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ORIGINAL PAPER

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Continuous marennin production by agar-entrapped *Haslea ostrearia* using a tubular photobioreactor with internal illumination

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Abstract The marine diatom *Haslea ostrearia* was immobilized in a tubular agar gel layer introduced into a photobioreactor of original design with internal illumination for the continuous synthesis of marennin, a blue-green pigment of biotechnological interest. Marennin was produced for a long-term period (27–43 days) and the volumetric productivity was maximum (18.7 mg day^{-1} l^{-1} gel) at the highest dilution rate (0.25 day^{-1}) and lowest agar layer thickness (3 mm). Heterogeneous cell distribution in the agar layer revealed diffusional limitation of light and nutrients. However, the 3 mm gel thickness led to a more homogeneous cell distribution during incubation and to an increase of the whole biomass in the agar gel layer.

Introduction

The pennate diatom *Haslea ostrearia* is able to synthesize and excrete a hydrosoluble, blue-green pigment named marennin, which is responsible for the greening of the oyster's gills. More recently, an anti-proliferative effect has been shown in the lung cancer model (Carbonnelle et al. 1999), with a prospect of pharmacological development.

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In order to produce this pigment for the greening of oysters at the industrial level, batch free-cell cultures are currently performed, using 6-m^3 tanks under non-axenic and partially controlled incubation conditions (Robert 1989). However, to standardize marennin production in terms of quantity and quality, efficient closed systems are required to allow more careful control of levels of nutrients such as nitrogen (Neuville and Daste 1972) and lighting (Schubert et al. 1995).

In the natural environment, the number of pigmented cells increased during the migration of algae from the planktonic to the benthic compartment and became maximum during the benthic stage (Robert 1983) where algal cells were immobilized in their own exopolysaccharides (Rincé et al. 1999). Artificial immobilization is a suitable technique to reproduce this natural phenomenon and seems the more adapted to microalgae, particularly to *H. ostrearia* cells, which are very sensitive to disturbance (Yang and Wang 1992; Rouillard 1996). Several microalgal species have been entrapped in various transparent polymer matrices for exometabolite production, and immobilized-cell techniques depend on the matrix composition. Polyurethane foams (Bailliez et al. 1988) and some cations used in alginate beads (Tamponnet et al. 1985) are particularly toxic for the majority of microalgae, while polysaccharides extracted from seaweed, such as sodium alginate (Hertzberg and Jensen 1989; Kannapiran et al. 1997), are suitable for diatoms, although phosphate in media from seawater led to partial dissolution of the alginate gel (Brouers and De Jong 1988; Travieso Cordoba et al. 1995). In a previous work (Lebeau et al. 1999), *H. ostrearia* was successfully entrapped in agar gel discs for long-term marennin production. About 8×10^{10} cells l^{-1} gel were reached, whereas the free-cell concentration did not exceed 1×10^5 cells l^{-1} medium, and the average specific productivities of immobilized cells were higher than those of free cells.

In studies dealing with immobilized microalgal cells, standard bioreactors derived from those devoted to non-

photosynthetic cells have been used, either packed-bed or fluidized-bed (Santos-Rosa et al. 1989; Travieso et al. 1992; Vilchez and Vega 1995). However, the hazards related to the limited transfer of light and nutrients into the matrices in which the microalgal cells are immobilized require suitable photobioreactors. To overcome these problems, the few photobioreactors devoted to immobilized microalgal cells are characterized by a high surface exchange between algal cells and nutrients and light (Wang et al. 1991; Chetsumon et al. 1993; Kaya et al. 1996) and/or an illumination device (Junter et al. 1989; Burgess et al. 1993).

In the present study, we made use of a bioreactor prototype dedicated to immobilized photosynthetic cells (Junter et al. 1989) – previously used for hydrogen gas production (Planchard et al. 1989) – for the production of marennin. *H. ostrearia* cells were entrapped in a layer of agar gel and cultured in continuous mode. To optimize the production of marennin, we tested the influence of the gel layer thickness (3 mm and 6 mm), the culture medium (F/2 and F/20) and the dilution rate (0.025 day^{-1} and 0.25 day^{-1}).

