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ORIGINAL ARTICLE

Microalgal cell immobilization for the long-term storage of the marine diatom *Haslea ostrearia*

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Abstract To preserve the characteristics of the marine diatom *Haslea ostrearia* during long term storage, particularly size and shape, the algal cells were immobilized in alginate beads and stored at 4 °C at reduced irradiance up to 4 months. Two clones of different size (Ho34, 63 μm and Ho40, 78 μm) were studied. With Ho34, a 10.4% decrease of the size was shown after 120 days, by using the conventional storage management, while it did not exceed 2.2% with immobilized cells. Consequently, *H. ostrearia* would have auxosporulated after 9 months compared to 52 months. At the same time, the rate of distortion (aberrant valve structure) free Ho34 cells reached 86% while no distorted immobilized cells were observed. Chorophyll content in cells showed that all the cells were alive up to 60 days and after this time cells immobilized in the core of the beads most probably suffered from the poor light diffusion. Culturability of the immobilized cells was tested immediately after their immobilization and after 60 and 120 days of storage. The delay (at least 5 days) before immobilized cells released from the beads decreased with the time of storage, because of the embrittlement of the beads during the storage. Once in fresh medium, the cells actively multiplied. We concluded that immobilization strongly slowed down the decrease in frustule size with time and allowed the storage of concentrated and calibrated inocula which could be inoculated directly in liquid culture medium without needing to dissolve the beads.

Keywords Alginate beads · Diatom · *Haslea ostrearia* · Immobilization · Preservation · Storage

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Introduction

One of the main tasks of microalgae culture collections is to provide viable inocula for research and for commercial purposes, e.g., larvae feeding in hatcheries, synthesis of active biomolecules. In the former case, microalgae provide a well balanced mixture of nutrients to higher organisms in the food web because they contain high, but variable percentages of the most important macronutrients (Becker, 2004; Volkman & Brown, 2006). During their storage in culture collections, they must be preserved as live feeds with all their potential key nutrients: proteins, carbohydrates, lipids and also essential micronutrients and antioxydants.

While the microalgae storage during a long period can save time and money–microalgae used for the early stages of growth of mollusc larvae, fish and crustaceans in the larval stages make up 30% of the total cost of

production (Benemann, 1992; Coutteau & Sorgeloos, 1992) – thanks to less frequent sub-culturings, the potential characteristics of the microalgal strains must be also preserved. Moreover, the case of diatoms is particularly tricky with a decrease of the size of the cells at each mitotic division. The consequence of the frustule size decrease can be the induction of distorted cells, as already shown with the marine diatom *Haslea ostrearia* (Robert, 1983) and the diatom *Pinnularia brebissonii* (Hostetter & Rutherford, 1976) which alter their physiological state. Now technological applications of microalgae make necessary to have at one's disposal inocula with the potential biochemical composition preserved.

