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Comparison of two membrane – photobioreactors, with free or immobilized cells, for the production of pigments by a marine diatom

N. Rossignol, T. Lebeau, P. Jaouen, J. M. Robert

Abstract The present study concerns the value enhancement of the microalga *Haslea ostrearia*. This marine diatom bears the peculiarity of synthesizing and secreting in the culture medium a blue-green pigment named "marennine". Anticancer research, cosmetics and aquaculture are the fields concerned with the utilizations of this hydrosoluble pigment. The aim of the study is to compare the pigment productivity obtained with two types of photobioreactors. In the first process, cells are free and recycled in a bioreactor combined with a membrane ultrafiltration equipment (external loop). In the second system, cells are entrapped in a tubular agar gel layer in a photobioreactor of original design. The influence of nitrate concentration and renewal rate is examined. Experiments, conducted on long term periods (up to 40 d) without any external contamination, revealed that marennine productivities of more than 5–7 mg 10^9 cell^{-1} d^{-1} can be reached with both bioreactors. The advantages and drawbacks of each process design are also discussed.

1 Introduction

Interest in microalgae has been growing over the last years. These micro-organisms represent a potentially important source of various valuable metabolites such as enzymes, fatty acids, polysaccharides, pigments and other bioactive compounds [1]. These products have found some recent commercial exploitation in dietetic, pharmaceutical and cosmetic fields [2, 3]. The productivity and yield of the process (mainly monospecific cultivation) depend to a large extent on the type and design of the bioreactor. The main parameters of production, i.e. nutriment addition, light and temperature should be controlled and optimized. With the aim to obtain maximum amounts of specific algal products, particularly when these products result from secondary metabolism, efficient closed systems are required. This can be achieved in photobioreactors in continuous controlled cultivation associated with a solid-liquid separation unit. Such a separation, often conducted in an external loop, should be considered as a first purification step of the removed metabolites [4, 5]. Cell immobilization, which allows high cell concentration in the bioreactor, is another means of producing metabolites and presents the advantage over free-cells of preventing culture contamination and shear stress of microalgal cells.

Haslea ostrearia is a marine diatom commonly found in the littoral waters of the Atlantic shore in France. This microalga has the unique peculiarity to synthesize at its extremities a blue-green hydrosoluble pigment named "marennine", which is released in the medium [6] and known to be responsible for the greening of oysters. For the time being, it is the only known industrial application of this diatom. On industrial site, the microalga was grown in batch culture under carefully-controlled conditions in 6 m^3-tanks [7]. Then, the culture was transferred to oyster ponds for the "greening" phase. This pigment should also find commercial applications as natural dye in the food and cosmetic industries, as phycobiliproteins already currently produced for this use [8]. Another interesting prospect concerns its antiproliferative properties, since aqueous extracts of the pigment show inhibitory effects both in vitro and in vivo against carcinoma lines [9].

Some forms of environmental stress factors lead to the biosynthesis of "marennine". It has been suggested that nutrient deficiency such as nitrates [6, 10] causes blueing of cells. High light intensity is required for the production of this pigment: marennine seems to act as a light shielding pigment in cells [11].

To perform the marennine production, processes associating a closed photobioreactor coupled with a separation system are studied. Two culture techniques are compared with free or immobilized cells. In the first system, the cells are free and recycled in a tubular photobioreactor combined with membrane ultrafiltration equipment. In the second system, cells are entrapped in a

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photobioreactor including a tubular agar gel layer lighted by a barrel of optical fibres.

Each system bears advantages and drawbacks for this application. Owing to the low microalgal cell concentration and metabolite productivity under natural conditions, these systems are promising. Bioreactors coupled with tangential micro- or ultrafiltration are widely used for fermentation processes with different configurations [12], and have been developed, more recently, for the production of a variety of compounds by different micro-organisms including microalgae. The major limitations of this continuous system are membrane fouling [13], and hydrodynamic shear stress on cells [14]. *Haslea ostrearia* being a particularly sensitive microalga, the pumps and throttling valves as well as the operating conditions of filtration have been chosen in order to minimize shear stress [15, 16]. In a previous work [17] we selected, for long-term operation, an organic ultrafiltration membrane which proved to be the most efficient in the particular conditions of low transmembrane pressure and low tangential velocity.

