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Research Article

Development of *Macrocystis pyrifera* from spores and gametes on artificial substrate. Algal production in a surface culture

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ABSTRACT. This study proposes establishing a methodology for generating algal biomass from spores and gametes of *Macrocystis pyrifera* (Phaeophyta, Laminariales) on nylon ropes that are 100 m long and 3 mm in diameter, installed on the sea surface. The algae are initially farmed in tanks with seawater of 11°-13°C, pH 8.2, salinity 28-33, radiation 75-100 $\mu\text{mol m}^{-2} \text{s}^{-1}$, aeration every 20 min, the addition of nutrients (NaNO_3 and Na_3PO_4), and a 12:12 photoperiod. Twenty days after beginning the cultivation, male gametophytes were 17 μm long by 2.5 μm in diameter, and female gametophytes were 10.5 μm by 7.5 μm . After 60 days of cultivation, the elongated laminar sporophytes were 412 μm by 103 μm . After 195 days, ropes with 2500 μm long sporophytes were installed in the sea at 1 m depth (intermediate cultivation phase), obtaining specimens 36 cm in length after 30 days. Of these specimens, 46 individuals between 30 and 40 cm in size were selected and tied to a 15 m long guide rope that was installed on the surface by means of buoys and anchored to the bottom. After three months, these specimens reached sizes of more than 3 m in length, with abundant laminate biomass surface, reaching an average of 7 kg per specimen, lacking stipes, and with holdfasts of a few centimeters. The surface technique used avoids the herbivory by crustaceans and sea urchins that occurs when the initial developmental stages are done on the seafloor.

Keywords: *Macrocystis pyrifera*, spores, gametes, artificial substrate, culturing, Chile.

Desarrollo de *Macrocystis pyrifera* en sustrato artificial a partir de esporas y gametos. Producción algal en cultivo de superficie

RESUMEN. Este estudio postula establecer una metodología para generar biomasa algal a partir de esporas y gametos de *Macrocystis pyrifera* (Phaeophyta, Laminariales), en cuerdas de nailon de 100 m de largo y 3 mm de diámetro, instaladas en la superficie del mar y cultivadas inicialmente en estanques con agua de mar a 11°-13°C, pH 8,2; 28-33 de salinidad, 75-100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ de radiación y aireación cada 20 min, con adición de nutrientes (NaNO_3 y Na_3PO_4) y fotoperiodo 12:12. A los 20 días de iniciado el cultivo se obtuvo gametofitos masculinos de 17 μm de largo por 2,5 μm de diámetro y gametofitos femeninos de 10,5 μm por 7,5 μm . A 60 días de cultivo, esporofitos laminares alargados de 412 μm por 103 μm . A los 195 días las cuerdas con esporofitos de 2500 μm , fueron instaladas en el mar a 1 m de profundidad (fase de cultivo intermedia), obteniéndose ejemplares de 36 cm de largo después de 30 días. De estos ejemplares se seleccionó 46 individuos de 30 a 40 cm de talla, los cuales fueron atados a una cuerda guía de 15 m de largo, instalada en la superficie mediante boyas y anclada al fondo. A los tres meses, estos ejemplares alcanzaron tallas sobre 3 m de longitud con abundante biomasa laminar en superficie logrando 7 kg promedio por ejemplar, ausencia de estipes y generación de discos de fijación de pocos centímetros. La técnica utilizada cultivando en superficie evita la herbivoría por crustáceos y erizos que ocurre cuando se efectúan cultivos de fondo en las etapas de desarrollo inicial.

Palabras clave: *Macrocystis pyrifera*, esporas, gametos, sustrato artificial, cultivo, Chile.

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INTRODUCTION

Macrocystis pyrifera (L) C. Agardh is distributed along the coasts of North and South America (Coyer

et al., 2001). In Chile, *M. pyrifera* (L) C. Agardh is present along the entire continental Chilean coast (17°29'-56°32'S) (Alveal *et al.*, 1982; Ramírez & Santelices, 1991) and has been exploited along the

northern and central zones of Chile. It is an integral part of both national and international demand for brown seaweeds, a demand that is not satisfied by algal species like *Laminaria*, *Durvillaea*, *Ecklonia*, *Sargassum*, *Lessonia* (Alveal *et al.*, 1990). *M. pyrifera* is used in Chile as a foodstuff in the cultivation of abalone (*Haliotis* spp.) (Fundación Chile, 2001), and exported, ground up, for the extraction of alginic acid.

After work by North (1971) regarding the biology of *Macrocystis* in California, Braud *et al.* (1974) carried out cultures of this species inoculating ropes with spores, which resulted in successful transplants, of plants reaching over 15 m in length in the coasts of France. Studies carried out by Candia *et al.* (1979), and Alveal *et al.* (1982), only achieved the development of the initial sporophyte stages with specimens from the area of Concepción (36°47'S, 72°58'W) and specimens from Beagle Channel (55°S, 66°W), Chile.

