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Intermediate renewal rates enhance the productivity of the cyanobacterium *Synechococcus* sp. in semicontinuous cultures

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Abstract — The cyanobacterium *Synechococcus* sp. isolated from a hypersaline pond in Venezuela was cultured semicontinuously, at rates of daily renewal of the culture volume of 20, 30, 40 and 50 %, in order to establish the optimal renewal rate for pigment, protein, carbohydrate and exopolysaccharide (EPS) production. Maximal steady-state cell density, protein, chlorophyll *a*, carotenoids and carbohydrate production, of 265.6 x10^6 cell ml^{-1}, 1.62 pg $cell^{-1}$, 34.62 fg $cell^{-1}$, 13.03 fg $cell^{-1}$ and 222.84 µg ml^{-1} respectively, were achieved with a renewal rate of 20 %. On the other hand, maximal EPS production, 173.13 µg ml^{-1}, was obtained at the 50 % daily renewal rate. Highest cell productivity, 84.49 x10^9 cell l^{-1} d^{-1}, was found with a daily renewal rate of 50 %. Protein, chlorophyll *a* and dry weight productivity, 93.51, 2.48 and 134 mg l^{-1} d^{-1} were achieved with a renewal rate of 40 % per day. Carbohydrate and EPS productivities of 95.56 and 86.56 mg l^{-1} d^{-1}, were higher with 50 %. This strain shows a high potential for biomass daily production enriched with EPS, chlorophyll and protein which is a function of the renewal rate in semicontinuous cultures.

carbohydrates / Cyanobacteria / EPS / pigments / productivity / proteins / renewal rate / semicontinuous culture / *Synechococcus* sp.

Résumé — Amélioration de la productivité de la Cyanobactérie *Synechococcus* par la variation des taux de renouvellement en culture semi-continue. La cyanobactérie *Synechococcus* sp., isolée d'un étang hypersalé au Vénézuela, a été cultivée de façon semi-continue avec des taux de renouvellement journalier du volume du milieu de culture de 20, 30, 40 et 50 %, dans le but d'établir un taux de renouvellement optimal pour la production de pigment, protéine, glucide et exopolysaccharide (EPS). L'état stable maximal de la densité des cellules, de la production de protéine, de chlorophylle *a,* de caroténoïdes et de glucides, respectivement de 265.6 x10^6 cell ml^{-1}, 1.62 pg $cell^{-1}$, 34.62 fg $cell^{-1}$, 13.03 fg $cell^{-1}$ et 222.84 µg ml^{-1}, est réalisé avec un taux de renouvellement de 20 %. D'autre part, la production maximale d'EPS, 265.6 x10^6 cell ml^{-1}, est obtenue au taux journalier de renouvellement de 50 %. La plus grande productivité cellulaire, 84.49 x 10^9 cell l^{-1} d^{-1}, est trouvée avec un taux de renouvellement journalier de 50 %. Les protéines, la chlorophylle *a,* et la production de poids sec, 93.51, 2.48 et 134 mg l^{-1} d^{-1}, s'obtiennent avec un taux de renouvellement de 40 % par jour. Les productions de protéines, glucides et EPS de 95.56 et 86.56 mg l^{-1} d^{-1}, sont les plus élevées avec 50 %. Cette souche montre un potentiel élevé pour la production journalière de biomasse enrichie en EPS, chlorophylle et protéine ; c'est le rôle du taux de renouvellement en cultures semi-continues.

culture semi-continue / Cyanobacteria / EPS / glucides / pigments / productivité / protéines / *Synechococcus* sp. / taux de renouvellement

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INTRODUCTION

The influence of several environmental factors on growth and physiology of many cyanobacteria such as *Oscillatoria agardhii* Gomont, *Anacystis nidulans* and *Synechococcus* sp. has been described in batch cultures (Sivonen, 1990; Van Liere *et al*., 1979; Utkilen, 1984). Likewise, growth of many strains of *Synechococcus*, has been described as a function of salinity (Díaz & Reyes, 1992), illumination (Beljanin & Trenkenšu, 1977; Koblizek *et al.*, 1997), temperature (Sakamoto & Bryant, 1998; Inoue *et al.*, 2000) and nutrients (Ritchie *et al.*, 1997; Sauer *et al.*, 1999). However, in batch cultures the cell composition changes with culture age. Thus, it is difficult to apply those techniques for obtain enriched biomass with a known composition.

