Appl Microbiol Biotechnol (1994) 41:137–141

Applied and Microbiology Biotechnology

Nitrite uptake by *Chlamydomonas reinhardtii* cells immobilized in calcium alginate

Carlos Vílchez, José M. Vega

Departamento de Bioquímica Vegetal y Biología Molecular, Facultad de Química, Apartado 553, E-41080 Sevilla, Spain

Received: 11 June 1993/Received revision: 13 September 1993/Accepted: 17 September 1993

Abstract. When an initial cell loading of about 30–40 μg chlorophyll (Chl)·g^{-1} gel and alginate suspension of 3% (w/v) were used for immobilization of *Chlamydomonas reinhardtii*, the resulting cell beads showed optimum nitrite uptake rate, at 30° C and pH 7.5, of 14 μmol NO_2^-·mg^{-1} Chl·h^{-1}, the photosynthetic and respiratory activities being about 120 μmol O_2 produced·mg^{-1} Chl·h^{-1}, and 40 μmol O_2 consumed·mg^{-1} Chl·h^{-1}, respectively. The nitrite uptake activity required CO_2 in the culture and persisted after 8 days of cells immobilization, or in the presence of 0.2 mM ammonium in the medium. Our data indicate that alginate-entrapped *C. reinhardtii* cells may provide a stable and functional system for removing nitrogenous contaminants from waste-waters.

Introduction

The presence of high concentrations of inorganic nitrogen (N) compounds decreases the quality of drinking water, thus the use of photosynthetic microalgae, which are able to consume these N compounds for growth, has been investigated with the practical purpose of contaminant bioelimination (Painter 1977; Jeanfils and Thomas 1986).

In these micro-organisms, nitrate and/or nitrite are reduced to ammonium by the successive action of nitrate and nitrite reductase activities, using reduced pyridine nucleotides and ferredoxin as electron donors. Ammonium is thereafter incorporated into carbon skeletons through the glutamine synthetase-glutamate synthase cycle, for the synthesis of L-glutamate, and then N is finally distributed thorough the biological material (Vega et al. 1991).

Immobilized microalgae have been used to catalyse conversions of practical interest, because of the high stability shown and the low cost of the process (Fukui and Tanaka 1982). Immobilization significantly facilitates the harvesting of cells and the function of semi-continuous or continuous systems (Proulx and de la Noüe 1988).

In this paper we study the nitrite uptake from water by *Chlamydomonas reinhardtii* cells immobilized in calcium alginate, as an initial step required to establish optimal conditions for the potential use of the system in bioreactors. We followed the guidelines for the characterization of immobilized biocatalysts as proposed by the Working Party on Immobilized Biocatalysts of the European Federation on Biotechnology (1983).

Materials and methods

Organisms and standard culture conditions. Wild type *C. reinhardtii* strain 21 gr, was grown at 25° C in 15 mM phosphate (pH 7.5)-buffered culture medium containing 10 mM KNO_3 as the nitrogen source (Vílchez et al. 1991). The standard cultures (about 200 ml), in 250-ml conical flasks, were bubbled with air containing 5% (v/v) CO_2 and continuously illuminated with white fluorescence lamps (250 μE·m^{-2}·s^{-1}, at the surface of the tube). The cells were harvested in the exponential phase of growth (15 μg Chl·ml^{-1}) by centrifugation at 5000 *g* for 5 min, washed and resuspended in Tricine buffer.

Immobilization of C. reinhardtii cells by entrapment in alginate. The cells were harvested, washed and resuspended (0.5–1%, w/v) in 20 mM Tricine-NaOH (pH 8.0)-buffered culture medium and they were throughly mixed with an equal volume of an alginate solution (6%, w/v) prepared by mixing 4% alginic acid and 2% alginate sodium salt and NaOH to reach pH 6.5–7.5. The final viscosity (7000 centipoises, cP) depended on the proportion of alginic acid and alginate mixed. Beads of about 3 mm diameter were obtained by dropping the alginate cell mixture into a solution of 0.1 M $CaCl_2$ or $BaCl_2$ at 4° C and after 5 h they were rinsed with fresh culture medium and were ready for use.

Measurement of photosynthetic and respiratory activities. The photosynthetic activity was determined using a Clark-type electrode to measure light-dependent O_2-production from the alginate-entrapped *C. reinhardtii* cells (ten beads) into 1.5 ml of 20 mM Tricine-NaOH (pH 8.0)-buffered culture medium. The measurements were made at 25° C under saturating white light illumination (1500 μE·m^{-2}·s^{-1}). The respiratory activity was

Correspondence to: C. Vílchez

determined by measuring the O_2 uptake in the dark by the immobilized cells under the conditions described above.