Several studies about marine ecological interrelationships have identified *M. pyrifera* as an important refuge environment from herbivory, for nurseries of invertebrates and fish (Macchiavello *et al.*, 2010). These studies were conducted by various national researchers in different parts of continental Chile (Santelices *et al.*, 1981; Moreno & Jaramillo, 1983; Ojeda & Santelices, 1984; Santelices & Ojeda, 1984a, 1984b; Dayton, 1985; Vásquez & Santelices, 1990; Lancellotti & Vásquez, 1999). Research regarding the social and economic importance of *M. pyrifera* has been carried out in Chile as well (Alveal, 1995; Vásquez, 2008; Westermeier *et al.*, 2011). Experimental and ecological works, as well as an understanding of the reproductive strategies of this species, have permitted the determination of population dynamic aspects of *M. pyrifera* in southern Chile (Buschmann *et al.*, 2006).

Furthermore, in previous studies it has been postulated that a correctly controlled culture of this species facilitates the building of biological barriers to attenuate wave impact along the shore (Westermeier & Möller, 1990). It has also been established that this algae can be useful in the reconstruction of zones that have been destroyed by anthropic activities (Celis & Alveal, 2003). Moreover, *M. pyrifera* is used as part of the diet of cultured mollusks, increasing its economical and commercial value (Stuart & Brown, 1994).

The central aim of this study was to generate a culturing methodology for *M. pyrifera* utilizing the implantation of spores and gametes on artificial substrates, through the manipulation of microscopic gametophytes and sporophytes in controlled hatchery conditions.

MATERIALS AND METHODS

Collection of fertile material

Algae were collected from natural populations found in Coliumo (36°32'S, 72°58'W) and in the area of Concepción Bay, Chile (36°39'S, 73°04'W). The reproductive specimens were transported to the laboratory in plastic containers, maintained at 12°C, and the fertile sporophylls were gently cleaned with brushes under flowing tap water before sporulation.

Inoculation and incubation process in tanks

The fertile sporophylls were placed on PVC frames, 50 x 50 cm square, with 100 m of nylon rope, 3 mm in diameter, and installed in 2000 L plastic containers with filtered seawater (0.45 µm). The fertile material was removed once the release of the spores occurred. Meanwhile, the frames with the inoculated substrate was moved to 350 L containers with aeration provided.

During the incubation and initial growing period, the culture was maintained at pH between 8.0 and 8.2, light intensity between 75-100 µmol m⁻² s⁻¹, salinity of the seawater between 28-33, temperature between 11-13°C, with addition of aeration for 20 min every 2 h (Fig. 1) and a 12:12 photoperiod.

The water was changed three times a week (Merrill & Gillingham, 1991), with seawater enriched in the laboratory with agricultural nutrients including NaNO₃ at a concentration of 0.06 g L⁻¹ and Na₃PO₄ at a concentration of 0.01 g L⁻¹ (Alveal *et al.*, 1997; Celis & Alveal, 2003). Growth was measured with an ocular caliper on a Zeiss Oberkochen microscope, obtaining photographs of the initial gametophytes and sporophytes with a Zeiss camera. With this species, the study enabled the generation of productive stages, with the development of biomass in the ocean surface.

Intermediate phase

Upon finalization of the laboratory cultivation in an intermediate culture medium (after 100-200 days) the substrate (nylon rope) was moved to the ocean for 30 days, from November to December. The frame was maintained at a depth of 1 m.

Suspended culture

At the end of the intermediate phase, 46 samples, with an average length of 36 cm, were selected and attached to ropes 2 cm in diameter and 15 m in length. This system was maintained on the sea surface with flotation devices anchored to the sea floor, and identified with a number to follow the unique development of each individual and to understand the

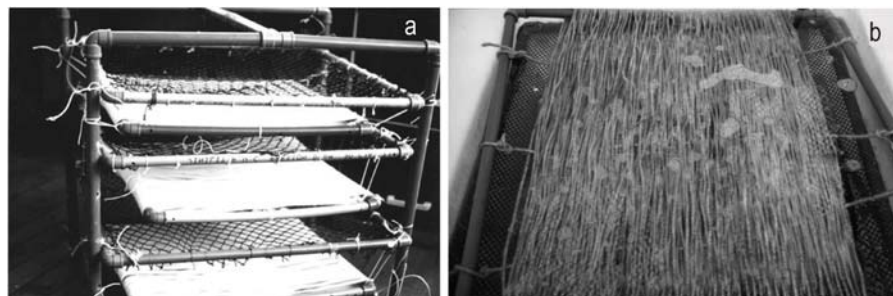


Figure 1. Culture system in tanks. a) Frame with nylon ropes, b) substrate in incubation and growth of the initial microthalli.

Figura 1. Sistema de cultivo en estanques. a) Bastidor con cuerda de nylon, b) sustrato en incubación y crecimiento inicial de microtalos.

developmental processes, both similar and different, to natural growth patterns. Inoculation in tanks facilitates the adhesion and development of more than 10 sporophytes per linear cm of rope and the latter possibility of obtaining samples for comparisons of morphological development. The sporophytes were maintained in the sea for three months.

