly evidenced nitrogen limitation for $m_T < 500$ mg/L and excess nitrogen inhibition above this threshold. The time behavior of the specific growth rate at variable urea feeding patterns allowed estimation of the time-dependent Gibbs energy dissipation for cell growth under the actual depletion conditions of fed-batch cultivations. Comparison of the yield of growth on Gibbs energy obtained using either urea or KNO_3 pointed to the preference of *S. platensis* for the former nitrogen source, likely owing to more favorable bioenergetic conditions.

Index Entries: *Spirulina platensis*; urea; fed-batch cultivation; kinetics; bioenergetics; microalgae production.

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Introduction

Among the filamentous cyanobacteria, *Spirulina* sp. is preferred for biomass production as human health food and animal feed, because of its relatively high cell growth rate, easy cell recovery *(1)*, ability to grow on alkaline and salty mineral media, reduced risks of external contamination *(2)*, and high level of γ-linolenic acid *(3)*. From time immemorial, it has been used as human food in some regions of Mexico and Africa *(4)*. Nutritional studies demonstrated that these microorganisms possess one of the highest protein contents, high nutritional value, good digestibility *(5)*, and all the essential amino acids in the proportions recommended by the Food and Agriculture Organization of the United Nations except methionine *(6)*.

The current designation of *Spirulina* for species belonging to the genus *Arthrospira* holds a more traditional, practical and technological meaning than a taxonomic one. *Spirulina* sp. includes pluricellular photoautotrophic blue-green algae, growing in colonies and having spiraliform and filamentous morphology, which prefer an aqueous habitat with high salinity. Individuals belonging to the *Spirulina* genus are constituted by mobile filaments with a 1- to 12-μm diameter containing cylindrical cells provided with helicoidal trichomes without ramification. They can grow rapidly in lukewarm, salty, and not very deep lakes and are among the most common microorganisms populating alkaline African and American lakes *(4)*.

The ultrastructure and morphology of *Spirulina platensis* are sensitive to environmental conditions and nutritional factors. In particular, temperature influences cell size, morphology, and degree of unsaturated fatty acids *(3,7)*, and an increased light intensity brings about corresponding increases in gas vesicle content and trichome size as well as a decrease in phycobilosome content *(8)*. Regarding the nitrogen source, *Spirulina* sp. is not able to fix N_2, with the exception of the thermophilic *Spirulina labyrinthyformis* species *(9)*. The composition of *Spirulina* sp. is influenced by the quality and amount of the nitrogen source *(1,5,10)*.

Nitrate was demonstrated to ensure the highest yields of *Spirulina* sp. biomass *(11)*, thus justifying the wide use of the media of Paoletti et al. *(12)*, Stanca and Popovici *(10)*, and Schlösser *(13)*, which employ KNO_3 and/or $NaNO_3$. Under conditions of nitrate shortage, cell growth stops and apoproteins and phycocyanins are metabolized *(11)*. However, the use of cheaper nitrogen sources such as urea or ammonium salts is particularly attractive.

According to Boussiba *(14)*, urea could serve as an excellent nitrogen source for blue-green microalgae growth, as an alternative to the well-accepted nitrate and mainly ammonia. Nitrogen deficiency in *S. platensis* affects growth *(1,5)*, influences fatty acid composition *(15)*, increases lipid content *(3)* and decreases both phycocyanine and chlorophyll production, with consequent loss of its characteristic blue-green color *(16)*. Urea is very cheap and recently was demonstrated to be the most effective nitrogen source for the biomass-associated production of γ-linolenic acid *(15)*.

Fed-batch culture allowed for cell concentration and phycocyanin production 5.1 and 2.8 times higher than batch culture, respectively *(17)*. In addition, an exponentially increasing feeding rate such as that selected in the present study to supply urea is suitable for those cultures in which microbial growth can be inhibited by some nutrient. In fact, the highest nutrient supply takes place just at the end of the run, when biomass concentration achieves its maximum value. In this way, despite the inhibiting effect of ammonia deriving from urea hydrolysis under alkaline conditions, the use of such a nitrogen source allowed *S. platensis* to reach a concentration comparable with that obtained with potassium nitrate in batch run *(18)*.