Microalgal storage has been studied for various possible applications. For larvae feeding, *Tetraselmis suecica* was stored more or less successfully depending on the preservation technique (Montaini et al., 1995) and served, for example, as food for *Crassostrea gigas* larvae (Robert et al., 2001). These authors showed that the microalgal preservation at low concentration was able to maintain the food value of this microalgae. D'Souza et al. (2002) showed that *Chaetoceros muelleri* or *Thalassiosira pseudonana* concentrated and stored for 6 weeks at 4 °C promoted similar survival rates of *Penaeus monodon* larvae as fresh algae, although the prawn development rates were slower. Microalgal storage has also been applied practically to water quality control (Cheng, 2003) and to ecotoxicological studies (Frense et al., 1998). By comparison with free cells, immobilization allows storage of cells for a longer period without any loss of viability than in free cells and the preparation of immobilized cellsis easier, cheaper and more readily available than other methods, such as cryopreservation (Romo & Pérez-Martínez, 1997). Tamponnet et al. (1985) showed that immobilized cells of *Euglena gracilis* retain 90% of their chlorophyll content after 3 months of incubation, whereas their free counterparts displayed pheophytinization (degradation of chlorophyll pigments by loss of the central magnesium atom) after only 7 days. Diatoms such as *Amphidinium carterae*, *Chaetoceros ceratosporum*, *Emiliana huxleyi*, *Phaeodactylum tricornutum*, *Skeletonema costatum*, *Thalassiosira pseudonana* (Hertzberg & Jensen, 1989), *Nitzschia obtusa* (Kannapiran et al., 1997)were stored in alginate matrices for several months. Hertzberg and Jensen (1989) cultivated immobilized cells of *Phaeodactylum tricornutum* at 4 °C with reduced light or in absolute darkness, with or without a liquid medium, to reduce the metabolic activity during the storage. Chen (2001, 2003) suggested that entrapped cells of *S. quadricauda* and *I. galbana* remained alive probably by consuming their reserve of pyrenoids during storage. It was shown that after concentration by flocculation the algal cells not only retained their viability and growth-up to 14 days with *Chaetoceros calcitrans* (McCaustand et al., 1999)–but also their appetence for larvae as above mentioned with the example of *C. muelleri* or *T. pseudonana* (D'Souza et al., 2002). Nell and O'Connor (1991) showed also that a combination of *P. Pavlova lutheri* and *C. calcitrans* concentrated to a paste and stored up to 14 days at 4 °C produced greater length increases of larvae of Sydney rock oyster than any other fresh or stored, single or combined diet tested. However, larvae fed *C. calcitrans* concentrate only had very poor survival and development.

Haslea ostrearia is commonly found in the littoral waters of the Atlantic shore in France. This marine diatom has the unique peculiarity to synthesize at its extremities a blue-green hydrosoluble pigment named "marennine" which is known to be responsible for the greening of oysters (Robert, 1983). This pigment should find also commercial applications as natural dye in the food and cosmetic industries as phycobiliproteins already currently produced for this use (Yaron & Arad, 1993). Some developments are also expected in medicine as aqueous extracts of marennine have an anti-proliferative effect both *in vitro* and *in vivo* against carcinoma lines (Carbonnelle et al., 1999). These various applications require to have available stock cultures whose morphological and physiological characteristics must be preserved. In a previous study, cells of this marine diatom were successfully immobilized for the first time and stored up to one month in the aim to place inocula of this microalgae with easy and long-term storage at the disposal of oyster breeders (Lebeau et al., 1998).

The aim of the present study was to preserve strains of *H. ostrearia* longer without any alteration of their characteristics, i.e., growth, chlorophyll content, algal size and frustule shape. We compared the conventional algal management of this diatom which consists in frequent sub-cultures of free cells, with immobilized cells which were stored at 4 °C and reduced light irradiance up to 4 months. The study was performed by using algal clones which differed in their length.

Materials and methods

Microalgal clones

The study was performed by using two axenic clones (Ho34 and Ho40) of *Haslea ostrearia* Simonsen isolated from oyster-pond waters of the Bouin district (Vendée, France). The clones were characterized by an average modal length of 63 μm for Ho34 and 78 μm for Ho40.

Preculture

Cells from the clone pools were first precultured for about 6–7 days in 250 mL Erlenmeyer flasks filled with 150 mL of the modified Provasoli (Provasoli, 1968) medium (ES 1/3; Lebeau et al., 1999). pH of the medium was set to 7.8, the incubation temperature was 15 °C and light intensity estimated with a quantameter (LI-COR, USA) was 3.0×10^{16} quanta cm^{-2} s^{-1} using neon lighting (36 W) with a 14-/10-h light/dark cycle. Then flask contents were inoculated in 2 L Erlenmeyer flasks containing 1 L of (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.

