CRYOPRESERVATION OF GAMETOPHYTES OF *LAMINARIA DIGITATA* (L) LAMOUROUX BY ENCAPSULATION DEHYDRATION

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Abstract

A cryopreservation procedure was developed for gametophytes of *Laminaria digitata* using an encapsulation-dehydration technique. Cells were encapsulated in calcium alginate beads pretreated for 6 h in liquid medium containing 0.3 to 0.5 M sucrose and then dehydrated. Freezing was performed in two steps, the first consisting of a slow temperature decrease from 19°C to -40°C and the second of direct immersion of samples in liquid nitrogen. Thawing was performed in a water bath at 40°C for 2 min. Survival rates were between 25 and 75% depending on age, sex and stress. The best results were obtained with alginate beads pretreated for 6 h in a 0.3-0.4 M sucrose solution and dehydrated down to 40% of the initial wet weight. Recovery time was about two weeks.

Key words: alga, *Laminaria digitata*, gametophyte, cryopreservation, encapsulation, dehydration.

Introduction

In France, the brown alga *Laminaria digitata* is used industrially to produce alginic acid, a phycocolloid which is favoured for its polymerization properties and stability and which requires no oxidation because of its natural white colour. To ensure control of reproduction, a "free-living" technique is now used for culturing gametophytes of *L. digitata* (6), i.e. cells are maintained in a slow-moving life state in suitable light and temperature conditions. This technique has certain drawbacks : risk contamination by pathogenic agents or competitors, high labour and chemical costs, the need for a thermostated room and the possibility that gametophytes will lose their reproductive capacity after a certain time.

Cryopreservation solves most of these problems. The encapsulation-dehydration technique, based on technology developed by the CNRS laboratory in Meudon (4) and originally used for artificial seeds (7), was applied by us to gametophytes of *L. digitata*. This work was funded within the scope of the European AIR III program to determine the "biological and genetical bases for the large scale cultivation and genetic improvement of *Laminaria digitata* (L.)".

Materials and Methods

Plant material

The gametophytes used in this study were obtained from sporophytes of *L. digitata* collected at Argenton (northern Brittany) in March 1995. They were cultured for 3 months by the "free-living" technique in a complex medium (6).

Cryopreservation method

Gametophytes were encapsulated in 3% alginate beads using a procedure developed in our laboratory which provides identical, perfectly rounded beads (Fig. 1).