Materials and methods

Algal strain and maintenance medium

The study was performed using an axenic strain of *H. ostrearia* Simonsen isolated from oyster pond waters of the Bouin district (Vendée, France). The cells used (strain HO1, ISOmer, Nantes, France) to maximize marennin production were characterized by an average modal length of 70 μm. The algal cultures were maintained by weekly transfer to fresh ES 1/3 medium (Table 1). The pH of the medium was set to 7.8, the incubation was at 15 °C and light (horticultural neon lighting, 36 W) was provided at 3×10^{16} quanta cm^{-2} s^{-1} with a 14-/10-h light/dark cycle.

Preparation of inocula

Algal precultures were performed applying a two-step procedure in the same culture conditions as for cell maintenance. Cells from the clone pool were first precultured for about 6 days in 250-ml Erlenmeyer flasks filled with 150 ml maintenance (ES 1/3) medium. Then the flask contents were inoculated in 2 l Erlenmeyer flasks containing 1 l (ES 1/3) medium. Algal inocula were collected by centrifugation (4000*g*, 6 min, 15 °C) from cultures in the exponential growth stage after incubation of these larger flasks for 6 days. Cell suspensions with concentrations ranging between

Table 1 Composition of ES 1/3 and F/2 media

ES 1/3 medium:		F/2 medium:	
Enrichment solution (see below)	7 ml	Vitamin solution (see below)	1 ml
Stock metasilicate solution (see below)	1 ml	Filtered seawater (salinity adjusted to 28 g/l with distilled water)	999 ml
Vitamin solution (see below)	2 ml	Sterilize at 121 °C for 20 min	
$NaHCO_3$	80 mg	Major nutrient solution	1 ml
Filtered seawater (salinity adjusted to 28 g/l with distilled water)	1000 ml	Trace metal solution	1 ml
Adjust pH to 7.8. Sterilize at 121 °C for 20 min			
		Major nutrient solution	
Enrichment solution		$NaNO_3$	7.5 g
$NaNO_3$	350 mg	Distilled water	100 ml
Sodium glycerophosphate ($C_3H_7O_6PNa_2 \cdot 6H_2O$)	50 mg	$NaH_2PO_4 \cdot H_2O$	0.5 g
Tris(hydroxymethyl)aminomethane (Tris buffer)	500 mg	Distilled water	100 ml
Fe-EDTA solution (see below)	2.5 ml	$Na_2SiO_3 \cdot 9H_2O$	3 g
Trace metal solution (see below)	25 ml	Distilled water	< 100 ml
Distilled water	100 ml	Sterilize each nutrient separately at 121 °C for 20 min	
Fe-EDTA solution			
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	3.51 g	*Trace metal solution*	
Na_2EDTA	3.50 g	Na_2EDTA	436 mg
Distilled water	500 ml	$FeCl_3 \cdot 6H_2O$	315 mg
		$CuSO_4 \cdot 5H_2O$ (98 mg/100 ml water)	1 ml
Trace metal solution		$ZnSO_4 \cdot 7H_2O$ (220 mg/100 ml water)	1 ml
H_3BO_3	570 mg	$CoCl_2 \cdot 6H_2O$ (100 mg/100 ml water)	1 ml
$FeCl_3 \cdot 6H_2O$	24.5 mg	$MnCl_2 \cdot 4H_2O$ (1800 mg/100 ml water)	1 ml
$MnSO_4 \cdot H_2O$	62.15 mg	$Na_2MoO_4 \cdot 2H_2O$ (63 mg/100 ml water)	1 ml
$ZnSO_4 \cdot 7H_2O$	11 mg	Distilled water	95 ml
$CoSO_4 \cdot 7H_2O$	2.4 mg	Sterilize at 121 °C for 20 min	
Na_2EDTA	500 mg		
Distilled water	500 ml	*Vitamin solution*	
		Thiamine – HCl	10 mg
Stock metasilicate solution		Biotin (5 mg/100 ml water)	1 ml
$Na_2SiO_3 \cdot 5H_2O$	10.607 g	Vitamin B_{12} (5 mg/100 ml water)	1 ml
Distilled water	500 ml	Distilled water	98 ml
Vitamin solution			
Thiamine	500 mg		
Biotin solution (20 mg/100 ml water)	1 ml		
Vitamin B_{12} solution (20 mg/100 ml water)	1 ml		
Distilled water	500 ml		

1×10^7 and 1×10^8 cells ml^{-1} (7.3–73 mg dry wt ml^{-1}) were obtained.