Immobilization is one way to overcome these disadvantages and seems to be adapted to microalgae, particularly to *Haslea ostrearia*. In natural environment, the number of pigmented cells increases during the migration of algae from the planktonic to the benthic compartment and becomes maximum during the benthic stage [6] where algal cells are immobilized in their own exopolysaccharides.

Several microalgal species have been entrapped in various transparent polymer matrices for exometabolite production. Suitable immobilization techniques depend on the matrix composition. Polysaccharides extracted from seaweed, as calcium alginates are convenient to diatoms [18, 19]. In a previous work [20], *Haslea ostrearia* was successfully entrapped in agar-gel discs for long-term marennine production.

Because of high cell concentration obtained, bioreactors with immobilized microalgae are generally small-volume units. They are used for water and effluent treatment: heavy metals, nitrates and phosphates elimination [21, 22] or for high added value metabolite production [23].

In the present paper, the diatom *Haslea ostrearia* was cultured in a continuous mode. Cells were fed on the basis of the same nutrient amount at various dilution rates (D = 0.025 d^{-1}, F/2 medium and D = 0.25 d^{-1}, F/20 medium) in order to compare two photobioreactors with free or immobilized cells for exocellular marennine productivity.

2 Materials and methods

2.1 Microalgal strain and preparation of inocula

The study was performed using an axenic strain of *Haslea ostrearia* isolated from oyster-ponds in the Bourgneuf Bay (Vendée, France). Cultures were maintained in 250 ml flasks filled with 150 ml of modified Provasoli medium (ES 1/3) according to Robert [6]. Algal preculture was performed applying a two-step procedure using 250 ml and 2-l Erlenmeyer flasks filled with ES 1/3 medium, with 6-d incubation time for each. Algal inoculum was collected by centrifugation (4000*g*, 6 min, 15 °C) from precultures in the exponential growth phase.

2.2 Photobioreactors and experimental set-up

Free-cells bioreactor (FCB)

The experimental set-up is shown in Fig. 1. The free-cells photobioreactor (FCB) consists of a glass cylinder (Hi-Flow, SGI, New Brunstick Scientific Co., Inc.) associated