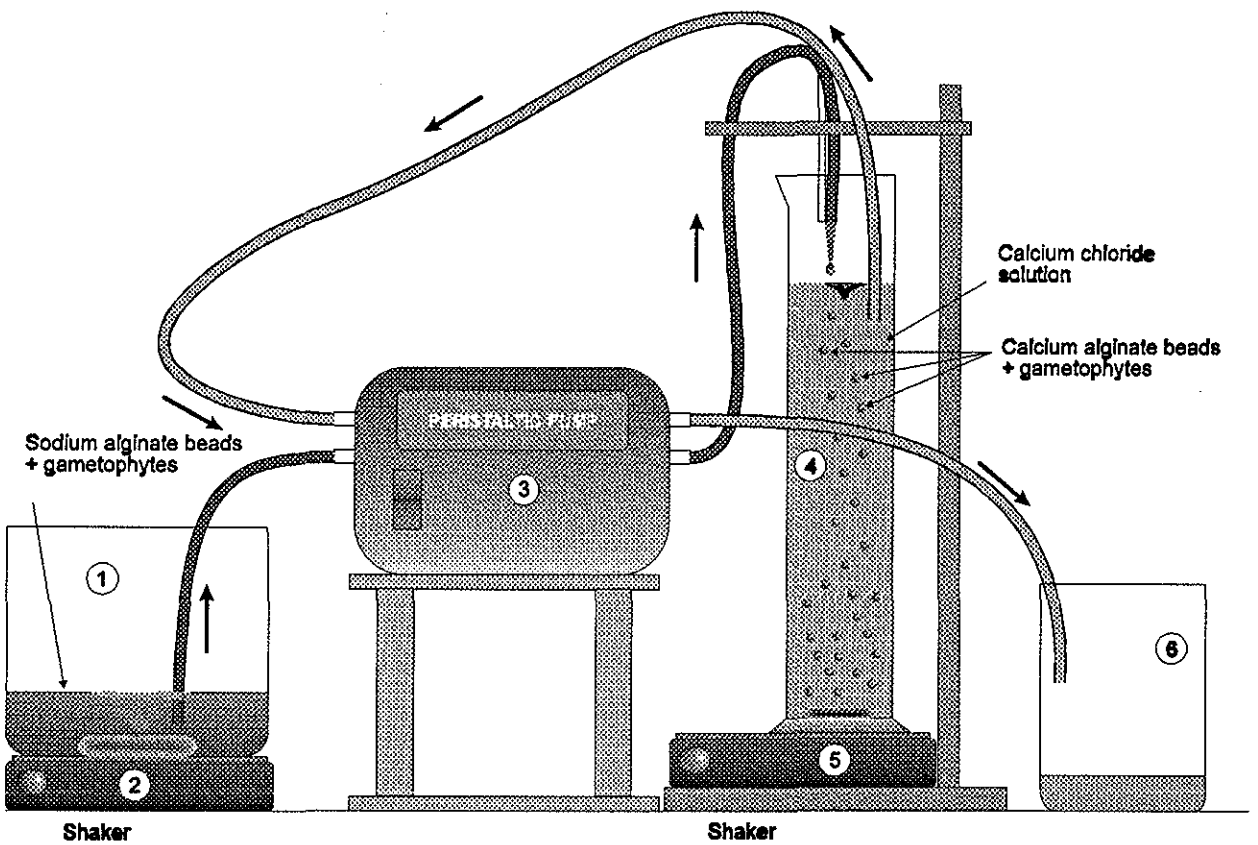

Figure 1: Diagram of the procedure allowing automatic production of alginate beads. Optimal conditions allowed the production of 60 beads per minute.

Encapsulated gametophytes were incubated at 19°C for 6 or 12 h in liquid medium with various sucrose concentrations (0, 0.3, 0.4 or 0.5 M). Prior to air drying water content ranges from more then 90% for 0 M sucrose to 70% for 0.5 M sucrose treated beads. Beads in 155-mm diameter Petri dishes were then dehydrated in the sterile air of a Holten HV 2448 laminar flow cabinet at the temperature of 19°C for 6 hours. Freezing was performed in two steps (2,3), the first consisting in a slow temperature decrease from 19°C to -40°C at the rate of 2°C min^{-1}, using a Minicool LC 40 (Air Liquide), and the second in direct immersion of the samples in liquid nitrogen (-196°C). Samples are then stored in special liquid nitrogen containers. After 3 days, the cryobiologic tubes were quickly thawed in a water bath at 40°C for 2 min. The gametophytes were then recultured at 19°C under continuous lighting (40 μmole.photon.$m^{-2}s^{-1}$) in 25 ml vials containing the same culture medium used for the "free-living" technique.

Determination of survival rate

Gametophytes observation by fluorescence microscopy using a excitation filter 390 nm and a Olympus BH2 microscope were started two weeks after reculturing. Their regeneration period (14 to 21 days) was similar to that for the conchocelis stage of the red alga *Porphyra linearis* (1). When the cells were excited by ultraviolet light (390 nm), the pigments contained

in the chloroplasts gave off a bright red light. Living cells appeared bright red, and dead cells white.

Survival rates were evaluated according to the following criteria: - = 0% survival (no living cells); + = 0 to 25% survival (some scattered living cells); ++ = 25 to 50% survival (many fluorescent cells but no entire gametophytes); +++ = 50 to 75% survival (many gametophytes, all with fluorescent cells); and ++++ = 100% survival (practically all fluorescent cells).

Capacity of gametophytes to produce gametes

The gametophytes which survived freezing were placed in conditions allowing gamete production. The temperature was progressively lowered (0.5°C per day) to 14°C. Observation by light microscopy indicated whether gametes had been released.

Results

Effet of sucrose concentration upon dehydration rate.

Dehydration is more important when beads have been precultured at a high sucrose concentration (Fig. 2). After 3 hours of dehydration residual water content falls down to more or less 40%. Thus dehydration rates varies and is greater for non treated beads than for treated ones.