Nitrite uptake conditions. Nitrite uptake experiments were carried out at 25° C, in small batch reactors (20 cm height and 4 cm diameter), containing 100 ml of 20 mM Tricine-NaOH (pH 7.5)-buffered culture medium supplemented with 600 μM nitrite as minimum, and 10% (w/v) of immobilized cells. Lower amounts of nitrite were not used because of rate-limiting conditions.

Analytical determinations. Chlorophyll was determined by extracting the free cells with acetone. For immobilized cells, the beads were extracted with acetone overnight. After removing the non-extracted material by centrifugation, the absorbance at 652 nm was determined in the supernatant ($\epsilon = 34.5$ $mg \cdot ml^{-1} \cdot cm^{-1}$). Further details are in Vílchez et al. (1991).

Nitrite in the medium was determined according to the colorimetric method of Snell and Snell (1949).

Results

Influence of immobilization conditions on the biological activities of C. reinhardtii

When *C. reinhardtii* cells are immobilized in alginate beads, Ca^{2+} or Ba^{2+} can be used as hardening cations, without significant changes in their biological activities (data not shown).

Cell loading is critical for work with immobilized photosynthetic microalgae. The nitrite uptake rate decreased with increasing cell loading in the beads, from 14 μmol $NO_2^- \cdot mg^{-1}$ $Chl \cdot h^{-1}$ at an initial cell loading of 20 μg $Chl \cdot g^{-1}$ gel, to a rate of 5.6 μmol $NO_2^- \cdot mg^{-1}$ $Chl \cdot h^{-1}$ at 100 μg $Chl \cdot g^{-1}$ gel. In parallel, the photosynthetic activity decreased from 150 to 70 μmol $O_2 \cdot mg^{-1}$ $Chl \cdot h^{-1}$, within the above range of initial cell loading, while the respiratory activity shown by the immobilized cells was in the range of 32–20 μmol $O_2 \cdot mg^{-1}$ $Chl \cdot h^{-1}$ (Fig. 1). These data suggest a significant influence of shading effect caused by the outermost cells of the beads. Unless otherwise indicated, the initial cell loading used for further experiments ranged between 30 and 40 μg $Chl \cdot g^{-1}$ gel.

The concentration of alginate suspension determines the diffusion of substrates through the matrix. In Fig. 1 we can see a decrease in the nitrite uptake rate, photosynthetic and respiratory activities of the immobilized cells when the alginate concentration was increased. The use of 3% (w/v) alginate in the cell suspension for the immobilization gave good levels of nitrite uptake rate and photosynthetic activity.

Effect of environmental and nutritional conditions on the biological activities of C. reinhardtii

The nitrite uptake rate shown by immobilized *C. reinhardtii* cells was higher at every temperature than that of freely suspended cells. In addition, this rate was high in the 20–40° C temperature range, reaching a maximum of 14 μmol $NO_2^- \cdot mg^{-1}$ $Chl \cdot h^{-1}$ at 30° C (Fig. 2). A maximum photosynthetic activity of 240 μmol $O_2 \cdot mg^{-1}$ $Chl \cdot h^{-1}$ was shown by immobilized cells at