Culture management of free and immobilized microalgal cells

Free cells: 250 mL Erlenmeyer flasks filled with 150 mL maintenance (ES 1/3) medium were inoculated with 2×10^3 cells ml^{-1} from each clone. 13 subculturings were performed in the same conditions at the rate of one per week. Temperature and irradiance were these of the preculture.

Immobilized cells: algal cells were entrapped in alginate beads according to the method of Hertzberg & Jensen (1989): sodium alginate (Prolabo; 1,5 g) was dissolved in a solution of NaCl (100 ml; 28% pH adjusted to 7.8 with 0.1 M NaOH). Alginate powder was sterilized under UV radiation for 16 h while other compounds and the vessel were autoclaved at 120 °C for 20 min before use. The cell suspension was mixed thoroughly with the sterile solution of alginate until the desired cell concentration, i.e., 1×10^6 cells mL^{-1} gel was reached. Beads of about 0.35 cm diameter were obtained by dropping the alginate cell mixture into a solution of 0.07 M $CaCl_2$ (salinity and pH were adjusted to 28% and 7.8 respectively) for less than 20 min using a peristaltic pump supplied with a calibrated needle. Beads remained in $CaCl_2$ for less than 20 min to harden and were then washed in marine natural water (28% pH 7.8). Beads were distributed amongst test-tubes filled with 25 mL liquid (ES 1/3) medium, each one holding 1 mL of beads. Temperature and light intensity were 4 °C and 2.0×10^{15} quanta cm^{-2} s^{-1} with a 14-/10-h light/dark cycle. Beads were incubated for 120 days. Fresh ES 1/3 medium was added after 60 days of incubation.

Analysis

Free- and immobilized- cells growth was estimated using two methods. In the direct method free cells were assessed by cell counts using a Nageotte-type haemocytometer. For immobilized cells, the samples consisted of 1 mL of alginate beads which was recovered and dissolved with 10 mM calcium citrate (25 mL), the salinity of which was adjusted to 28%. The indirect method consisted in estimating the number of viable free- or immobilized-cells by chlorophyll *a* determination. Chlorophyll *a* was extracted from the free or immobilized cells after plunging them into an aqueous solution of acetone 90% (v/v) at 4 °C in obscurity 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).

The morphological characteristics of microalgae were determined by estimating the length of the cells, using an ocular micrometer, and by determining the proportion of distorted cells.

After various periods of storage, all beads from the tubes were rinsed with (ES 1/3) medium and introduced into 2 L Erlenmeyer flasks containing 1 L of the same culture medium, and the same incubation temperature and light (intensity and cycle) as for the preculture were used. The culturability of the cells which were released from the beads was determined by measuring their growth rate, μ (day^{-1}), calculated as followed:

$$\mu = \frac{\mathrm{Log}C2 - \mathrm{Log}C1}{\mathrm{Log}_2(T2 - T1)}$$

where C1 and C2 are cell concentrations (cells mL^{-1}) at time T1 and T2 (day).

Table 1 Growth characteristics of the strains Ho34 and Ho40 cultivated in a free mode before (T0) and after several sub-culturings

	T0	T70
Ho34		
Lag time (day)	1	0
Maximum growth rate (day^{-1})	1.34	1.44
Maximum cell concentration ($\times 10^5$ mL^{-1})	2.29	2.61
Ho40		
Lag time (day)	1	0
Maximum growth rate (day^{-1})	1.23	1.06
Maximum cell concentration ($\times 10^5$ mL^{-1})	1.39	1.83

Statistical analysis

All the experiments were performed in triplicate. Statistical analysis of data included Student's *t*-test. Statistical significance was determined at $P = 0.05$.

Results

Characteristics of the free cells after successive sub-culturings

Table 1 and Fig. 1 show the effect of the successive free cell sub-culturings on growth, microalgal size and the rate of distorted cells. The Ho40 clone showed only slightly lower growth rate and maximum cell concentration than Ho34 .