Cell immobilization

Microalgae were entrapped in an agar gel layer (Diagnostic Pasteur; Pastagar A, 15 g l^{-1} seawater, the salinity of which was adjusted to 28 g l^{-1} with distilled water), tubular in shape (internal diameter, 15 cm; height, 19 cm; outer surface for 0.6 cm gel layer thickness (experiments A and B), 967 cm^2, or, for 0.3 cm gel layer thickness (experiment C), 931 cm^2, according to the following protocol: after heating the agar suspension to 100 °C, the solution was cooled to 35 °C, to keep cells alive, and mixed thoroughly with cell suspension until the desired cell concentration was reached, i.e., 2×10^6 cells ml^{-1} gel (1.46 mg dry wt ml^{-1}). The mixture was then introduced into a mould delimited by an inner glass cylinder directly in contact with the barrel of optical fibres and an outer cylindrical stainless-steel grid covered with an aluminium sheet until gel hardening was achieved (after about 30 min at 4 °C) after which aluminium sheet was removed.

Reactor and experimental setup

Figure 1 shows the design of the immobilized-cell photobioreactor in simplified form (Mignot et al. 1989). The photobioreactor was filled with 10 l F/2 (Table 1; Guillard 1982) or F/20 (Rouillard 1996) medium. F/20 consists of F/2 diluted ten times with salinity adjusted to 28 g l^{-1} with distilled water. In the two media, 80 mg $l^{-1}NaHCO_3$ was added.

Immobilized cells were fed continuously: (1) F/2 at the dilution rate (D) value of 0.025 day^{-1} during experiment A, (2) F/20 at $D = 0.25$ day^{-1} during experiments B and C.

To reduce nitrate concentration within the gel during experiments, F/2 or F/20 medium without nitrate (F/2 0N or F/20 0N) were selectively used.

The photobioreactor was placed in an air-conditioned room with the temperature adjusted to 15 °C. The pH value of the medium was adjusted to 7.8 at the beginning of each experiment and during the incubation with HCl 1 *N*. The immobilized-cell layer was illuminated by the barrel of optical fibres connected to a 150-W light generator (model Lux 150 S, Fort, Dourdan, France) and light intensity was at 3×10^{16} quanta cm^{-2} s^{-1} (at the surface of the agar layer directly in contact with the source of light) with a 14-/10-h light/dark cycle.

Analytical procedures

Marennin concentrations (hydrosoluble pigment) were determined by measuring the optical density (OD) at 663 nm (Robert and Hallet 1981) of a membrane-filtered broth sample.

NO_3^- concentrations were determined with NitraVer 5 kit (Hach, Loveland, Colo., USA) for immediate analysis to quickly adjust the concentration level to a defined one (nitrate is reduced to nitrite, the concentration of which is determined by measuring the OD at 400 nm after reaction between nitrite and sulfanilic acid and gentisic acid). More accurate analysis was done a posteriori using a SKALAR auto-analyser (SKALAR Analytical, Technicon, Delft, Holland).

The final cell biomass was determined at the end of the incubation run. The algal population of immobilized-cell cultures was assessed by two methods. With the direct method, the algal population was assessed by cell counts using a Nageotte-type haematocytometer. Samples consisted of 1 ml of the cell-agar mixture obtained after heating of the whole agar layer, or one of the three slices obtained after the agar layer was cut off, and suitable dilution in distilled water (to reduce the sample viscosity and avoid too low sedimentation). The indirect method consisted in estimating the number of immobilized viable cells by chlorophyll a determination: the pigments were extracted from the agar gel layer after plunging it into an aqueous solution of acetone 90% (v/v) at 4 °C in darkness for 24 h. After centrifugation (1500*g*, 15 min), the absorbance at 665 nm before and after acidification with HCl 2 M was determined in the supernatant according to Lorenzen (1967).