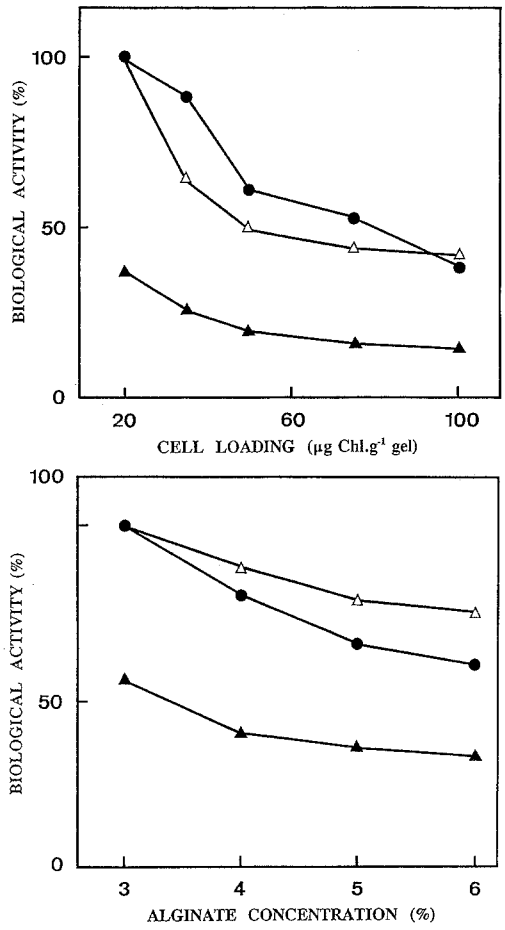

Fig. 1. Effect of cell loading and alginate concentration in the beads on biological activities of immobilized *Chlamydomonas reinhardtii*. Cells collected from standard cultures, washed and resuspended in 20 mM Tricine-NaOH (pH 8.0) buffer were used to prepare algal suspensions with different concentrations of chlorophyll (Chl) and alginate. The cell immobilization, nitrite uptake rate (●), photosynthetic (△) or respiratory (▲) activities were performed as indicated in Materials and methods: 100% was 14 μmol $\cdot mg^{-1}$ $Chl \cdot h^{-1}$ for nitrite uptake, and 150 μmol $O_2 \cdot mg^{-1}$ $Chl \cdot h^{-1}$ for either photosynthetic and respiratory activities

40° C, whereas freely suspended cells showed better activity at 35° C.

The effect of pH on the biological activities of *C. reinhardtii* cells is shown in Fig. 3. The nitrite uptake rate shown by the immobilized system was high in the pH range 6.5–8.0, reaching a maximum at pH 7.5, corresponding to the optimal photosynthetic activity in the cells. At this pH the values shown by the free cells were significantly lower, especially for nitrite uptake rate.

In carbon-depleted *C. reinhardtii* cells the nitrite uptake rate was negligible. When CO_2 was newly supplied to cultures, immobilized cells recovered their activity after a lag phase of 10 min and reached the maximum after 40 min of CO_2 addition (Fig. 4). The curve shown by the free-cell system was slightly different, lacking a lag phase in recovering their activity, but reached a lower maximum than the immobilized cells.

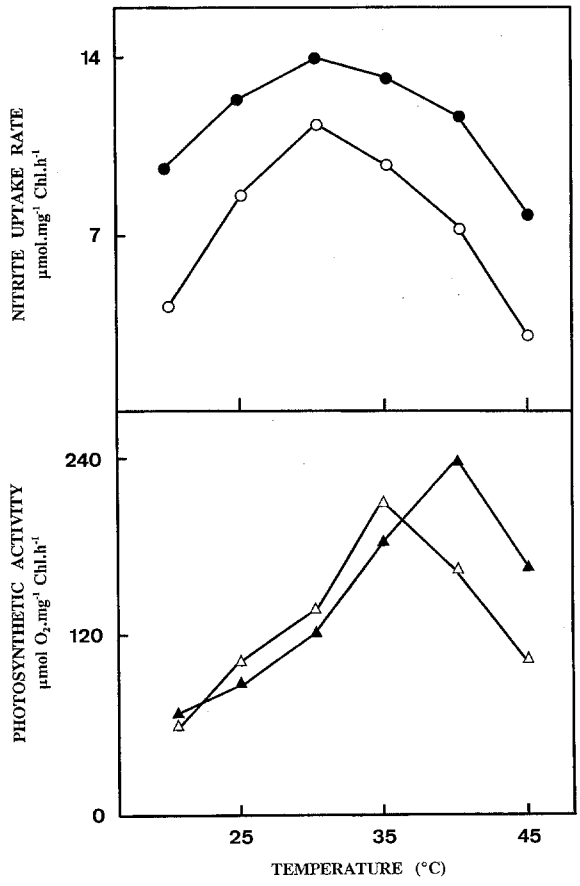

Fig. 2. Effect of temperature on biological activities of *C. reinhardtii*. Cells collected from standard cultures, washed and resuspended in the Tricine buffer to 30 μg Chl·ml^{-1} were used as the free system. Immobilized cell systems, prepared as indicated in Fig. 1, contained 30 μg Chl·g^{-1} gel. The nitrite uptake rate by free (○) or immobilized cells (●), and photosynthetic activity by free (△) or immobilized cells (▲) were determined as indicated in Materials and methods at different temperatures