The cell growth of the two microalgal Ho34 and Ho40 clones was not modified significantly, except the lag time, after several sub-culturings (Table 1). Indeed one day lag time was observed for cells from the clone pool without any sub-culturing history which corresponds to their acclimatation to the new culture conditions, whereas no lag time was observed after several sub-culturings. Conversely the algal size quickly decreased during successive sub-culturings (Fig. 1a, b). A linear 10.4% and 8.4% decrease in the frustule size of Ho34 and Ho40 was shown after 120 days (17 sub-culturings).

Visible outcome of the decrease in frustule length was the alteration of the frustule shape with an increase of the distorted cells rate. This phenomenon was all the more significant since cells were small-sized . The rate of distorted Ho34 cells ranged from 2% before sub-culturing up to 86% after 17 sub-culturings (Fig. 1a). This rate increased exponentially when the size of the cells was less than 60 μm while at same time the decrease in size was linear. With Ho40, it reached only 4% (Fig. 1b) since frustule size was still 71 μm after 120 days of storage.

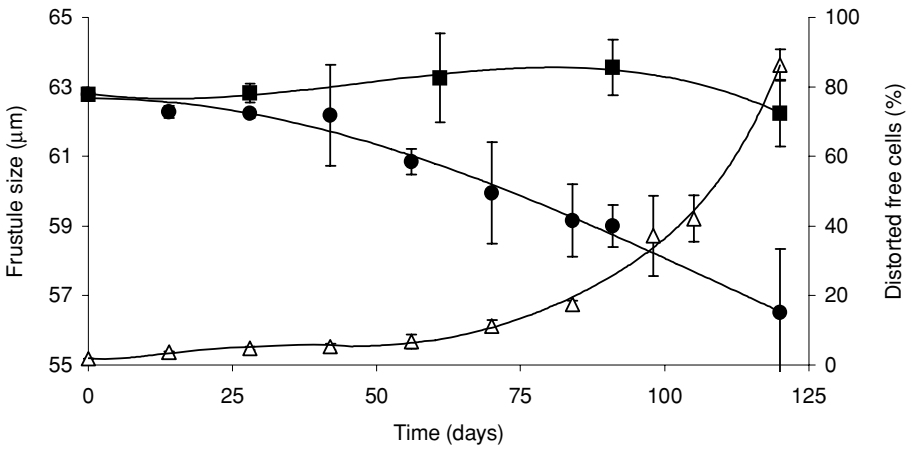
Characteristics and survival of the immobilized cells in the course of the storage

Contrary to free cells, the size of immobilized cells did not decreased significantly during the 120 days of storage (Fig. 1) whatever the clone might have been, and no distorted cells were observed (data not shown). Indeed, immobilized cells grew at a low rate (Fig. 2), i.e., 0.066 and 0.03 day^{-1} with Ho34 and Ho40 respectively, comparatively to their free counterparts (Table 1). Consequently the frustule size decreased at a lower rate, i.e., 0.9% and 2.2% for Ho34 and Ho40 respectively. Figure 3 indicates the algal cell viability by measuring chlorophyll *a* (Chl*a*) content during the storage of free and immobilized algal cells. Chl*a* concentration in beads immobilizing cells (Fig. 3a) was closely connected to the cell concentration (Fig. 2) for 25 and 60 days of storage for Ho34 and Ho40 respectively. The assumption that Chl*a* cell content was unchanged for a given clone during the storage showed that all the cells were alive. The consequence was the almost unchanged specific Chl*a* content (Fig. 3b) which is defined as the Chl*a* content to the algal biomass ratio. However after 25 days for Ho34 and 60 days for Ho40, specific Chl*a* content decreased.