The results of tests performed alternately varying the total added mass of urea and the total feeding time were used in the present study to carry out a bioenergetic study of the growth of the cyanobacterium *S. platensis*.

Materials and Methods

Microorganism

S. platensis UTEX 1926 was obtained from the University of Texas Culture Collection (Austin, TX). The microorganism was maintained and cultivated in the mineral medium suggested by Paoletti et al. *(12)*, of which the nitrogen source consisted, according to circumstances, 2.57 g/L of potassium nitrate (batch reference tests) or variable amounts of urea (fed-batch tests). Erlenmeyer flasks (1.0 L) containing 200 mL of this medium were aseptically inoculated with 1.0 mL of *S. platensis* suspension. The microorganism was then grown in rotary shakers (100 rpm) at 30°C using a light intensity of 6.0 klux.

The inoculum was prepared by recovering by centrifugation at 4000 rpm *S. platensis* cells from the exponential growth phase and resuspending them in the medium, so as to ensure a starting biomass concentration of 50 mg/L (dry weight). Suspension volumes never exceeded 10% of the open miniponds' working volume. The starting amount of urea was 80 mg/L.

Equipment and Cultivation Conditions

Among the available systems of algaculture, lengthened polyvinyl chloride miniponds, each having a total area of 1250 cm^2 and containing 5.0 L of the aforementioned media, were preferred because of the excellent performance demonstrated by this reactor configuration under different cultivation conditions *(19)*. Culture mixing at 23 rpm was ensured by paddle wheels. Starting pH was adjusted at 9.7 ± 0.1 by means of a 6 *N* NaOH solution. Distilled water was added daily to the ponds to replace water lost by evaporation. Temperature was maintained at the optimum value of 25°C *(20)* by means of a set of heating elements controlled by thermostats. Light intensity was regulated at 6.0 klux by means of 40-W fluorescent lamps.

Urea solutions were employed concentrated enough to prevent valuable variation in the reaction volume. The nitrogen source was fed by exponentially increasing the feeding rate as previously described *(18)*. The starting mass of urea added per unit reactor volume, m_o, was 80 mg/L, and the added overall amount of urea, m_T, and the total feeding time, t_T, were varied from time to time.

Analytical Techniques

Dry cell mass concentration was determined by optical density measurements using a calibration curve. Light intensity was measured with a Minolta TL-1 illuminance meter (Osaka, Japan).

Medium samples, preliminarily filtered through membranes with a 10-µm pore diameter, were made alkaline (pH 13.0) with 6 *N* NaOH and immediately analyzed for total ammonia concentration with an Orion model 710-A potentiometer (Beverly, MA) using an Orion model 95-12 selective ammonia electrode. Ammonia concentration was determined from electric potential measurements by means of standard curves daily prepared by five serial dilutions of an alkalinized solution of 1.0 *M* NH_4Cl. To determine the total nitrogen concentration, expressed as ammonia, tubes containing samples were located within a digester where urea was completely hydrolyzed by 6 *N* H_2SO_4. After about 10 h, the solutions were analyzed using the same procedure as for ammonia. Residual urea during cultivations was determined as the difference between total nitrogen and total ammonia, considering that the decomposition of 1.0 mol of urea leads to the formation of 2.0 mol of ammonia.

All measurements, during either batch (KNO_3) or fed-batch (urea) runs, were performed in triplicate and the corresponding data expressed as mean concentrations. SDs were always <6% of the mean values, thus making statistical analysis unnecessary.

Theory

The specific growth rate, µ, was calculated as the logarithm of the ratio of final and starting biomass concentrations divided by the time interval under consideration.

The Gibbs energy dissipation for cell growth ($1/Y_{GX}$) was estimated according to Heijnen by the equation (*21*):

$$\frac{1}{Y_{GX}} = \frac{1}{Y_{GX}^{max}} + \frac{m_G}{\mu} \tag{1}$$

in which

$$m_G = 4.5 \exp\left[-\frac{E^*}{R}\left(\frac{1}{T} - \frac{1}{T^o}\right)\right] \tag{2}$$