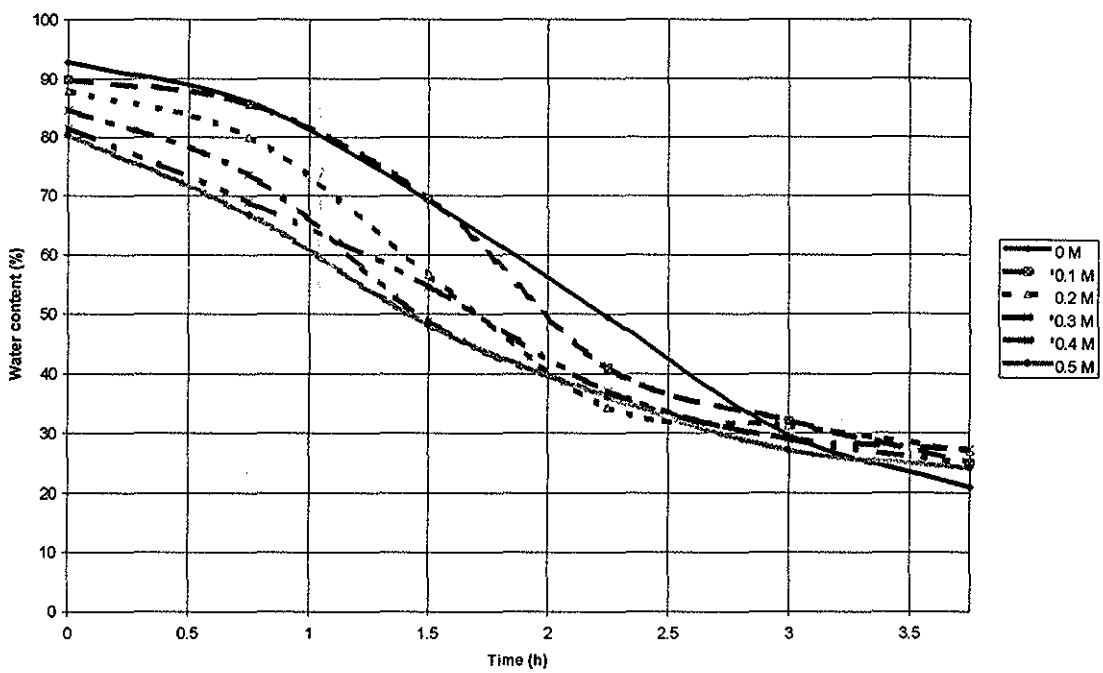

Figure 2: Evolution of the water content of calcium alginate beads during dehydration after 6 hours preculture in 0M to 0.5M sucrose solutions

Cryopreservation

All gametophytes not subjected to dehydration showed no survival after low temperature treatment (Tables 1 and 2).

Culture treatment with a sucrose concentration of 0.5 M was detrimental to gametophyte survival.

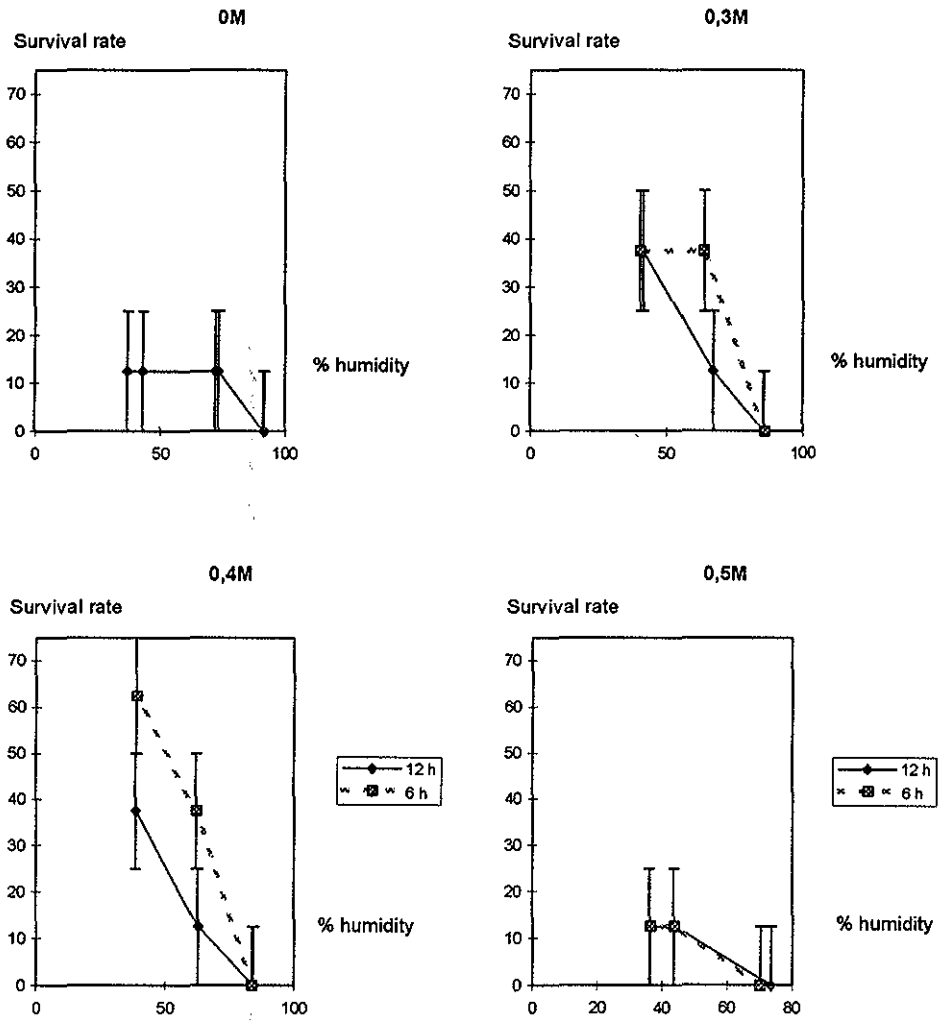

Figure3 : Survival rates of gametophytes for different residual water contents, cryoprotection concentrations and preculture times. Bars indicate the observed range of the survival rate and not a standard error. In these conditions, 25 to 75% of cells survived. The use of a 0.5 M saccharose solution was relatively harmful for gametophytes.