Previous studies have demonstrated the potential importance of the use of semicontinuous cultures for the production of marine microalgae because of the simplicity of operation of the system and the possibility of manipulating the productivity and the biochemical composition of the microalgal biomass through changes in culture parameters such as renewal rate, nutrient concentration or irradiance (Sukenik *et al.*, 1993; Otero *et al.,* 1997).

Batch cultures have been traditionally considered as optimal systems for the production of secondary metabolites but when the productivity of batch cultures is calculated by dividing the concentration in stationary phase by the number of days to reach it, the productivity obtained with the semicontinuous culture is much higher; in many cases are higher than those obtained in continuously operated photobioreactors (Fábregas *et al.*, 1998; Fábregas & Otero, 1998).

For example, *Tetraselmis suecica* enhanced the protein and lipid production in semicontinuous cultures at a daily renewal rate of 50 % and at a concentration of 8 mmol N l^{-1} (Fábregas *et al.,* 1995b). In contrast, eicosapentaenoic acid productivity was stimulated at high renewal rates in *Porphyridium cruentum* (Fábregas *et al.,* 1998).

Very little information is available on the responses in the metabolic activity and biochemical composition of cyanobacteria in semicontinuous cultures. However, an increase in phycocyanin, chlorophyll *a* and EPS has been already described in semicontinuous cultures of *Anabaena* sp. PCC 7120 (Morales *et al*., 2002). In addition, the influence of nitrogen starvation on the cell cycle, and pigment and DNA contents have been reported for *Synechococcus* WH7803 cultured continuously (Liu *et al.*, 1999).

It is necessary to undertake the isolation and characterization of autochthonous cyanobacterial strains from hypersaline environments for physiological studies in order to evaluate their potential for the production of biotechnologically valuable compounds, as for example, with the production of EPS with industrial value in the marine cyanobacterium *Cyanothece* sp. (Shah *et al.*, 2000).

In the last decade, many strains of *Synechococcus* have been used successfully in the biotechnological field for the production of various metabolites such as chlorophyll, carotenoids and exopolysaccharides, as well as the production of other compounds like absisic acid, ethanol and halogens (Marsalek *et al.*, 1992; Takano *et al.*, 1995; Scarrat & Moore, 1997; Deng & Coleman, 1999) and in aquaculture for shrimp feeding (Gallager, 1988; De Mott, 1998).

In this paper, we report the cellular, chlorophyll *a*, carotenoids, protein, carbohydrate and EPS productivity as a function of renewal rate in semicontinuous cultures in *Synechococcus* sp., a species which shows high potential for biomass production enriched with biotechnologically valuable compounds.

MATERIALS AND METHODS

The cyanobacterium *Synechococcus* sp. was isolated from a hypersaline pond (Salina Rica) north of Maracaibo, Venezuela. Cultures, in triplicate, were maintained in 350 ml flasks with 150 ml culture medium composed of sterilized seawater (salinity 35 ‰) enriched with nutrients (Fábregas *et al*., 1984) at a concentration of 8 mmol (nitrate) N l^{-1}; with a N:P ratio of 20:1. Flasks were inoculated with 15 x 10^6 cell ml^{-1} and maintained at 27 ± 2°C under a 12h-light/12h-dark cycle with a photon flux of 156 mmol of photons m^{-2} s^{-1} and constant aeration of 4.95 ± 0.03 ml s^{-1}.

The semicontinuous regime started once the culture reached the peak of the exponential growth phase, by renewing either 20, 30, 40 or 50 % of the culture volume daily. The renewal of the cultures was made during the first hours of the light period with sterilized seawater enriched with nutrients at the initial concentration. Cultures were kept in this semicontinuous regime for 15 days after stabilization without significant variation in steady-state cell density. The control was a batch culture maintained under the same conditions described above.