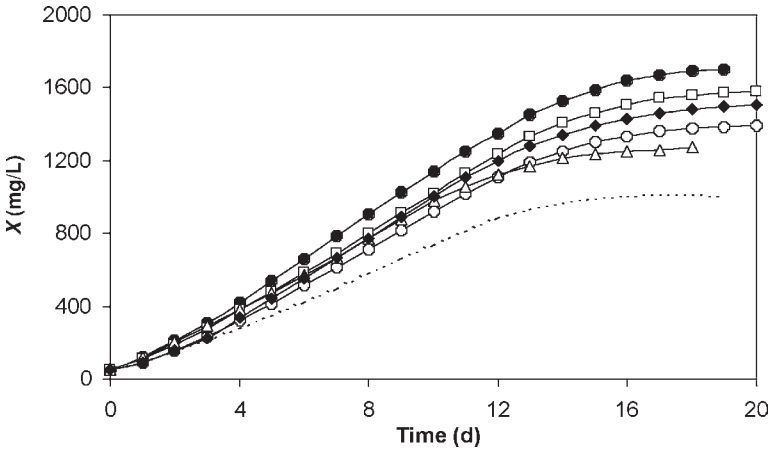

Fig. 1. Time behavior of cell mass concentration during cultivations of *S. platensis*. (□) t_T = 12 d, m_T = 275 mg/L; (●) t_T = 12 d, m_T = 500 mg/L; (○) t_T = 12 d, m_T = 725 mg/L; (△) t_T = 9 d, m_T = 500 mg/L; (◆) t_T = 15 d, m_T = 500 mg/L; (– – –) reference test with KNO_3.

is the specific rate of Gibbs energy dissipation for cell maintenance,

$$1/Y_{GX}^{\max} = 200 + 18(6 - n_c)^{1.8} + \exp[(3.8 - \gamma_S)^{0.32}(3.6 + 0.4n_c)] \qquad (3)$$

the theoretical value that $1/Y_{GX}$ approaches for $\mu \to \infty$; that is, under ideal growth conditions, E^* = 69.000 J/mol is the activation energy, T is the absolute temperature, $T°$ = 298.16 K is its standard value, R = 8.314 J/(mol·K) is the ideal gas constant, γ_S is the reduction degree *(22)*, and n_c is the number of carbons per molecule of the carbon source.

Results and Discussion

The dashed curve in Fig. 1 shows the average data of biomass concentration, X, collected along batch *S. platensis* cultivations carried out using KNO_3 as reference nitrogen source. The other curves deal with additional tests carried out either feeding daily for 12 consecutive days the selected overall amounts of urea per unit fermentor volume ($275 < m_T < 725$ mg/L) or feeding 500 mg/L of urea for increasing total feeding time ($9 < t_T < 15$ d).

The kinetic results of these tests are listed in Table 1 in terms of average volumetric growth rates either at the end of cultivation, Q_T, or after half cultivation time, $Q_{T/2}$. The latter parameter reached a maximum value at m_T = 500 mg/L, which means that nitrogen source limitation and inhibition took place below and over this threshold value, respectively. An opposite influence seemed to be exerted by the total feeding time. As suggested by the nearly coincident values of Q_T obtained with tests 2 and 4, owing to the exponential feeding of urea, the deleterious effect of nitrogen source shortage on the growth disappeared at the end of cultivations. On the other hand, the inhibition owing to excess urea persisted even at the end of the run performed at the highest m_T value (725 mg/L), whereas the quickest one (t_T = 9 d) exhibited inhibition at the start and limitation at the end.

Table 1
Average Rates of *S. platensis* Growth Obtained by Alternately Varying Total Feeding Time (t_T) and Overall Supplemented Amount of Urea (m_T)

Test no.	m_T (mg/L)	t_T (d)	$Q_{T/2}$ [a] (mg/[L·d])	Q_T [b] (mg/[L·d])
1	500	9	93	68
2	500	12	109	75
3	500	15	95	73
4	275	12	97	76
5	725	12	87	67

[a]Volumetric growth rate calculated after half cultivation time.
[b]Volumetric growth rate calculated at the end of cultivation.

Since the variation in volume was negligible under the selected operating conditions, it is reasonable to consider the process, for modeling purposes, as a batch cultivation *(23)*. The changes in feeding conditions can then be assumed to influence the medium composition and, consequently, the specific growth rate. Therefore, a bioenergetic study was performed just on the effects that the different feeding conditions exert on this parameter.