With all concentrations, survival was consistently lower after 12 h than 6 h culture. The highest survival rates were obtained with sucrose concentrations of 0.3 and 0.4 M with a 6 h preculture time and a residual water content of 40% (Fig. 3).

Fluorescence microscopy showed that most of the cells which survived freezing were female gametophytes, which are larger than male ones. Moreover, the tests determined that younger gametophytes (1.5 months) are not resistant to freezing.

Gamete emission

Gametophytes which survived freezing showed that they had retained their reproductive capacity by emitting gametes three weeks after the beginning of temperature decrease.

Thus, this first use of the encapsulation-dehydration technique for macroalgae is promising, notably in the perspective of creating a gametophyte bank.

Discussion and Conclusion

This study constitutes the first attempt at using the encapsulation-dehydration technique to cryopreserve marine microalgae. The major aim of encapsulation combined with sucrose is to reduce the overall water content in the cells and to lower dehydration rate as it seems that the slower this rate is the better the results are. The best results were obtained with 6 h preculture of cells in a liquid medium with a surcrose concentration of 0.3-0.4 M, dehydration to obtain a bead residual water content of 40%, two-step freezing, and rapid thawing at 40°C for 2 min.

Because of its net structure alginate limits and regulates dehydration . It is also thought that it minimizes the osmotic shock as the beads are poured in the preculture medium and avoids superficial withering of the gametophytes. After cryopreservation, gametophytes retained their ability to produce gametes despite the stress experienced. Large cells as female gametophytes seem to be more resistant than male gametophytes indicating the importance of the physiological stage of the cells. Specific cultures only made of males or females should be used to determine whether the sex and the innercell structure are of importance.

Although the results obtained in this study are encouraging, their variability could be reduced by improving the conditions for dehydration and cryoprotection. Gametophytes of *L. digitata* can only resist temperatures above 20°C for a short time. However, in the absence of a room thermostated at 19°C, they were subjected to temperatures of 24-25°C for 3 h during dehydration. More precise temperature control would allow a closer correlation of the dehydration period and bead water content in order to standardize experimental conditions and improve reproducibility.

Administration of the cryoprotector in a single dose during the preculture period might cause osmotic shock despite the use of an alginate coating. Progressive doses might be less aggressive for the cells. Moreover, the preculture step would be easier to control if the nature of sucrose action were clearly determined. Electron microscopy studies could indicate the precise moment when damage occurs, whether during the slow temperature decrease or when the gametophytes are immersed in liquid nitrogen or when the cells are thawed in a water bath.

Comparison of younger (3 weeks) and older (3 months) cells showed a marked increase in wall thickness for the latter, which also had better survival rates. The disorganisation of cellular organelles such as thylakoids has already been demonstrated for the gametophytes of *Undaria pinnatifida* (5).

References

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Table 1 : Evaluation of survival rate as a function of residual water and saccharose concentration for a preculture time of 6 h (- = 0%; + = up to 25%; ++ = 25 to 50%; +++ = 50 to 75%; ++++ = 75 to 100%).

	Dehydration 0h			Dehydration 2h			Dehydration 3h		
Saccharose concentration (%)	Water content (%)	Control	Cryo sample	Water content (%)	Control	Cryo sample	Water content (%)	Control	Cryo sample
0 M	91.84	+++	-	73.47	++	+	37.14	+	+
0.3 M	86.08	+++	-	63.93	++	++	40.48	++	++
0.4 M	83.74	+++	-	62.06	+++	++	38.90	++	+++
0.5 M	70.24	++	-	43.68	++	+	36.29	++	+

Table 2 : Evaluation of survival rate as a function of residual water and saccharose concentration for a preculture time of 12 h (- = 0%; + = up to 25%; ++ = 25 to 50%; +++ = 50 to 75%; ++++ = 75 to 100%).

	Dehydration 0h			Dehydration 2h			Dehydration 3h		
Saccharose concentration (%)	Water content (%)	Control	Cryo sample	Water content (%)	Control	Cryo sample	Water content (%)	Control	Cryo sample
0 M	91.45	+++	-	72.04	++	+	43.18	++	+
0.3 M	85.73	++	-	67.23	++	+	41.55	++	++
0.4 M	84.39	++	-	62.68	++	+	38.63	++	++
0.5 M	73.60	++	-	43.67	+	+	36.02	++	+