Cell density was determined by microscope counting using an improved Neübauer haematocytometer. Biomass was harvested by centrifugation at 18,000 x g for 15 min. Frozen biomass, stored at – 20 ºC, was used for all the biochemical analyses, except for pigments content, for which fresh biomass samples were used. The protein content was determined by the Folin-Lowry method (Lowry *et al.*, 1951) modified by Herbert *et al.* (1971). Pigments were extracted in methanol (99 %) at 4 °C overnight and determined spectrophotometrically using the equations of Marker *et al.* (1980) for chlorophyll *a* and Strikland & Parsons (1972) for carotenoids. Carbohydrates were measured by the phenol-sulfuric acid method (Kochert, 1978). Exopolysaccharides (EPS) were quantified by the same method after clarification by centrifugation. Dry weight was determined using a Millipore© filtration system, with 0.45 µm fiberglass filters, by the method of Utting (1985).

The cell and metabolites productivity corresponds to the number of cells or concentration of metabolite obtained per liter of culture per day, and is related to the renewal rate applied to the culture (expressed as cell (or mg) l^{-1} d^{-1}), using the equation of Otero (1994).

Statistical analyses were performed with StatMost for Windows 3.0, using analysis of variance (ANOVA) and Sheffé's test to examine differences in cellular density and biochemical composition between different renewal rates.

RESULTS

Growth of *Synechococcus* sp. was stable during the semicontinuous regime, but in contrast, population growth in batch culture presented a short stationary phase and then started to decrease on 16th day of culture (Fig. 1).

Steady-state cell density decreased with renewal rate. The highest biomass value of 265.6 ± 8.8 x 10^6 cell ml^{-1} was reached at a 20 % renewal rate (Tab. 1). Cell densities at daily renewal rates of 20 % and 30 % were stabilized within 6 and 8 days after starting the semicontinuous regime, and within 10 days at 40 % and 50 % (Fig. 1).

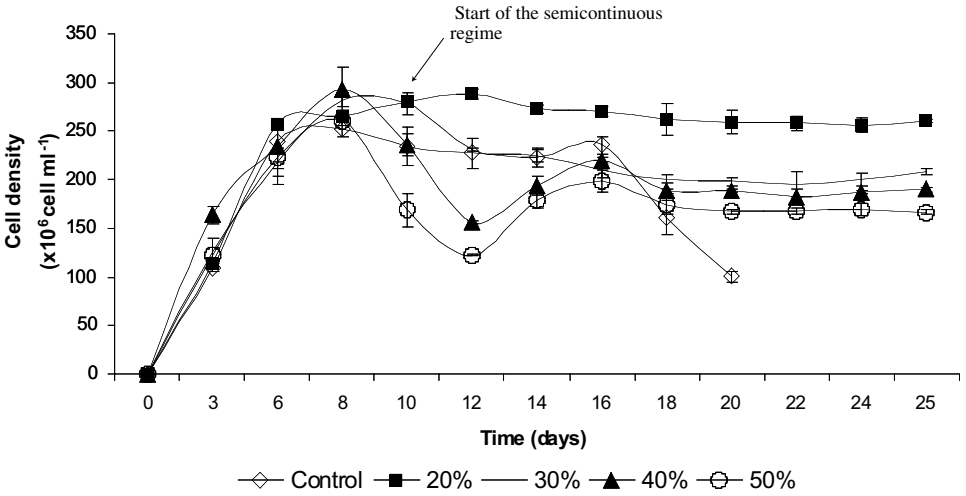

Fig. 1. Growth of *Synechococcus* sp. in semicontinuous cultures at different daily renewal rates.

Tab. 1. Cell density (x 10^6 cell ml^{-1}) and chlorophyll *a* (fg $cell^{-1}$), carotenoids (fg $cell^{-1}$), protein (pg $cell^{-1}$), carbohydrate (pg $cell^{-1}$) and dry weight (µg $cell^{-1}$) production in semicontinuous cultures at different renewal rates (%) of *Synechococcus* sp.