To estimate the diffusion of the light through the agar layer, we determined a ratio I_t/I_i using a quantameter (LI-1000 Data Logger, LI-COR, Lincoln, Neb., USA). The incident light (I_i) was measured at the surface of the agar layer directly in contact with the barrel of optical fibres and the transmitted light (I_t) was measured at the surface of the agar layer opposite to the light source.

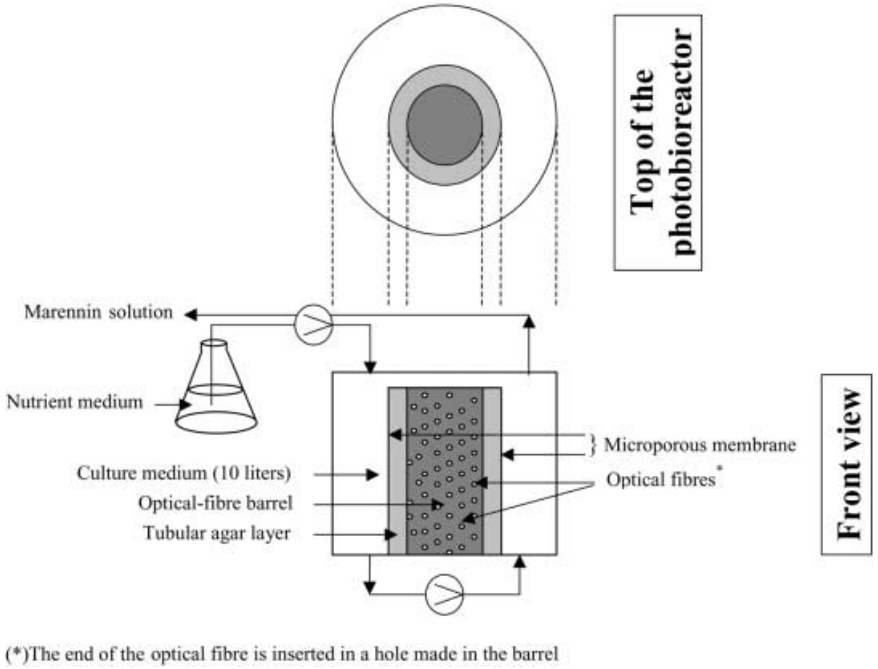

Fig. 1 Immobilized-cell photobioreactor used for marennin production

Results

Figure 2 shows continuous marennin production and nitrate uptake using the prototype photobioreactor with the agar layer loaded with 2×10^6 cells ml^{-1} gel. Whatever the culture conditions (F/2, 0.025 day^{-1} or F/20, 0.25 day^{-1}) and gel layer thickness (3 mm or 6 mm) used, marennin was synthesized for 27–43 days and incubations were stopped deliberately. To reduce nitrate concentration, periods that consisted of F/2 or F/20 medium without nitrate (Fig. 2a, b) were selectively alternated with short batch periods. During incubation, marennin concentrations began to appear as soon as nitrate concentrations decreased. In spite of the addition of $NaHCO_3$ to the medium, pH rose by 0.5 unit in 5–10 days during incubations (data not shown), requiring to be regulated regularly.

Comparison between experiments A and B – based on the same nutrient supply per day and the same agar layer thickness – showed the best marennin production to occur with the highest dilution rate (Table 2): maximum volumetric productivity during each of these experiments increased by about 4.5 whilst the maximum marennin concentration only decreased from 4.5 to 2.0 mg l^{-1} with a ten-fold higher dilution rate. If we compare against this experiments B and C, the reduction of the agar layer thickness, which roughly halved the volume of the agar layer, led to a slight decrease in the maximum volumetric productivity (including the volume of the liquid medium