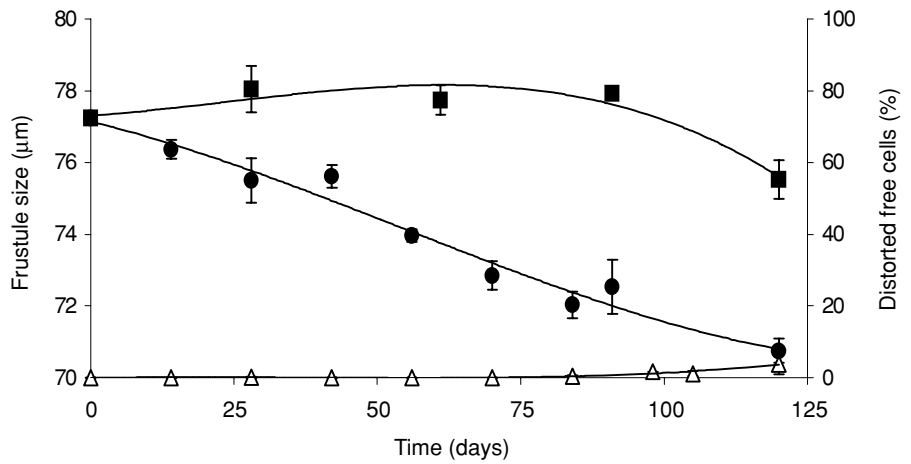
In spite of their low growth rate, immobilized cells released from beads and multiplied in the liquid medium (Fig. 2). Thus, free cells represented 70% of the whole cells in the tubes with the Ho40 clone, i.e., 6.6 $\times$ 10^5 cells mL^{-1} and 18.6% with the Ho34 one. This release was the result of the addition of fresh culture medium 60 days after incubation. Conversely, without any substrate supply (data not shown) cell release felt to less than 2.3% whatever the clone might have been (data not shown).

Culturability of immobilized cells after various periods of storage

The culturability of immobilized cells was tested immediately after their immobilization in alginate beads and after 60 and 120 days of storage (Fig. 4). Immobilized cells in beads– free released cells were ignored– were collected from each tube and introduced in one



(a)



(b)

Fig. 1 Cell size during the storage of immobilized cells (■) and during the successive sub-culturings of free cells (●), and percentage of distorted free cells (△). (a) clone Ho34; (b) clone Ho40. Bars show confidence interval

litre of a fresh liquid ES1/3 medium. Cell growth (Fig. 4a) was observed only for the non-stored microalgae. Their maximum growth rates were respectively 0.23 and 0.29 day^{-1} with Ho34 and Ho40. Opposite results were shown in the liquid medium (Fig. 4b). Free cell growth – includes the cells released from beads and their growth in the liquid medium– exhibited a 15 days lag phase with non-stored Ho34 and Ho40 which did not exceed 5 or 6 days respectively with beads previously stored during 60 and 120 days. Considering the beads which were stored during 60 and 120 days, maximum growth rate of the released cells ranged from 0.47 day^{-1} (Ho34 cells, 120 days of storage) up to 2.07 day^{-1} (Ho40 cells, 60 days of storage).

Discussion

Two clones of *H. ostrearia* that differed by their length (Ho34, 63 μm; Ho40, 78 μm) were studied in this work. Ho40 showed a slightly lower growth than Ho34 owing to its 20%-higher size, as already shown by Williams (1964) who showed a highly significant negative correlation between the growth rate of salt marsh diatoms and their cell volume.

In general, irradiance and temperature levels used for the storage of free diatom cells, including *H. ostrearia*, promote cell growth and the appearance of distorted cells as a consequence. Beyond distorted cells drawback, this culture management is also time-consuming since it involves sub-culturing each