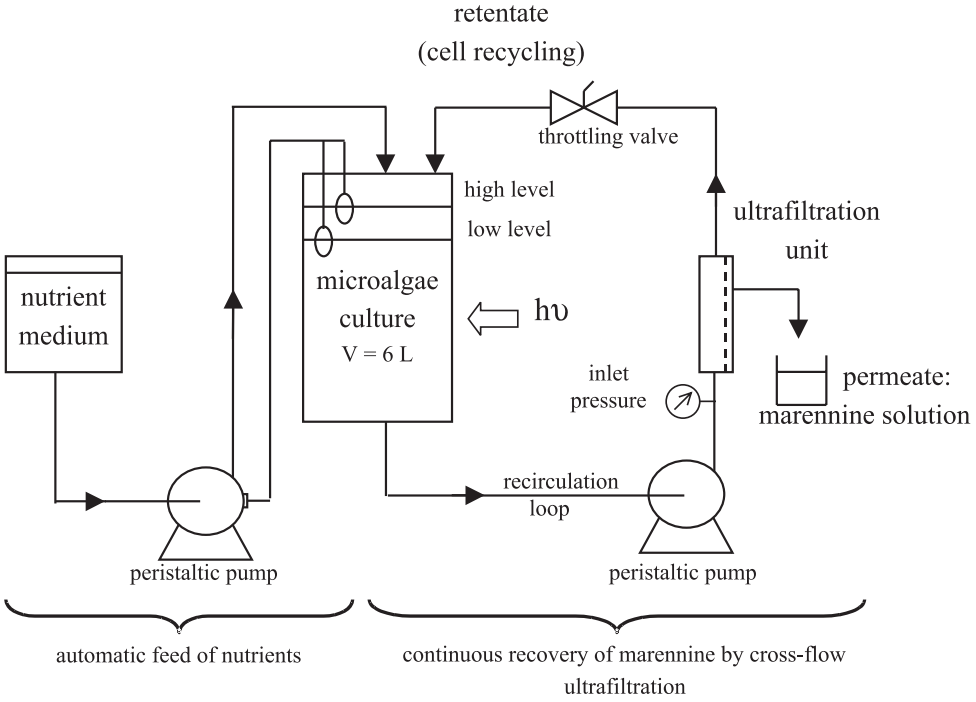

Fig. 1. Schematic flowsheet of membrane free-cells bioreactor (FCB)

with an external flat membrane module to perform tangential filtration (Rayflow, Rhodia-Orelis Co., Miribel-France). According to previous work [17], a plane polyacrylonitrile ultrafiltration membrane (IRIS 3038 Rhodia-Orelis Co., Miribel-France) with molecular weight cut-off of 40 kDa and 100-cm^2 area has been selected for this application. Although the retentate loop is closed (total biomass recycling), it is however possible to take samples punctually. In order to keep a constant volume, sterile nutritive solution is added into the bioreactor, as the permeate is extracted, by an annex peristaltic pump monitored by automatic control level.

Immobilized-cells bioreactor (ICB)

Figure 2 illustrates a simplified schematic representation of the immobilized-cells bioreactor (ICB) [24]. The ICB consists of a 10-l glass- and stainless-steel cylinder.

Both photobioreactors were incubated with the following inoculum: one part was introduced into the membrane photobioreactor (FCB) filled with 6 l of sterile medium F/20 [25]. The salinity was adjusted to 28 g l^{-1} NaCl and complemented with 80 mg l^{-1} $NaHCO_3$ (carbon supply). The pH was maintained at a value between 7.8 and 8.2 through daily acid addition (HCl, 0.1 N). The other part was entrapped in a tubular agar gel layer (surface: 955 cm^2 and volume: 550 cm^3) according to the method described by Mignot et al. [26] and adapted to *Haslea ostrearia*. In particular, the solution of agar was cooled to 35 °C to keep cell alive. Then, tubular gel was introduced into the photobioreactor (ICB) filled with 10 l of the same medium (F/20) as FCB.

Initial cell concentrations, for all experiments, were adjusted to 100×10^6 cells per litre of culture medium.

Photobioreactors were placed in an air-conditioned room with temperature of 15 °C. Light was 80 μmol photons m^{-1} s^{-1} at the surface of the agar layer and inside the FCB (LI-1000 Data Logger quantameter) with the following cycles: 14 h light/10 h dark. Bioreactors, tubes and nutritive medium were autoclaved before incubation, non-autoclavable membranes and ultrafiltration module were flushed with ethanol (95%) and rinsed with sterile desionized water. Two peristaltic pumps were used to ensure homogenization and renewal of medium.

Analysis

Marennine concentrations were determined by measuring the optical density (OD) at 663 nm [27] referring the OD values of calibration curve. Nitrate concentrations were daily determined with NitraVer (Hach) kit for immediate analysis. Cell concentration (expressed as number of cells per litre of culture medium) was estimated using a Nageotte cell with optical microscope.