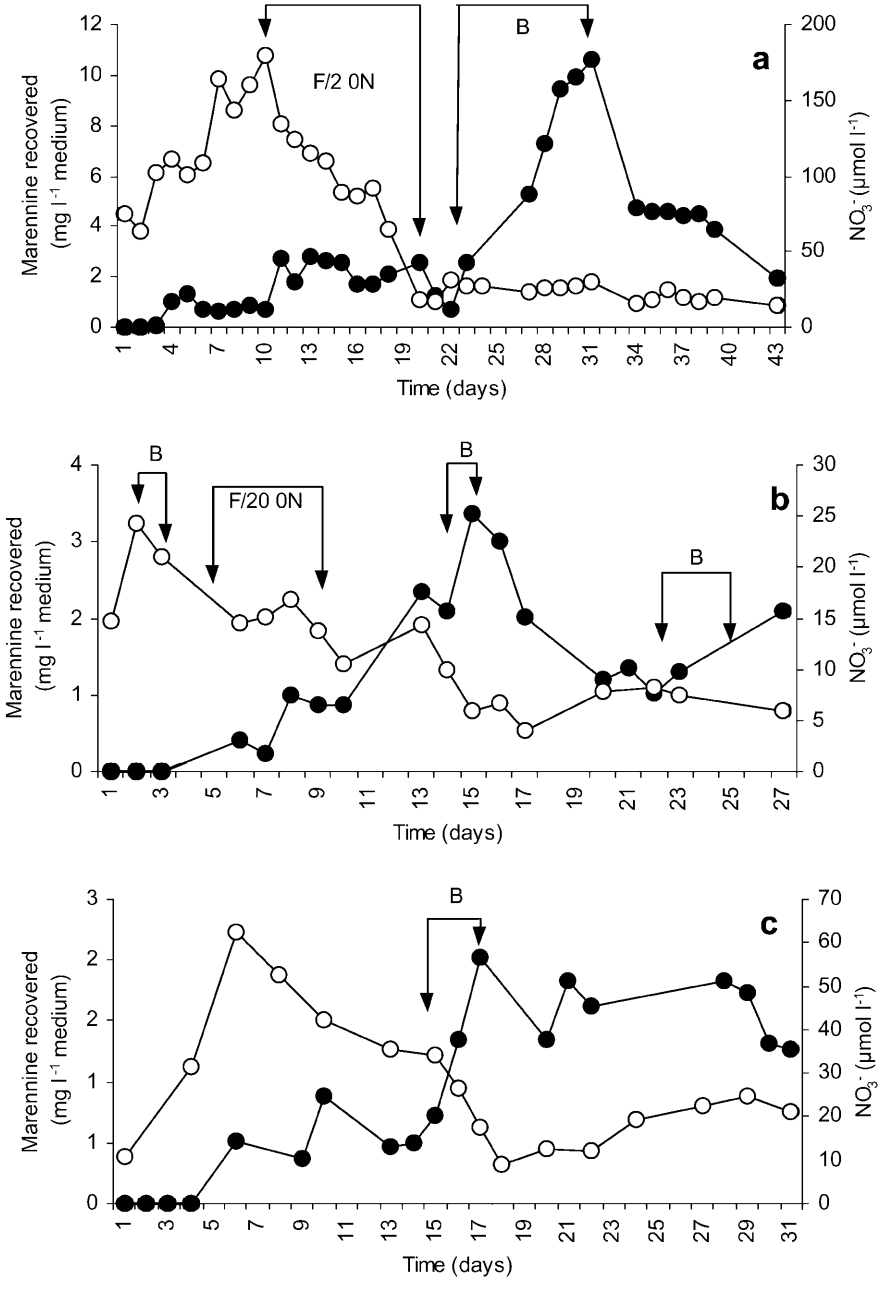

Fig. 2a–c Kinetics of marennin production (●) and nitrate uptake (○) by agar-entrapped cells of *Haslea ostrearia* (initial cell concentration, 2×10^6 cells ml^{-1} gel): **a** experiment A, F/2 medium, 0.025 day^{-1}, gel layer thickness 6 mm; **b** experiment B, F/20 medium, 0.25 day^{-1}, gel layer thickness 6 mm; **c** experiment C, F/20 medium, 0.25 day^{-1}, gel layer thickness 3 mm. *B* Batch period