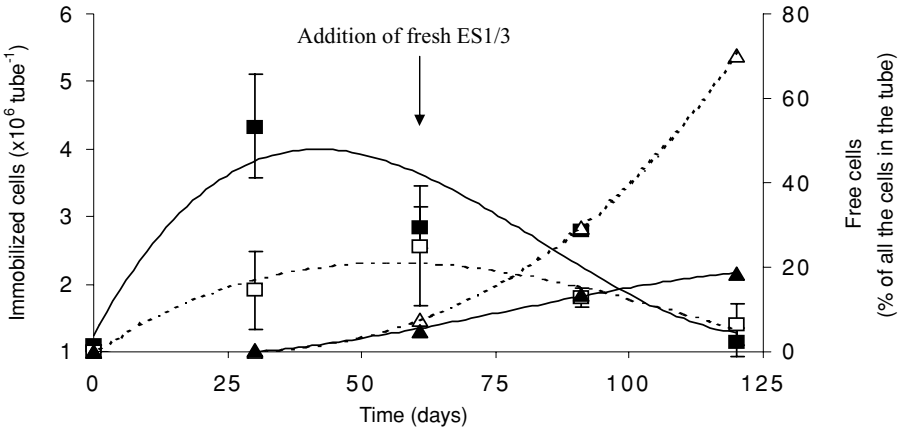


Fig. 2 Growth of *H. ostrearia* cells immobilized in alginate beads (■, □) during the storage (fresh culture medium was added after 60 days) and free cells in the medium (▲, △) resulting from both the cell leakage from the beads and the growth in the medium. Clone Ho34 (solid symbols and solid lines) and clone Ho40 (open symbols and dotted lines). Bars show confidence interval

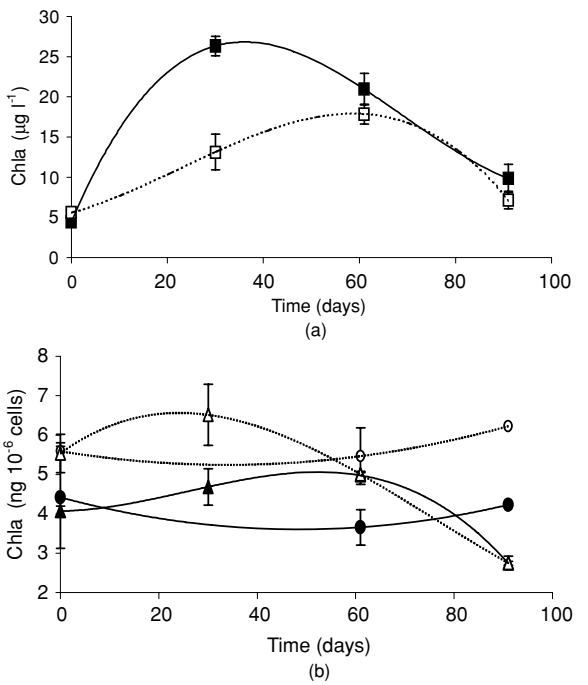


Fig. 3 Viability of the clones Ho34 (solid symbols and solid lines) and Ho40 (open symbols and dotted lines) during the storage estimated by the chlorophyl *a* (Chl*a*) content in the cells. (a) concentration of Chl*a* in the beads (■, □); (b) specific Chl*a* content of the cells stored in a free (●, ○) and immobilized (▲, △) mode. Bars show confidence interval

7 days otherwise pheophytinization occurs in the absence of culture medium renewal. This phenomenon is the consequence of the cellular death concerning most of microalgae. However few *H. ostrearia* cells can survive longer by metabolizing the organic products released by the dead cells which derived from the previous generations. *H. ostearia* is known, like many benthic diatoms, to have the capability to take up dissolved organic nutrients (Robert et al., 1982; Maestrini & Robert, 1987). However, the microalgal stock cultures cannot be managed in this way.

