Growth of *Synechococcus* sp. immobilized in chitosan with different times of contact with NaOH

Bily Aguilar-May · M. del Pilar Sánchez-Saavedra ·
Jaime Lizardi · Domenico Voltolina

Received: 15 May 2006 / Accepted: 8 September 2006 / Published online: 29 November 2006
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Abstract The thickness of the walls of the capsules of chitosan-immobilized *Synechococcus* cultures was dependent on the time of contact with NaOH and was directly related to culture growth. After an initial lag phase, probably caused by cell damage, the capsules obtained after 80 s in a 0.1 N NaOH solution showed better growth than that of free cell cultures (6.9 and 5.2 divisions in 10 days, respectively).

Key words *Synechococcus* sp. · immobilization · chitosan · growth

The most common substrates for the immobilization of live cells are carrageenan and alginate (Cohen 2001), but the first is expensive and the temperature required for immobilization may be too high for some

species (Varlop and Klein 1987), whereas alginate is less expensive but has a low stability in presence of citrate and phosphate, which are used as buffering or chelating agents of growth media (Kierstan and Coughlan 1985).

Chitosan is soluble in acid solutions but insoluble in neutral and alkaline solutions, which allows the formation of gel networks at room temperature (Varlop and Klein 1987). In addition, it has other desirable properties, being biodegradable, non-toxic, stable at high temperatures and biocompatible (Tan and Lee 2002).

Synechococcus is a cosmopolite, non-toxic cyanobacteria with a good potential for aquaculture and biotechnology (Matsunaga et al. 1988; Sasikala and Ramana 1994), because of its physiological characteristics and biochemical composition. In this study, we determined the adequate conditions for the immobilization and growth in chitosan of a local strain of *Synechococcus* sp. isolated from the effluent of a shrimp farm, and kept in non-axenic cultures in *f* medium (Guillard and Ryther 1962) under the same conditions used for the growth experiments ($27 \pm 0.1^\circ\text{C}$ and $35 \mu\text{E m}^{-2} \text{s}^{-1}$).

Chitosan was obtained from shrimp exoskeletons and its characteristics were: <0.7% ash, <0.1% protein, 90% deacetylation (determined by Fourier Transform Infrared Spectroscopy) and intrinsic viscosity ($[\eta]$) of 6.49 dL g^{-1} .

In preliminary trials, a 2.0% w/v chitosan solution in 1.0% v/v acetic acid at pH 4 was dripped from a

B. Aguilar-May · M. del Pilar Sánchez-Saavedra (✉)
Departamento de Acuicultura, Centro de Investigación
Científica y de Educación Superior de Ensenada,
Kilometro 107 carr. Tijuana a Ensenada,
Apartado Postal 2732, Ensenada,
Baja California, Mexico 22800
e-mail: psanchez@cicese.mx

J. Lizardi
Laboratorio de Biopolímeros,
Centro de Investigación en Alimentación y Desarrollo,
Apartado Postal 1735, Hermosillo, Sonora, Mexico 83000

D. Voltolina
Laboratorio de Estudios Ambientales UAS-CIBNOR,
Centro de Investigaciones Biológicas del Noroeste,
Apartado Postal 1132, Mazatlan 82000, Sinaloa, Mexico

Figure 1 Mean values of optical density of cultures of *Synechococcus* sp. immobilized in chitosan, with 80 s (—) 140 s (---) and 300 s (···) of contact time with 0.1 M NaOH. Bars indicate one standard deviation.

25 mL buret with a 0.5 mm tip into 7.14 μM , 0.03 M, 0.3 M solutions of NH_4OH and 0.05 M and 0.1 M solutions of NaOH. These preliminary trials showed that only the 0.1 M NaOH solution allowed continuous capsule production, probably because of the volatility of NH_4OH at high pH, whereas the lower concentration of NaOH did not allow a complete gelling. Therefore, *Synechococcus* sp. cultures in exponential growth were centrifuged at 2,465 g for 30 min and 0.5 mL aliquots (with a cell concentration of 1.4×10^8 cells mL^{-1}) were resuspended in 10 mL of chitosan solution and dripped into 100 mL of 0.1 N NaOH, allowing contact times of 80, 140 and 300 s. A new 100 mL NaOH solution was used for each 10.5 mL of chitosan-cells mixture.

The capsules were concentrated on a sterile 1 mm sieve, the residual NaOH was eliminated by repeated washings in sterile filtered seawater until pH 8, and the capsules were incubated during 12 days in triplicate test tubes with 10 mL of *f* medium. Daily optical density (OD) readings at 550 nm served to determine the contact time that gave the best growth. New capsules prepared with 80 s contact time were kept in triplicate cultures in 250 mL Erlenmeyer flasks with 150 mL of *f* medium, and their growth was compared to that of similar cultures of free cells.