Equation 1, which contains one term for the growth and another one for the Gibbs energy dissipation for maintenance, is of general validity and covers a wide range of microbial growth systems (e.g., heterotrophic, autotrophic, aerobic, anaerobic) and temperatures. By Eq. 2 we estimated, for the system under consideration, an m_G value coincident with that of the heterotrophic growth at 25°C (4.5 kJ/[C-mol$_X$·h]) *(24)*. This result suggests that the aerobic metabolism differs from the photosynthetic one, from a bioenergetic viewpoint, for the fact that the Gibbs energy comes in the former case from the oxidation of the carbon source and in the latter from the light absorption. On the other hand, Eq. 3 allowed estimation of, in the case of CO_2 as carbon source ($\gamma_S = 0$; $n_c = 1$), a Gibbs energy dissipation for the growth of 986 kJ/C-mol$_X$.

By combining the previous equations and using the experimental data of cell concentration, it was possible to investigate the time behavior of Y_{GX} during the different tests, carried out keeping constant either the total feeding time (t_T = 12 d) or, alternately, the overall amount of urea added per unit reactor volume (m_T = 500 mg/L) (Fig. 2). Note that the yield of biomass on Gibbs energy dissipation decreased with increasing time, i.e., with the culture medium's depletion. The estimated values of Y_{GX} are remarkably lower than those of the heterotrophic growth (about 4 C-mmol$_X$/kJ) *(24)*, which is in agreement with the well-known higher energy requirements for the autotrophic growth on CO_2 as a carbon source, owing to the involvement of a greater number of anabolic pathways.

Comparison of the different growth curves reveals that the largest differences in such a bioenergetic parameter occurred mainly during the

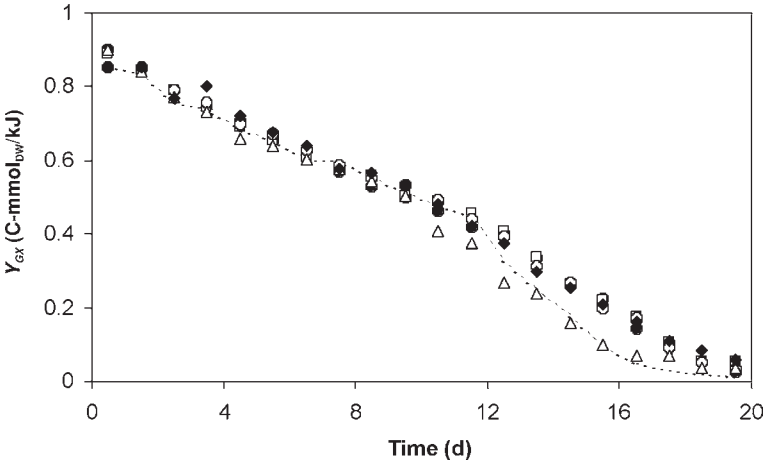

Fig. 2. Yield of biomass on Gibbs energy dissipation vs time during cultivations of *S. platensis*. (□) t_T = 12 d, m_T = 275 mg/L; (●) t_T = 12 d, m_T = 500 mg/L; (○) t_T = 12 d, m_T = 725 mg/L; (△) t_T = 9 d, m_T = 500 mg/L; (◆) t_T = 15 d, m_T = 500 mg/L; (– – –) reference test with KNO_3.

final phases, during which the reference culture exhibited the lowest growth rate. To explain this unfavorable bioenergetic situation when using KNO_3, one should consider that the high cell concentration at the end of cultivations might lead to a low energy supply particularly concerning the deepest zones of the reactor. Therefore, the use of KNO_3 as nitrogen source, which requires the preliminary energy-consuming reduction to ammonia for assimilation *(24)*, could have brought about a condition of energy limitation. A better situation could have taken place in the presence of urea, because of its spontaneous tendency to release ammonia by hydrolysis under alkaline conditions. The unsatisfactory results obtained with fed-batch addition of urea at m_T = 500 mg/L and t_T = 9 d can then reasonably be ascribed to inhibition at the start, owing to excessively rapid feeding of the nitrogen source, and limitation at the end, because of the excess amount of urea lost by hydrolysis and volatilization for $t > t_T$.