Storage management that made use of immobilized *H. ostrearia* cells showed that this culture technique was able to preserve both microalgal characteristics and viability over a long period as already demonstrated by several authors with other diatoms (Hertzberg & Jensen, 1989; Kannapiran et al., 1997). Chen (2001) even observed that *Scenedesmus quadricauda* was alive

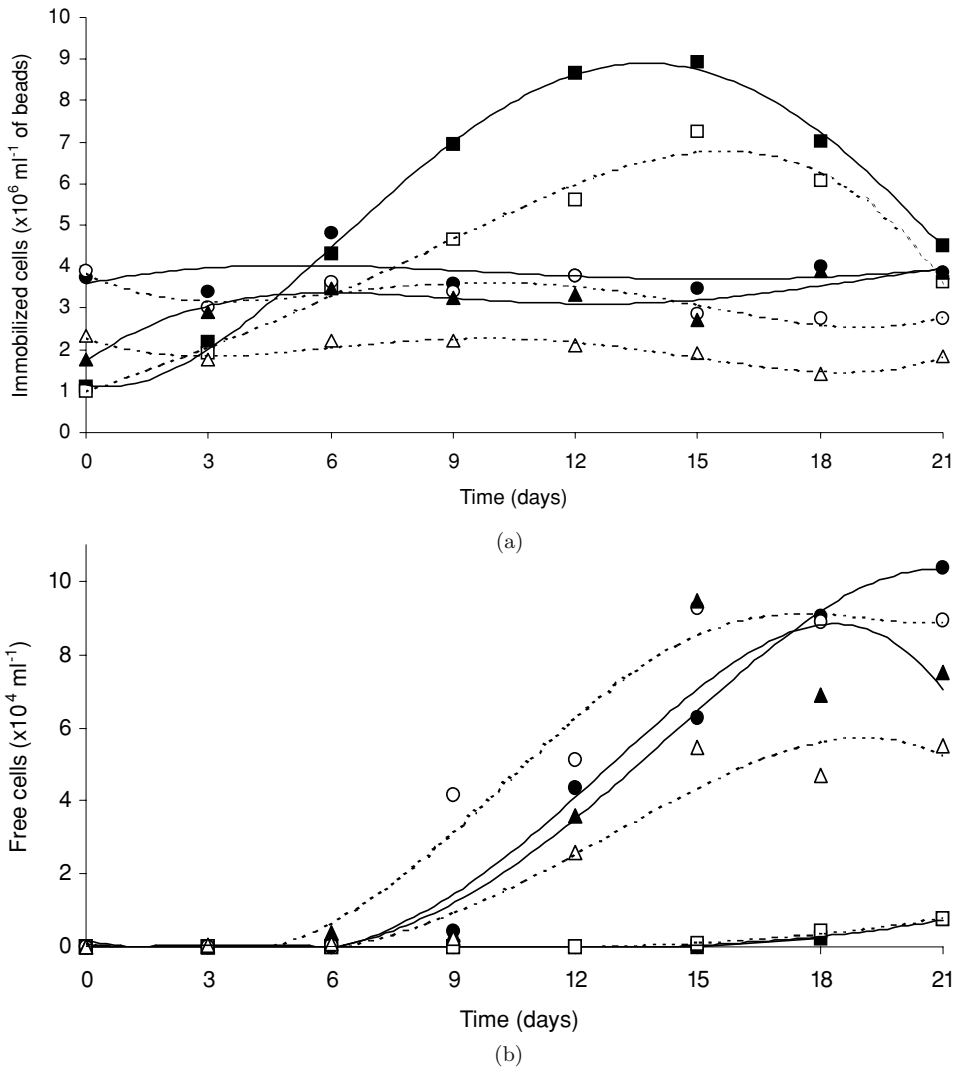


Fig. 4 Growth of the Ho34 cells (solid symbols and solid lines) and Ho40 (open symbols and dotted lines) previously immobilized and cultivated in a fresh ES 1/3 medium after various periods of storage: 0 (■, □), 60 (●, ○) and 120 days (▲, △). After the period of storage, the beads were tranferred from the tubes to fresh ES 1/3 culture medium. (a) immobilized cells; (b) free cells resulting from both the cell leakage from the beads and from the growth in the medium.