The initial concentration of all cultures was 0.07×10^8 cells mL^{-1} and the concentration of each culture obtained by daily counts with a haemocytometer was used to calculate its daily growth rate (μ) after $\log_2$ transformation. This was added to that of the previous day to obtain the accumulated growth rate ($\Sigma\mu$) that, when plotted against the age of the

cultures, pinpoints the end of exponential growth as a change of its slope (Nieves et al. 2005).

All data were normal and homoscedastic (Lilliefors' and Bartlett's tests). Therefore, differences in growth caused by the contact time in NaOH were determined using one-way analysis of variance (ANOVA) and Tukey's multiple comparison test. Differences between the initial and final mean concentrations of free and immobilized cell, and of the respective mean values of μ and $\Sigma\mu$, were determined using Student's *t* tests. The significance level was $\alpha=0.05$ in all cases.

Possibly because the high pH of the NaOH solution caused some cell damage, OD decreased in all treatments during the first day of the first experiment. After this, OD increased slightly until days 4–5 in the capsules prepared with contact times of 300 and 140 s, and bleaching was observed in 60% of the capsules exposed for 300 s. The increase continued until day 9 in the capsules prepared with 80 s in the NaOH solution and the final mean OD reading was significantly higher ($P<0.05$) than those of the other treatments (Figure 1).

The mean thicknesses of the walls of the capsules obtained after 80 and 140 s in NaOH, measured in sections obtained with histological techniques, were 0.10 ± 0.01 and 0.19 ± 0.02 mm, whereas after 300 s the capsules were completely solid. This confirms that the growth of immobilized cells is inversely related to the thickness of the capsule wall, which affects nutrient and metabolite exchanges with the external medium (Hirano et al. 1980).

The end of exponential growth of the free cell cultures was day 4, as indicated by the change of

Figure 2 Mean number of total cell divisions ($\Sigma\mu$) of cultures of *Synechococcus* sp. cells, free (---) or immobilized in chitosan (—). Bars indicate one standard deviation.

slope of the mean accumulated growth rate, after which the daily growth rates decreased progressively until the end of the experiment. This trend and the final concentrations and mean growth rates are comparable to those obtained by Campa-Ávila (2002) under similar culture conditions. As a result, the free cell cultures did not show a lag phase, whereas the immobilized cells showed a lag of 1 day ($\Sigma\mu=0$), but increased with an almost constant rate, close to 1 division day⁻¹ during the following 6 days (Figure 2). This lag phase, observed by Chevalier and De la Noüe (1985) and Lau et al. (1998) in carrageenan-immobilized cells of *Scenedesmus obliquus* and *Chlorella vulgaris*, is the most probable effect of the new chemical and physical conditions inside the polymer matrix (Mallick 2002).

Probably because of cell damage during the immobilization process, the initial concentration of viable cells was significantly lower ($P<0.05$) in the cultures with immobilized cells. However, in agreement with similar studies (Rai and Mallick 1992), the final concentration (3.33×10^8 cells mL⁻¹), the mean exponential growth rate (0.96 divisions day⁻¹) and the total accumulated growth rate (6.90 divisions in 10 days) of the immobilized cultures were significantly higher ($P<0.05$) than those of the free cell cultures (Table 1).

These high cell concentrations and growth rates show that 80 s in 0.1 M NaOH are an adequate contact time to immobilize *Synechococcus* sp. cells in chitosan. This substrate meets the conditions suggested as desirable by Mallick (2002), that include non-toxicity of the polymer matrix, as well as transparency, stability in the culture medium, retention of cell biomass and high diffusivity of nutrients and gases.

Table 1 Mean initial and final cell concentrations (initial and final N, in 10⁸ cells mL⁻¹), mean growth rate during the exponential phase (μ) and mean number of cell divisions ($\Sigma\mu$) during the 10 days of culture of immobilized and free cells of *Synechococcus* sp. Mean $\pm$ SD; different letters indicate significant differences (Student's *t* tests; $\alpha=0.05$)

Growth parameters	Immobilized cells	Free cells
Initial N	0.03 $\pm$ 0.006 a	0.07 $\pm$ 0.001 b
Final N	3.33 $\pm$ 0.350 b	2.41 $\pm$ 0.037 a
μ (divisions day ⁻¹)	0.96 $\pm$ 0.050 b	0.75 $\pm$ 0.037 a
$\Sigma\mu$ (divisions in 10 days)	6.90 $\pm$ 0.245 b	5.16 $\pm$ 0.132 a

Acknowledgements Work supported by Centro de Investigación Científica y de Educación Superior de Ensenada (CICESE Project 7073) and by Consejo Nacional de Ciencia y Tecnología (CONACyT Project 45844). The first author acknowledges a CONACyT PhD scholarship. S. Fierro-Reséndiz gave technical assistance and M. Segovia, M.I. Castro-Jiménez and L. Salinas-Flores revised the initial draft of the manuscript.

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