The marennine volumetric r and specific q productivities were calculated from dilution rates and daily marennine and biomass concentration determinations, according to the marennine mass balance:

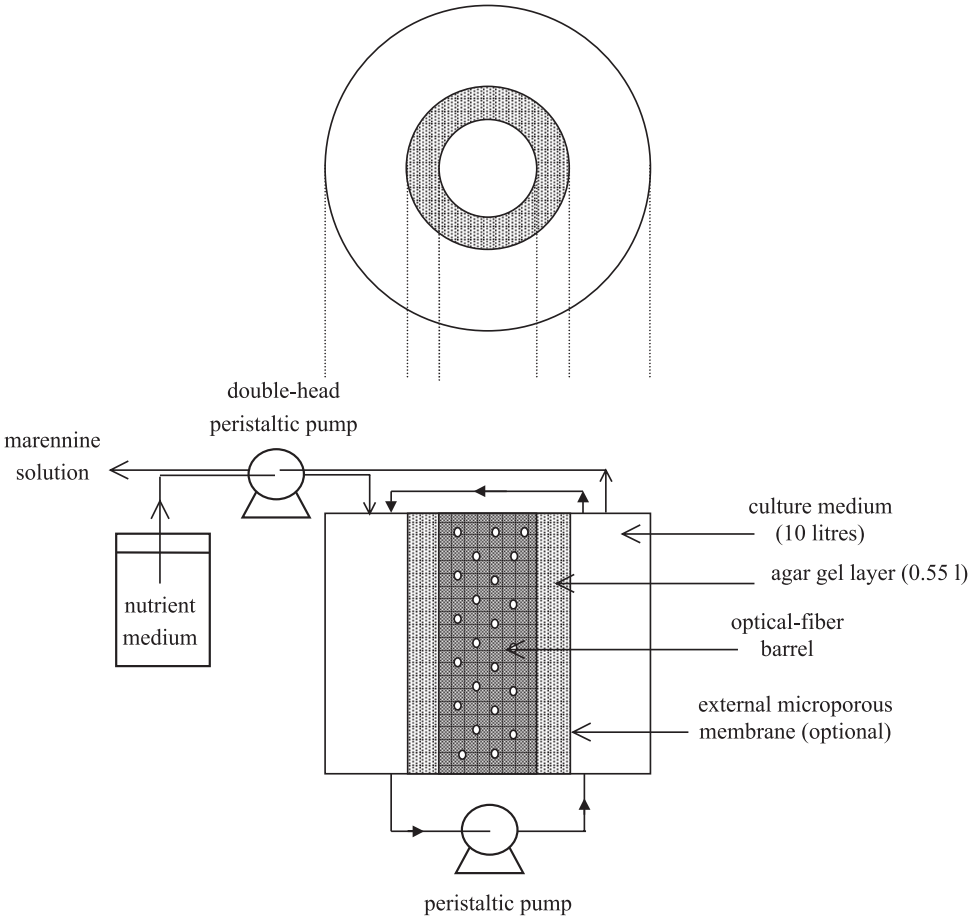

Fig. 2. Schematic representation of immobilized-cells photobioreactor (ICB)

Marennine production rate, i.e. volumetric productivity r (mg l^{-1} d^{-1}) of marennine:

$$r = \frac{C(t_i) - C(t_{i-1})}{t_i - t_{i-1}} + DCm \quad i \geq 1 \ . \tag{1}$$

Specific productivity q (mg $cell^{-1}$ d^{-1}) of marennine:

$$q = \frac{r}{Nm} \ , \tag{2}$$

with D: dilution rate (d^{-1}), t_i: time after incubation (d) $C(t_i)$ and C (t_{i-1}): marennine concentrations (mg l^{-1}) at times t_i and t_{i-1}, Cm: average marennine concentration between times t_i and t_{i-1} (mg l^{-1}), Nm: average cell concentration (cells l^{-1}) between times t_i and t_{i-1}.

3
Results and discussion

3.1
Influence of nitrate concentration in culture medium on exocellular marennine production

In this experiment spanning 40 d, the renewal rate chosen is $D = 0.025$ j^{-1} (F/2 medium feed). Insofar as marennine synthesis makes up for a lack of nitrogen, the supply of that nutrient varied during the experiment, alternating feeding periods using nitrate-free F/2 medium with periods without renewal ($D = 0$). The influence of nitrogen deficiency on the amount of marennine released is studied for both culture modes (Fig. 3).