and maintained its physiological activity over 3 years of storage. The slow algal size decrease was most probably the consequence of the culture conditions during the storage, i.e., low irradiance and temperature which led to less frequent cell divisions and not the culture technique, as reported by Lebeau and Robert (2006)

Which resulted in low frustule distortion rates. Too quickly auxosporulation phenomenon was then avoided. Indeed auxosporulation usually occurs under a 50 μm frustule size (Rouillard, 1996) and *H. ostrearia* recovers its initial frustule size. Since the decrease of the microalgal size is almost linear, free Ho34 and Ho40 cells would have auxosporulated after less than 9 and 16 months respectively up to 52 and 72 months with immobilized cells. These results show that the conventional stock culture management using free cells does not allow maintaining the microalgal clones for a long time without any fundamental physiological changes (transition from the vegetative division to the auxosporulation) while most of the technological applications require microalgae with constant characteristics.

During storage, specific Chl*a* content of immobilized cells decreased, as compared with free cells, while

some authors observed the contrary (Bailliez et al., 1986; Robinson et al., 1986), i.e., an increase that ranged from 10% (Pane et al., 1998) up to 200% (Lau et al., 1998). Thus it indicates cell mortality inside beads in our experimental conditions.

Immobilized cells most probably suffered from the self-shading effect, as already observed by several authors (Urrutia et al., 1995; Vilchez et al., 1997; Lebeau et al., 2000) because of the low diffusion of light in the core of the beads due to both the amount of cells which were immobilized (about 15 times that of the free cells) and the growth of cells during incubation, Nutrient exhaustion may also explain the cell mortality since culture medium was renewed once only, 60 days after the beginning of incubation. Additionally, released cells from beads most probably intensified this phenomenon by growing in the culture medium to the detriment of their immobilized counterparts, by taking advantage of mass transfer limitation in nutrients. Conversely, the weekly addition of fresh medium to free cell cultures most probably explained that their specific Chl*a* content was holding steady over the successive sub-culturings.

Despite the decrease of the immobilized cells viability after several weeks of storage, most of them remained culturable. The length of the lag time was surprisingly inversely correlated with that of the storage and was correlated with the cell growth in beads while opposite results were usually observed in this latter case (Bréant et al., 2002; Jézéquel et al., 2005). In our experiments, modification of the mechanical resistance of beads with time most probably explain this result. Indeed, integrity of the non-stored beads was not modified by cell growth and no cell release was observed until 15 days. Conversely cell release from beads previously stored was shown after only 6 days although almost no growth occurred inside the beads. The embrittlement of beads during the storage was most probably due to phosphate in seawater used for the ES 1/3 medium which was shown to partially dissolve the alginate beads (Brouers and De Jong, 1988, Travieso et al., 1995). Thus the delay before cell growth occurred in the culture medium included both the delay for the cells to be released and the metabolic adaptation of the released cells to the culture medium as a function of the lenght of storage.

To conclude, cell immobilization is well adapted to the long term storage of viable diatoms all the more since the size of microalgal cells is close to the critical one below which microalgae sporulate. Additionally, the culturability of immobilized cells was preserved without being necessary to dissolve the beads. Indeed cells were naturally released in a few days followed by their growth in the culture medium. Since no cell growth was observed in beads previously stored for 60 and 120 days, calibrated inocula with almost identical size, morphological and physiological characteristics can be provided for various applications. For example, calibrated inocula can be useful for the inoculation of ponds. In case of *H. ostrearia*, it could be thinking of placing viable inocula with easy and long term storage at the breeders of oyster's disposal with the intention of inoculating ponds for the greening of oysters at the right moment. The high cell concentration in beads, i.e., about 15 times the free cell ones, allows also using smaller culture chambers . If necessary, culture medium can be renewed easily without any loss of microalgae thanks to the almost instantaneous deposit of beads at the bottom of the tubes. Finally this storage management is less-time consuming as well as less expansive and it contributes to reduce the contamination risk thanks to less frequent handlings.

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