	Control	*20*	*30*	*40*	*50*
Cell Density	238.1 ±19.6	265.6 ±8.8	201.6 ±8.09	187.6 ±5.4	168.9 ±5.8
Chlorophyll *a*	44.56 ±2.82	34.62 ±3.41	33.19 ±3.29	31.06 ±1.81	25.00 ±2.57
Carotenoid	17.42 ±1.42	13.03 ±0.98	12.69 ±0.81	10.02 ±0.62	7.20 ±0.71
Protein	2.13 ±0.07	1.62 ±0.15	1.54 ±0.13	1.29 ±0.04	0.96 ±0.10
Carbohydrate	1.42 ±0.005	0.83 ±0.04	0.80 ±0.03	0.94 ±0.04	1.18 ±0.01
Dry Weight	2.56 ±0.32	1.86 ±0.19	2.23 ±0.19	1.61 ±0.10	1.31 ±0.07

Chlorophyll *a*, carotenoid and protein production was also inversely proportional to renewal rate, with highest values of protein (1.62 ± 0.15 pg $cell^{-1}$), chlorophyll *a* (34.62 ± 3.41 fg $cell^{-1}$) and carotenoid (13.03 ± 0.98 fg $cell^{-1}$) being found at the lowest (20 %) daily renewal rate. The highest dry weight was obtained in the control culture and at the 30 % daily renewal rate, between which there were no significant differences ($p > 0.05$) (Tab. 1).

EPS production was directly proportional to the daily renewal rate, increasing from 33.38 ± 3.22 µg ml^{-1} at 20 % to 173.13 ± 2.47 µg ml^{-1} at 50 %. These values were 2.8 and 5 times higher than in batch cultures and at the lowest (20 %) renewal rate. However, carbohydrate production decreased with increasing renewal rate, with the peak value of 222.3 ± 11.71 mg ml^{-1} at a daily renewal rate of 20 % and the lowest value of 191.1 ± 15.79 mg ml^{-1} at 50 % (Fig. 2).

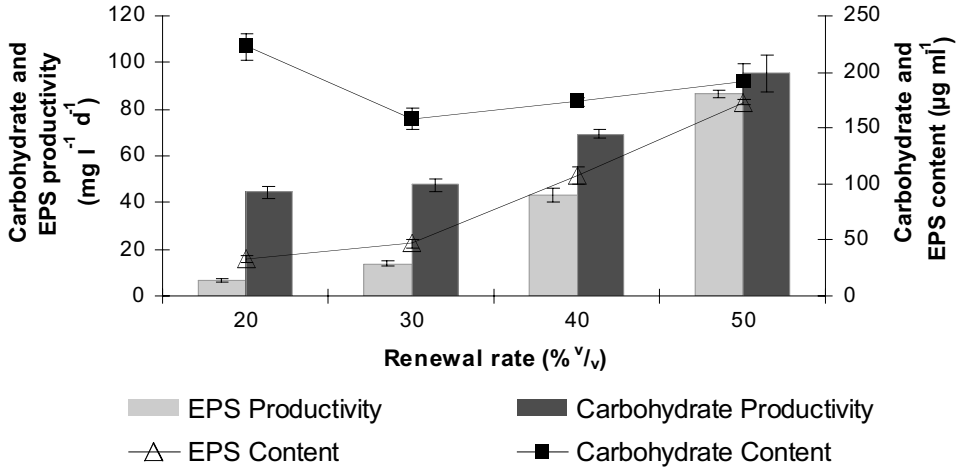

Fig. 2. Production of carbohydrate and EPS in semicontinuous cultures at different daily renewal rates of *Synechococcus* sp.