Table 2 Parameters for marennin production by immobilized *H. ostrearia*

Medium	Experiment A[a] F/2	Experiment B[a] F/20	Experiment C[a] F/20
Gel layer thickness (mm)	6	6	3
Volume of the gel agar layer (ml)	559	559	274
Dilution rate (day^{-1})	0.025	0.25	0.25
Marennin concentration (mg l^{-1}liquid medium)	1.0–4.5	1.0–2.0	1.0–2.0
Marennin volumetric productivity			
(mg day^{-1} l^{-1} liquid medium)	0.03–0.11	0.25–0.50	0.25–0.50
(mg day^{-1} l^{-1} gel)	0.47–2.1	4.7–9.4	9.4–18.7
Marennin specific productivity At time 0[b]			
[mg day^{-1} (10^8 cells)$^{-1}$]	0.02–0.1	–	0.47–0.94
[mg day^{-1} (g dry wt)$^{-1}$]	0.31–1.4	–	6.4–12.8
At final time[c]			
[mg day^{-1} (10^8 cells)$^{-1}$]	0.05–0.23	–	0.09–0.21
[mg day^{-1} (g dry wt)$^{-1}$]	0.71–3.15	–	1.23–2.9

[a] Each experiment (A, B, C) combined a medium (F/2 or F/20), a gel layer thickness (3 or 6 mm) and a dilution rate (0.025 or 0.25 day^{-1})
[b] Cell concentration was 2×10^6 cells ml^{-1} gel for experiments A and C
[c] Cell concentration was 0.9×10^6 cells ml^{-1} gel for experiment A and 9.9×10^6 cells ml^{-1} gel for experiment C

and the volume of the gel), but the latter was about two-fold higher (18.7 mg day^{-1} l^{-1} gel) if we consider the volume of the gel that contained biocatalyst. Marennin specific productivity was higher in experiment C and ranged from 1.23 to 12.8 mg (g dry wt)$^{-1}$.

At the end of incubation, average cell concentrations were 0.9×10^6 cells ml^{-1} gel (5×10^8 cells in total) in experiment A and 9.9×10^6 cells ml^{-1} gel (2.7×10^9 cells in total) in experiment C, where the gel layer had a volume of 274 ml instead of 559 ml. Cell concentration increased in the gel during incubation (Fig. 3), but only in the area of the gel close to the liquid medium in experiment A (3.4×10^6 cells ml^{-1} gel, Fig. 3a), whereas in experiment C the cell concentration increased in the whole gel layer (Fig. 3b), from 4.4×10^6 cells ml^{-1} gel to 24×10^6 cells ml^{-1} gel in the gel area directly in contact with the liquid medium.

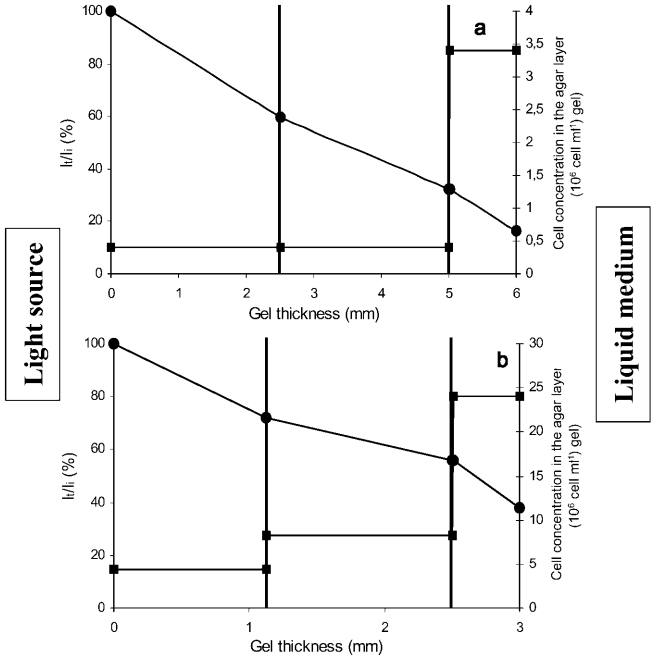

Fig. 3a, b Distribution of *H. ostrearia* cells in the gel layer (■) and percentage of incident light (I_i) transmitted (I_t) through the gel (●) at the end of the incubation run: **a** experiment A; **b** experiment C. Initial cell concentration was 2×10^6 cells ml^{-1} gel

The analysis of microalgal cells at the end of incubation of experiment C, using chlorophyll *a* determination (data not shown), showed living microalgal cells in the whole gel. Microscopic observation confirmed this but revealed, in the case of experiment A, dead cells with a high marennin content in the area close to the light source.

During incubations, leakage in the medium was observed because no microporous membrane was placed around the external grid of the structure. However, the leakage did not exceed 3000 cells ml^{-1} medium.