By comparing these trends with those in Fig. 1, it becomes clear that the yield of biomass on Gibbs energy dissipation, although significant for the energetic characterization of a biosystem, puts into evidence the most marked variations in behavior just under conditions of strongly limited growth at the end of cultivations. For this reason, future studies will deal with investigating additional bioenergetic and thermodynamic parameters, in order to provide more information about the metabolic behavior of this biosystem.

References

1. Piorreck, M., Baasch, K. L., and Pohl, P. (1984), *Phytochemistry* **23,** 207–213.
2. Walach, M. R., Bazin, M. J., Pirt, S. J., and Balyuzi, H. H. M. (1987), *Biotechnol. Bioeng.* **29,** 520–528.

3 Mahajan, G. and Kamat, M. (1995), *Appl. Microbiol. Biotechnol.* **43,** 466–469.
4. Richmond, A. (1983), in *Biotechnology*, vol. 3, Rehm, H.-J. and Reed, G., eds., Verlag Chemie, Weinheim, Germany, pp. 109–143.
5. Cohen, Z. (1997), in *Spirulina Platensis (Arthrospira)*: *Physiology, Cell-Biology and Biotechnology*, Vonshak, A., ed., Taylor & Francis, London, pp. 175–204.
6. Durand-Chastel, H. (1980), *Production and Use of Spirulina in Mexico*, Elsevier Biomedical, Amsterdam, The Netherlands.
7 Deshnium, P., Paithoonrangsarid, K., Suphatrakul, A., Meesapyodsuk, D., Tanticharoen, M., and Cheevadhanarak, S. (2000), *FEMS Microbiol. Lett.* **184,** 207–213.
8 Babu, T. S., Kumar, A., and Warma, A. K. (1991), *Plant Physiol.* **95,** 492–497.
9 Ciferri, O. and Tiboni, O. (1985), *Microbiologia (Italy)* **39,** 503–526.
10. Stanca, D. and Popovici, E. (1996), *Rev. Roum. Biol.* **41,** 25–31.
11 Cornet, J. F., Dussap, C. G., Cluzel, P., and Dubertret, G. (1992), *Biotechnol. Bioeng.* **40,** 826–834.
12. Paoletti, C., Pushparaj, B., and Tomaselli, L. (1975), in *Atti del 17 Congresso Nazionale della Società Italiana di Microbiologia*, Padova, Società Italiana di Microbiologia, Rome, Italy, pp. 833–839.
13. Schlösser, U. G. (1982), *Ber. Deutsch Bot. Ges.* **95,** 181–276.
14 Boussiba, S. (1989), *Plant Cell Physiol.* **30,** 303–308.
15 Funteu, F., Guet, C., Wu, B., and Trémoliéres, A. (1997), *Plant Physiol. Biochem.* **35,** 63–71.
16 Pirt, S. J., Walach, M. R., and Bazin, M. J. (1987), *Biotechnol. Bioeng.* **24,** 520–528.
17 Chen, F. and Zhang, Y. (1997), *Enzyme Microb. Technol.* **20,** 221–224.
18 Danesi, E. D. G., Rangel-Yagui, C. O., Carvalho, J. C. M., and Sato, S. (2002), *Biomass Bioenergy* **23,** 261–269.
19. Belay, A. (1997), in *Spirulina Platensis (Arthrospira)*: *Physiology, Cell-Biology and Biotechnology*, Vonshak, A., ed., Taylor & Francis, London, pp. 131–158.
20 Lynch, D. V. and Thompson, G. A. (1984), *Plant Physiol.* **74,** 193–197.
21. Heijnen, J. J. (2001), in *Basic Biotechnology*, 2nd ed., Ratledge, C. and Kristiansen, B., eds., Cambridge University Press, Cambridge, UK, pp. 45–58.
22. Roels, J. A. (1983), *Energetics and Kinetics in Biotechnology*, Elsevier Biomedical, Amsterdam, The Netherlands.
23. Carvalho, J. C. M. and Sato, S. (2001), in *Biotecnologia Industrial*, vol. 2, Schmidell, W., Lima, U. A., Aquarone, E., and Borzani, W., eds., Edgar Blücher, São Paulo, Brazil, pp. 205–218.
24. Hatori, A. and Myers, J. (1966), *Plant Physiol.* **41,** 1031–1036.