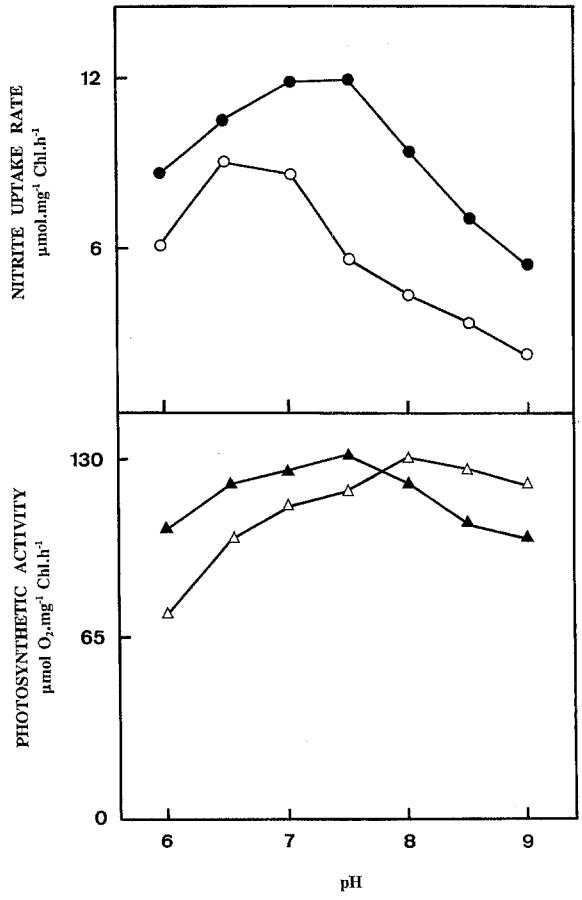

Fig. 3. Effect of pH on biological activities of *C. reinhardtii*. The cell systems used were prepared as indicated in Fig. 1. The nitrite uptake rate by free (○) or immobilized (●) cells, and photosynthetic activity by free (△) or immobilized cells (▲) were determined as indicated in Materials and methods at different pH

Kinetic studies

The substrate saturation constant (K_s) for nitrite uptake shown by immobilized *C. reinhardtii* cells, as deduced from the Lineweaver-Burk treatment of the rate data obtained at different nitrite concentrations, was 80 μM (data not shown). This value is higher than the 1.5 μM for freely suspended cells (Córdoba et al. 1986).

In order to determine the capacity of the immobilized cells for elimination of nitrite from a water source it is important to check its nitrite uptake capacity in the presence of ammonium, an inhibitor of this process in freely suspended cells (Florencio and Vega 1983). Figure 5 shows that ammonium inhibition decreased with increasing cell loading, indicating that immobilized cells of *C. reinhardtii* are an attractive system for nitrite elimination.

The storage stability of the immobilized system is another interesting property to be considered in this biotechnological process. Figure 6 shows that immobilized cells retain 80% of their nitrite uptake capacity

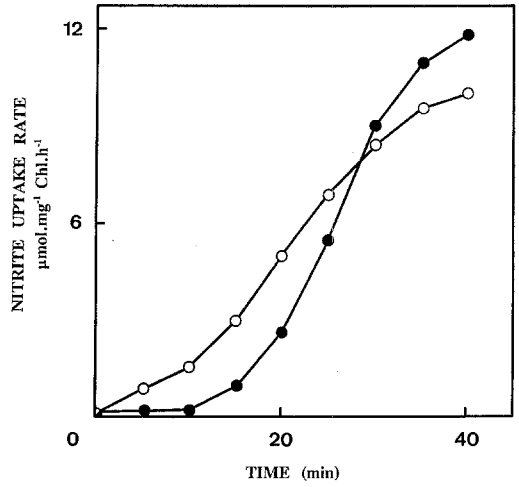

Fig. 4. Effect of CO_2-stressed conditions on nitrite uptake by *C. reinhardtii*. The cell systems used were prepared as indicated in Fig. 2. Free (○) or immobilized (●) cells were bubbled with CO_2-free air for 1.5 h. Then CO_2 was newly supplied according to the standard growth conditions, and nitrite uptake rate was determined as indicated