Curves obtained from the operation of the two systems roughly present the same evolution, following four successive phases:

- Phase 1: both incubation and renewal media contain nitrates (88 μmol l^{-1}). Nitrate content increases in the culture medium. Cells therefore need nutrients in lower amounts than those provided, hence a nutrient build-up in the medium. Thus, the amount of dosed marennine is low: 1–2 mg l^{-1}.
- Phase 2: in order to weaken the culture medium, the F/2 solution supplied is no longer enriched with nitrates. Cells consume nitrates, thereby allowing their concentration to decrease from 180 to 35 μmol l^{-1} in 14 d for both reactors, i.e. ≈10 μmol of nitrate ions consumed per litre of reactional medium every day. At the same time, a larger amount of marennine is released: 2–6 mg l^{-1} in the FCB and 1–3 mg l^{-1} in the ICB, which corresponds to a marennine yield three times as large for an amount of nitrates 6 times smaller in the medium.
- Phase 3: systems are no longer renewed ($D = 0$). Almost immediately, external marennine concentration increases up to $C \approx 12$ mg l^{-1} in each of the reactors after 10 d.
- Resuming volumetric renewal (phase 4) causes the medium to dilute in marennine. Concentration in the FCB drops back to that corresponding to phase 2: $C \approx 6$ mg l^{-1}. Concentration in the ICB gets stabilized to a slightly higher level than that of phase 2: $C \approx 5$ mg l^{-1}. Nitrate concentrations are then lower than 50 μmol l^{-1}.

Whether culture is free or immobilized, both systems react the same way to different medium changes: marennine production begins as soon as nitrate concentration decreases, and it builds up during periods when dilution rate is nil. Nitrogen components consumption also seems identical in both photobioreactors. It can be assumed that nutrients diffusion within the gel is not a productivity-limiting factor. The relative stability of nitrate concentration in phase 3 may be due to a fresh release of cell content resulting from dead cells.

As only FCB technology allows to get to the biomass, the evolution of cell population is only monitored in that photobioreactor (Fig. 4).

In the FCB cell concentration tripled during experiment: from 100×10^6 to 300×10^6 cells l^{-1} (Fig. 4). Total biomass increases up to a plateau from 10th day of culture. This stationary state may be explained by a cell renewal in which the number of living cells makes up for the number of dead cells.

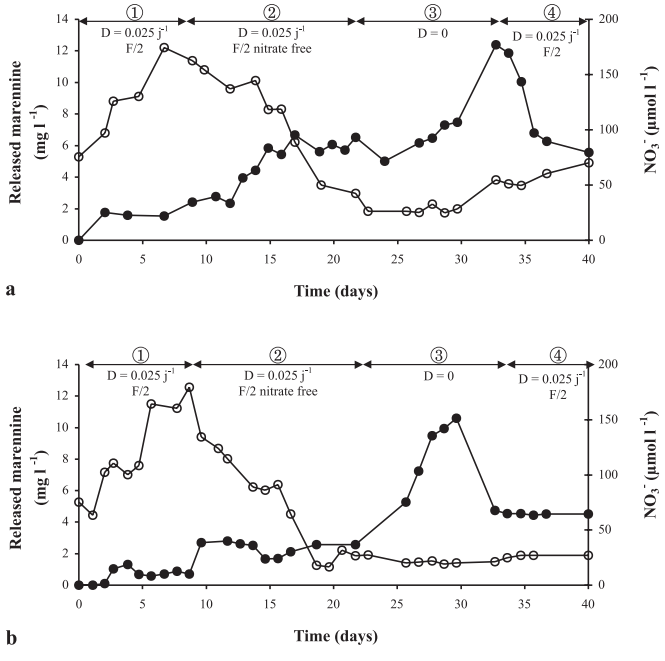

Fig. 3a, b. Kinetics of marennine production (●), nitrate concentration (○), $D = 0.025$ d^{-1}, F/2 medium: by free cells **a** and agar-entrapped cells **b** of *Haslea ostrearia*