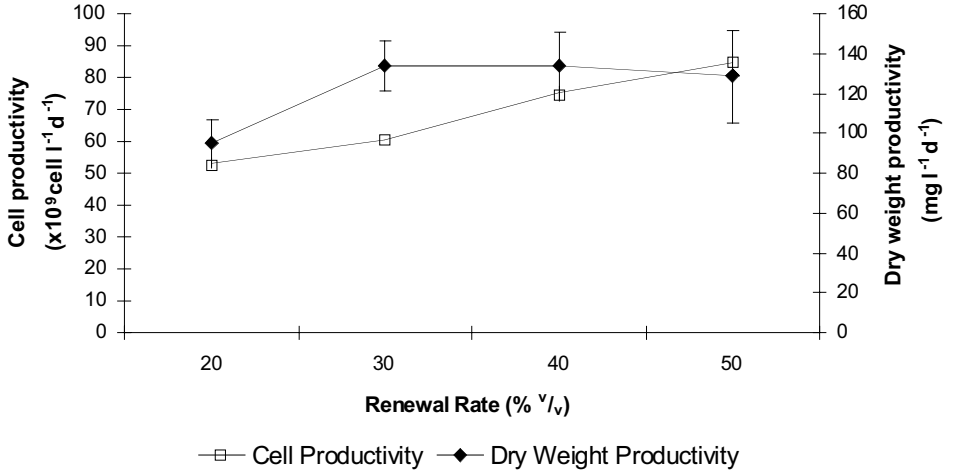

Fig. 3. Cell and dry weight productivity in semicontinuous cultures at different daily renewal rates in cultures of *Synechococcus* sp.

Highest carbohydrate (95.56 mg l^{-1} d^{-1}) and EPS (86.56 mg l^{-1} d^{-1}) productivity was obtained at a 50 % renewal rate. These were 2 and 12.9 times higher than the productivity reached at 20 % (Fig. 2).

Maximum cell productivity peaked at the 50 % renewal rate, with 84.49 x 10^9 cell l^{-1} d^{-1}, and was 1.5 times higher than that at 20 %. Highest protein (93.51 mg l^{-1} d^{-1}), dry weight (134.4 mg l^{-1} d^{-1}), chlorophyll *a* (2.48 mg l^{-1} d^{-1}) and carotenoid (0.84 mg l^{-1} d^{-1}) productivity was produced in cultures at 40 %, although there are no statistical differences ($p > 0.05$) between the remaining values (Figs 3 and 4).

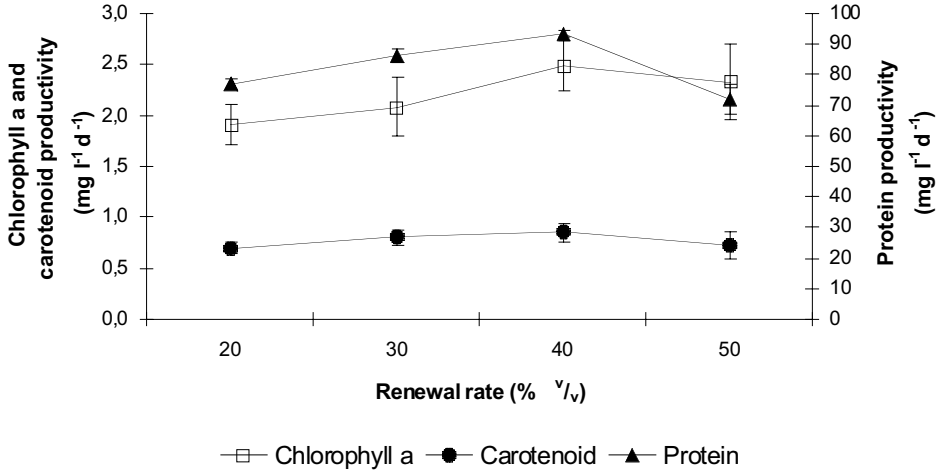

Fig. 4. Protein, chlorophyll *a* and carotenoid productivity in semicontinuous cultures at different daily renewal rates in cultures of *Synechococcus* sp.

DISCUSSION

This study demonstrates that it is possible to maintain a daily biomass production of this cyanobacterium in semicontinuous cultures, as compared to batch systems. The latter have been traditionally considered as the optimal systems for the production of secondary metabolites but when the productivity of batch cultures is calculated by dividing the concentration in stationary phase by the number of days to reach it, the productivity obtained with the semicontinuos culture is much higher (Fábregas *et al.*, 1998).

In the semicontinuous regime, the culture may be manipulated with daily inputs of nutrients, according to the renewal rate. With this process, the physiological condition of cells remains similar to that found in exponential phase (Fábregas *et al.*, 1995a).

The renewal rate is a valuable tool to control biomass and metabolite productivity in microalgae and cyanobacteria during steady-state in semicontinuos cultures. Every renewal rate modulates the metabolic activity of the cells in relation to nutrient availability and the effective light intensity. The high efficiency in biomass production of the cyanobacterium at different renewal rates, enables sufficient growth rate to regenerate the cell population every 24 h. This sustained production applies equally to the remaining metabolites.