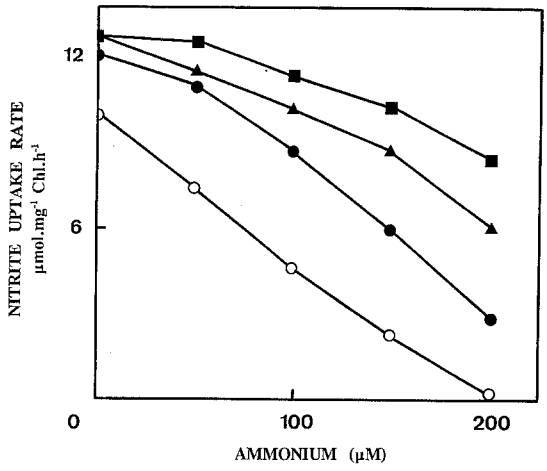

Fig. 5. Nitrite uptake inhibition by ammonium in *C. reinhardtii*. The cell systems used were prepared as indicated in Fig. 2, but using the identicated cell loading. Nitrite uptake rate by free (○) or immobilized cells (●, 15; ▲, 60; ■, 100 μg Chl·g^{-1} gel) was determined in the presence of different concentrations of ammonium in the medium

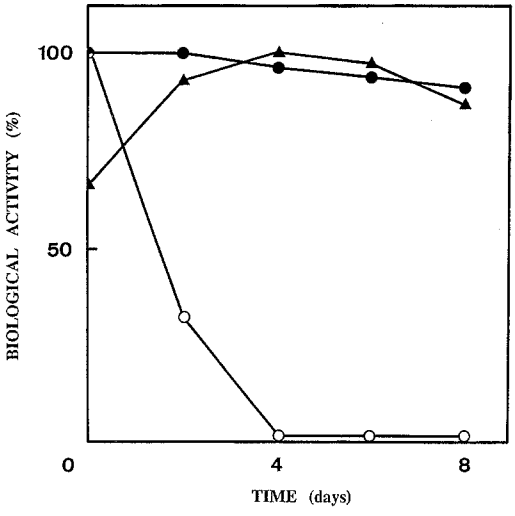

Fig. 6. Effect of storage time on the biological activities of *C. reinhardtii*. The cell systems used were prepared as indicated in Fig. 2. Nitrite uptake rate by free (○) or immobilized (●) cells, and photosynthetic activity by immobilized (▲) cells were determined after different periods of storage at 4° C

after 8 days storage at 4° C, whereas the activity of freely suspended cells was negligible after the 4th day.

Discussion

C. reinhardtii cells immobilized in calcium alginate beads may provide a continuous biological system for eliminating nitrogenous contaminants from waters. Since inorganic N assimilation by microalgae is closely dependent on photosynthetic electron flow (Vega et al. 1991), we have fixed the optimal conditions of the system using the nitrite uptake rate and photosynthetic activity as basic parameters.

Initial cell loading is a critical factor in this immobilized system because growth of the alga during culture produces two kinds of effects: (a) Shading of cell colonies located near the surface of the bead over the internal ones (Vílchez et al. 1991), which produces a limitation of the photosynthetic efficiency, thus decreasing the nitrite uptake rate, and (b) problems for substrate diffusion through the bead because the cells occupy a significant fraction of the useful volume of the bead, and also because the exit route for molecules is longer (Tanaka et al. 1984; Westrin and Axelsson 1991).

Particularly important is the enrichment of photosynthetically produced O_2 in the beads, determining an O_2/CO_2 ratio in the beads environment that increases cell photorespiration, thus decreasing their viability. Therefore, it seems very suitable to use an initial small charge of cells, such as 30–40 μg Chl·g^{-1} gel, in order to retard the shading effect and increase in photorespiration activity, allowing cell growth for a few days. This is particularly active near the bead surface, thus favouring an efficient distribution of cells in the matrix (Wijffels et al. 1991). These considerations are consistent with those reported in *Nitrobacter agilis* (Tramper and de Man 1986) and *Streptomyces clavuligerus* (Scott et al. 1988).

Problems of substrate diffusion also depend on the alginate concentration used for cell immobilization (Garbisu et al. 1991; Bailliez et al. 1986). Our data indicate that 3% (w/v) alginate in the cell suspension is adequate for minimizing this problem and allowed us to obtain beads with a physical consistency that avoids cells leakage and/or disruption of the system. In addition the cells retain good viability.

Particularly interesting from a biotechnological point of view are the specific properties of immobilized systems compared to the freely suspended ones:

1. The cells show high resistance to nitrite toxicity, as described in *N. agilis* (Tramper and de Man 1986) or in *Phormidium laminosum* (Serra et al. 1990).
2. The ammonium-dependent inhibition of nitrite assimilation by *C. reinhardtii* cells is minimized.
3. The high stability of the system, mainly concerning the biological viability of the cells.