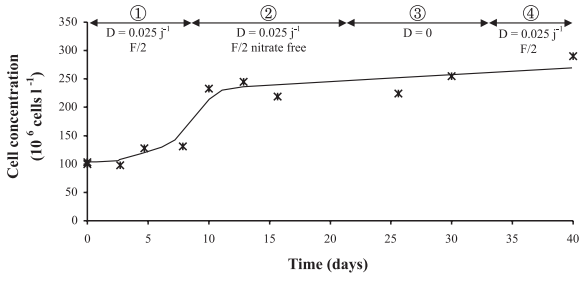

Fig. 4. Kinetic of growth of *Haslea ostrearia* (free cells) in membrane photobioreactor (FCB)

As the immobilized biomass of the ICB is not accessible, at the end of experiment the agar gel was cut into 3 slices thicknesswise. After gel dissolution (by heating at 35 °C), total biomass counting was performed. It could be observed that cell concentration only increased within the first millimetre of gel toward the culture medium with 3.4×10^6 cells per ml of gel, while cell concentrations are 0.4×10^6 cells per ml of gel in the other two slices closer to the light source: the assumption can be made that the intensity of radiating light in the immediate proximity of optical-fiber barrel is excessive. Overall cell concentration within the gel decreased, shifting from 2×10^6 down to 0.9×10^6 cells per ml of gel, i.e. from 100×10^6 to 45×10^6 cells per litre of culture. Cell release out of the gel into the culture medium is negligible (maximum 1–2%). Moreover, Vilchez and Vega [28] using green algae *Chalmydomonas reinhardtii* also observed cell concentration decrease at the core of an alginate matrix. They attribute the phenomenon to both nutrients and light diffusion difficulties inside gel thickness.

3.2 Influence of dilution rate

Marennine concentration largely depends on nitrate concentration in the medium. So as to stress microalgae from the start, the initial incubation medium of the second experiment (Fig. 5) is not nitrate-enriched, renewal being then performed using F/20 medium containing nitrates. In both photobioreactors, nitrogen nutrients concentration in the medium during experiment remained lower than 30 μmol l^{-1}. To improve the volumetric productivity of marennine, a dilution rate 10 times as large as the previous one ($D = 0.25\ j^{-1}$, F/20 medium) is tested for both reactors. An increase of medium renewal rate would allow to avoid a possible inhibiting effect due to marennine overconcentration [29] and to stimulate its synthesis as a light shielding for the cell [11]. A comparison of concentration evolutions released marennine both ICB and FCB culture systems (Fig. 5), shows that after a short period when production is not yet detectable (area 1 for 4 d), marennine amounts increase exponentially (area 2 for 4 d) before reaching a plateau (area 3) at concentrations ranging from 9 to 12 mg l^{-1} in the FCB and from 1 to 3 mg l^{-1} in the ICB.

A synthesis of experimental results for both series of experiments ($D = 0.025\ j^{-1}$ and $D = 0.25\ j^{-1}$) is displayed in Table 1. Calculated values correspond to periods of relatively stable maximum marennine production, i.e. to the last days of phases 2 and 4 of the first experiment (Fig. 3) and to the stationary area (area 3) of the second (Fig. 5). Specific productivities are calculated from final cell concentrations which, after 25 d of culture in the second experiment, amount to 400×10^6 and 45×10^6 cells l^{-1} respectively for FCB and ICB.

A comparison of both culture systems reveals that exocellular marennine yields are much larger when cells are free (concentrations and volumetric productivities). However, as cell population increases in the FCB and decreases in the ICB, specific productivities, calculated from the overall cell number at the end of culture, are higher in the reactor with immobilized cells.

A high volumetric renewal rate ($D = 0.25\ j^{-1}$), which ensures better supply homogenization, an elimination of inhibiting substances and a reduction of marennine-induced light shielding, will improve metabolite productivities in the two bioreactor geometries (volumetric productivity multiplied twentyfold in the FCB and eightfold in the ICB with $D = 0.025\ j^{-1}$).