In this study, the highest cell density stabilization (obtained at 20 %) agreed with the results reported for the microalga *Dunaliella tertiolecta* (Fábregas *et al.*, 1995a), which yielded high stabilization densities at daily renewal rates between 10 and 30 %.

In general, productivity is maximal at intermediate renewal rates (30 % and 40 %) and lower at higher rates (Otero *et al.*, 1997). Our results showed a peak at the highest renewal rate. Evidently, *Synechococcus* sp. is able to maintain an elevated growth rate, and consequently significant cell productivity, at the 50 % daily renewal rate.

Decreases in pigment, chlorophyll *a* and carotenoids content with increased renewal rate have been previously described in semicontinuous cultures of microalgae. Elevated chlorophyll synthesis at low renewal rates is due to limited light (Beardall & Morris, 1976). At higher renewal rates, more light is available due to decreased self-shading within cultures. Thus, the amount of pigments needed to optimize the photosynthesis rate is low, resulting in lower pigment content (Fábregas *et al.,* 1998).

Improved production of protein by the cyanobacterium under a semicontinuous regime depends on cell density. That is, at the lowest (20 %) renewal rate, with the lowest growth rate and nutrient concentration, protein production is higher. Conversely, at a daily renewal rate of 50 %, cells duplicate at higher rates and in high nutrient conditions, hence, the protein content is lower. A decrease in protein content with renewal rate, under nitrogen saturating conditions, has also been observed in the microalga *Nannochloropsis gaditana* (Maseda, 2002).

A decrease in carbohydrate production with renewal rates has also been described in the microalgae *Tetraselmis suecica* and *Phaeodactylum tricornutum* (Fábregas *et al.*, 1995b; 1996). In cultures at low renewal rates, cells start to accumulate reserve substances in the form of sugars, just as in mass cultures with low nutrient concentrations (Ben-Amotz, 1987).

Likewise, the high protein productivity obtained in *Synechococcus* at the moderate daily renewal rate of 40 %, has also been reported in the microalgae *Porphyridium cruentum, Dunaliella tertiolecta, Chlorella autotrophica, Tetraselmis suecica* and *Isochrysis galbana*, for daily renewal rates between 30 and 40 % (Otero, 1994).

Our observation that carbohydrate productivity and renewal rate are directly proportional conflicts with early studies, where the trend is for carbohydrate production to decrease with renewal rate, due to their use as a primary energy source (Otero *et al.,* 1997). For example, carbohydrate productivity decreases with renewal rate in the microalga *Phaeodactylum tricornutum,* maintained with sufficient nitrogen and in semicontinuous culture (Fábregas *et al.,* 1996).

However, the increase in carbohydrate synthesis in our strain of *Synechococcus*, may be related to increased exopolysaccharide production with increased renewal rate, as a physiological strategy to maintain sugar levels available for EPS synthesis.

The sulfated polysaccharide production in the microalga *Porphyridium cruentum* was stimulated at the highest renewal rate, and therefore with increasing nutrient availability and effective light intensity in the semicontinuous cultures at 50 % (Fábregas *et al.*, 1998). Thus, these two factors seem to favor exopolysaccharide synthesis in *Synechococcus* sp., even though some studies relate nitrogen limitation to exopolysaccharide production (Beljanin & Trenkenšu, 1977; Moreno *et al.*, 1998; Giroldo & Vieira, 2002).

The direct effect of nitrogen availability on exopolysaccharide production had been reported previously in batch cultures with high nutrient concentrations (Arad *et al.,* 1988; Friedman *et al.,* 1991). Here, *Synechococcus* was cultured in nutrient-sufficient conditions (8 mmol N l^{-1} $NaNO_3$); which could stimulate the exopolysaccharide production in semicontinuous cultures.

The results suggest that our strain of *Synechococcus* may be used at elevated renewal rates of 40-50 % for daily production of pigments, proteins, carbohydrates and EPS, in comparison to batch cultures, which may be used only during the stationary phase. In addition, we demonstrated that renewal rate exerts control on productivity and biochemical composition of semicontinuous cultures.

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