We have studied the environmental and nutritional conditions for optimum operation of immobilized *C. reinhardtii* cells in assimilating nitrite. According our data immobilized cells may assimilate nitrite at 14 μmol·mg^{-1} Chl·h^{-1}, at 30° C and pH 7.5. The system may operate for a few days, requires CO_2 and shows high resistance to toxicity by an excess of nitrite or inhibition by ammonium. These observations suggest good possibilities for this system in bioreactors.

Acknowledgements. This work has been supported by Research Grant no. PB90-0880 from the Comisión Interministerial de Ciencia y Tecnología. One of us (C. V.) was the recipient of an F.P.I. fellowship from the Ministerio de Educación y Ciencia. We wish to thank Dr. Cecilia Gotor for a critical reading of the manuscript.

References

Bailliez C, Largeau C, Berkaloff C, Casadevall E (1986) Immobilization of *Botriococcus braunii* in alginate: influence on chlorophyll content, photosynthetic activity and degeneration during bath cultures. Appl Microbiol Biotechnol 23:361–366

Córdoba F, Cárdenas J, Fernández E (1986) Kinetic characterization of nitrite uptake and reduction by *Chlamydomonas reinhardtii*. Plant Physiol 82:904–908

Florencio FJ, Vega JM (1983) Utilization of nitrate, nitrite and ammonium by *Chlamydomonas reinhardtii*. Planta 158:288–293

Fukui S, Tanaka K (1982) Immobilized microbial cells. Annu Rev Microbiol 36:145–172

Garbisu C, Gil JM, Bazin MJ, Hall DO, Serra JL (1991) Removal of nitrate from water by foam-immobilized *Phormidium laminosum* in bath and continuous-flow bioreactors. J Appl Phycol 3:221–234

Jeanfils J, Thomas D (1986) Culture and nitrite uptake in immobilized *Scenedesmus obliquus*. Appl Microbiol Biotechnol 24:417–422

Painter HA (1977) Microbial transformations of inorganic nitrogen. Prog Water Technol 8:8–29

Proulx D, Noüe J de la (1988) Removal of macronutrients from wastewater by immobilized algae. In: Moo-Young M (ed) Bioreactor immobilized enzymes and cells. Fundamentals and applications. Elsevier, London, pp 301–310

Scott RI, Wills SJ, Bucke C (1988) Oxygen uptake by carrageenan entrapped *Streptomyces clavuligerus*. Enzyme Microb Technol 10:151–155

Serra JL, Arizmendi JM, Blanco F, Martínez-Bilbao M, Alaña A, Fresnedo O, Urkijo I, Llama MJ (1990) Nitrate assimilation in the non N_2-fixing cyanobacterium *Phormidium laminosum*. In: Ullrich WR, Rigano C, Fuggi A, Aparicio PJ (eds) Inorganic nitrogen metabolism. Springer, Berlin Heidelberg New York, pp 196–202

Snell FD, Snell CT (1949) Colorimetric methods of analysis, vol 3. Van Nostrand, New York, pp 804–805

Tanaka H, Matsumura M, Veliky IA (1984) Diffusion characteristics of substrates in Ca-alginate gel beads. Biotechnol Bioeng 26:53–58

Tramper J, Man AWA de (1986) Characterization of *Nitrobacter agilis* immobilized in calcium alginate. Enzyme Microb Technol 8:472–476

Vega JM, Menacho A, León J (1991) Nitrate assimilation by macroalgae. Trends Photochem Photobiol 2:69–111

Vílchez C, Galván F, Vega JM (1991) Glycolate photoproduction by free and alginate-entrapped cells of *Chlamydomonas reinhardtii*. Appl Microbiol Biotechnol 35:716–719

Westrin BA, Axelsson A (1991) Diffusion in gels containing immobilized cells: a critical review. Biotechnol Bioeng 38:439–446

Wijffels RH, Gooijer CD de, Kortekaas S, Tramper J (1991) Growth and substrate consumption of *Nitrobacter agilis* cells immobilized in carrageenan: part 2. Model evaluation. Biotechnol Bioeng 38:232–240

Working Party on Immobilized Biocatalysis of the European Federation on Biotechnology (1983) Guidelines for the characterization of immobilized biocatalysts. Enzyme Microb Technol 5:304–307