The light measurement through the gel at the end of incubation revealed a gradient of light intensity inversely proportional to the cell concentration gradient (Fig. 3). The percentage of light transmitted (I_t/I_i) through the agar layer was only 16.5% in experiment A (Fig. 3a); in experiment C it was 38%, due to the reduced thickness of the gel layer (Fig. 3b).

Discussion

Immobilization of cells in agar layer is a method well suited to *H. ostrearia* species, improving the surface exchange between algal cells and nutrients and light, while benthic cultures of *H. ostrearia*, characterized by the sedimentation of microalgae at the bottom of the culture vessel during incubation, endured limitation of light (Rincé et al. 1999). Our previous results (Lebeau et al. 1999) showed that marennin production in the photobioreactor was effective with regard to long-term operational stability without biomass regeneration. In the present work, however, the marennin level did not

stabilize during the incubation runs and was extremely dependent on the nitrate concentration and its evolution (Fig. 2) in respect of nutritional stress, in accordance with the findings of Neuville and Daste (1972).

Cell leakage from gel matrices is a well-known phenomenon, as already described by Bailliez et al. (1988) and Vilchez et al. (1997). Here, F/20 medium was chosen because leakage remained very reasonable (Lebeau et al. 1999) and cell leakage, ranging from 1000 to 3000 cells ml^{-1} medium, was very moderate. During the incubations, there was no apparent deterioration of the top surface of the gel, which certainly explained the low leakage rate, and the gel layer, abrasion of which is likely to be related to fatigue of the gel materials, was not affected by mechanical stress – in contrast to alginate beads, which are knocked together in a stirred medium (Dos Santos et al. 1997). To allow perfect cell-product separation, a microporous membrane may nevertheless be bonded to the face of the agar layer that is directly in contact with the liquid medium (Planchard et al. 1989) of the photobioreactor. However, to avoid complicating the assembly of the photobioreactor, we deliberately first used it without a microporous membrane.

To consider experiments A and B (identical amount of nutrients supplied daily), volumetric productivity was higher with the highest dilution rate, which most likely reduced marennin accumulation in the gel, with possible limitation of a toxic effect for cells in consequence (Robert and Turpin 1993) and self-shading (Urrutia et al. 1995; Vilchez et al. 1997). Maximum marennin productivity during experiment C – 18.7 mg day^{-1} l^{-1} gel – was comparable to that achieved in our previous work (Lebeau et al. 1999) using batch-incubated beakers. Conversely, specific productivity was lower in the present study and the volumetric productivity was made up by higher growth, with a biomass at least five-fold the biomass reached in our previous study.

Heterogeneous cell distribution in the gel revealed diffusional limitation of nutrients and light, as already shown by Lebrun and Junter (1993) using the same "composite" immobilized-cell structure. In the present work, cells were in two different states. (1) In the area near the liquid medium, cell concentration was high and cells were alive and weakly pigmented, revealing, in agreement with Schubert et al. (1995), no cell stress from either light or substrate, particularly nitrogen, as already shown by Neuville and Daste (1972). (2) In the opposite, substrate-limited area close to the light source, blue-pigmented cells were mixed with dead cells, particularly in experiment A, as already shown by Vilchez et al. (1995) with *Chlamydomonas reinhardtii* cells immobilized in alginate beads, where cells immobilized in the core of the beads were dead owing to a considerable limitation of substrate and also of light. During experiment C, the reduced thickness of the gel, giving a more suitable diffusion of nutrient and light through the gel, may explain the improved cell growth and preservation of cell viability throughout the agar layer.

The photobioreactor used in the present study was well suited to marine microalgal cultures, due to its large surface-to-volume ratio and efficient lighting provided by the optical fibres evenly distributed around the surface of the barrel. Moreover, this "cold" light source allowed the use of *H. ostrearia* cells, whose cultivation temperature is quite low. The limitation of diffusion into the gel should be overcome by reducing the gel thickness even further, or by using other technology such as hollow-fibre reactors (Wang et al. 1991). It would also be interesting to evaluate the influence of light intensity (Burgess et al. 1993) on marennin production, high levels of which are related to high-intensity lighting (Schubert et al. 1995).

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