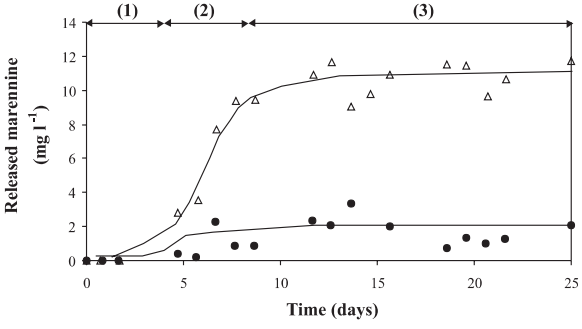

Fig. 5. Comparison of marennine productions by FCB (△) and ICB (●), $D = 0.25\ j^{-1}$, renewal medium: F/20, initial medium: F/20 nitrate free

4 Conclusion

These results show that both systems are adapted to continuous culture of *Haslea ostrearia* in photobioreactor. Cultures react in a similar fashion to modifications in the composition as well as in medium renewal. When composition is stable, constant volumetric productivity is achieved.

Studies were carried out so that operating conditions be as close to each other as possible in order to allow a comparison of both photobioreactors performances. The comparison, however, has its limits. On the one hand, the quality of light is different ("neon" white light for free

Table 1. Comparison of marennine productivities for cultures of *Haslea ostrearia* in membrane photobioreactors with free-cells (FCB) and immobilized-cells (ICB)

	Dilution rate: $D = 0.025\ j^{-1}$ (F/2 medium)		Dilution rate: $D = 0.25\ j^{-1}$ (F/20 medium)	
	FCB	ICB	FCB	ICB
Marennine concentration: C (mg l^{-1})	5–7	2–5	9–12	1–3
Volumetric productivity: r (mg l^{-1} j^{-1})	0.12–0.17	0.05–0.12	2.2–2.7	0.3–0.7
Specific productivity: q (mg 10^9 $cell^{-1}$ d^{-1})	0.50–0.68	1.1–2.6	5–7	6–15

cells, "halogenous" for immobilized cells); on the other hand, light intensity gradients are characteristic of each photobioreactor. Immobilized cells are concentrated in a gel of reduced volume compared with the total volume of the reactor, while free cells adhere to glass walls or settle at the bottom of the bioreactor.

Furthermore, measurements allowing to calculate specific productivities, expressed here according to cell concentration, present uncertainties. Actually, microscope counting indifferently includes either living cells or dead ones; another parameter assessing active biomass, such as chlorophyll a concentration would add another criterion to compare the specific productivities of the two populations.

In the case of the free-cells photobioreactor, the marennine solution recovered in the permeate is free from any cell or debris. Access to the biomass allows to control the state of the culture and possibly to withdraw or partly renew it. As nutrient supply is low in these two experiments, maximum and stationary biomass does not reach beyond 450×10^6 cells l^{-1}; in this case, partial biomass extraction proves unnecessary. Biomass yield is, however, about 100×10^6 cells l^{-1} greater than that of a batch culture.

As for the bioreactor with immobilized cells, once the matrix has been set up, it presents the advantage of forming a compact unit, the maintenance of which is simpler than that of membrane bioreactors with external tangential filtration loop, which also limits contamination risks. Yet cell back-release out of the gel, however small, requires an additional solid/liquid separation device.

A solution to the problem is to place a plane microporous membrane around the gel mantle, thereby suppressing any back-release of microalgae out of the gel [26]. By reproducing natural immobilization of *Haslea ostrearia*, the culture would have a time-constant metabolic activity favourable to marennine synthesis.
The system can also be improved by reducing gel thickness, thereby enhancing nutrients and marennine diffusions. In fact, overconcentration of the metabolite in the matrix may inhibit its synthesis and limit cell growth.

Finally, the geometry of the free-cells photobioreactor could be modified so as to increase the surface/volume ratio and then improve nutrient and luminous transfers without disturbing the natural benthic behaviour of microalgae. The design of an immersed membrane photobioreactor currently under investigation would enable us to overcome these problems, at least partly [30].

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