RESULTS

Inoculation and incubation in the hatchery phase

The release of the spores occurred 2 h after the start of the experiment. This was followed by their settlement on the nylon rope and the commencement of gametophyte development between 24 and 72 h. The formation of the lateral tube reached an average of 43.4 μm and the two-celled structure reached an average of 63 μm in length at 100 h. After five days the gametophytes consisted of 4 to 6 cells. The female gametophytes, 7.5 μm in diameter, appeared strongly pigmented while the male gametophytes were less pigmented and 2.5 μm in diameter. The gametophytes measured an average of 62.6 μm in length at nine days of development, 105 μm at 13 days, and 132 μm at 20 days (Fig. 2).

During this stage and after 20 days of culture, the male gametophytes developed antheridia, while fertilized female cells developed into zygotes that divided into 2 cells (an upper and lower one). The first division is always parallel to the substrate. The sporophytes develop on a hyaline stalk that corresponds to the area of the embryo-spore of the female gametophyte. At first, it had a columnar and oval shape, strongly pigmented, with two rows of cells along the same plane in clear apical cell (Fig. 3).

The sporophytes were 133 μm in length at 24 days. They gradually acquired a laminar shape after 39 days

achieving 287.9 μm in length, with a wide apical section containing 4 to 5 long thin and hyaline rhizoids. At 52 days they had reached an average length of 535 μm .

After 98 days of hatchery culture the size of the sporophytes was on the average 552.42 μm in length, with rhizoids fixed to the nylon substrate, which favors good growth. At 148 days, they reached an average length of 1436 μm , and at 195 days, the end of the laboratory phase, the sporophytes measured 2750 μm , with fronds that tended to be flattened and with a clear development of the holdfast disk. Likewise, lamina, stipes and initial holdfasts were identifiable (Figs. 4 and 5). The growth of this stage is fast and the formation of the holdfast system is notable, obviously seeking a strong adherence to the substrate, as well as the generation of tissue to form the assembly of the initial lamina upon which the definitive specimen will be structured. In the hatchery at 6.5 months the specimens showed an average length of 0.275 cm (Fig. 5).

Sea culture stage (intermediate phase)

The inoculated ropes were moved to the sea, initiating the intermediate phase, until they reached an average length of 36 cm, growing between 16 and 122 cm during the one month of cultivation. After this phase 46 specimens were selected, on the average, between 30 and 40 cm in size, and fixed to a rope 15 m in length, installed on the water surface.

Suspended culture

The development of the suspended adult sporophytes began at 7.5 months, with an initial wet weight of 90.4 g and size of 36 cm. After 17 days they reached 52.3 cm and 88.1 cm in average length by 33 days of development. In the suspended culture phase, the developing stipes formed rhizoids in the upper

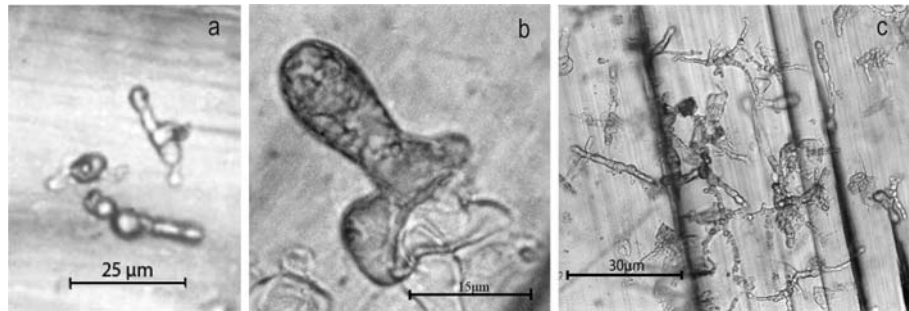


Figure 2. Incubation phase in hatchery. a) Male gametophytes, b) female gametophytes, c) female and male gametophytes on nylon substrate (20 days).

Figura 2. Fase de incubación en hatchery. a) Gametofitos masculino, b) gametofito femenino, c) gametofitos femeninos y masculinos sobre sustrato de nylon (20 días).

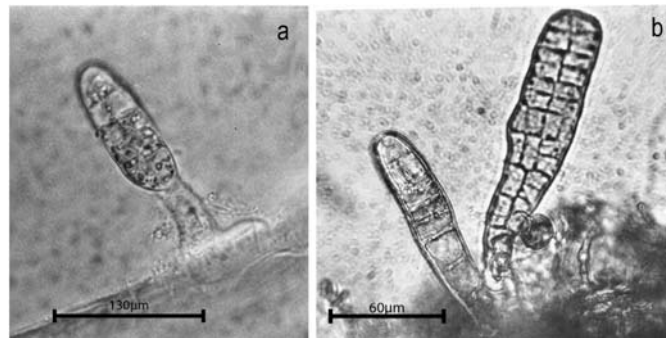


Figure 3. a) Initial development of the sporophytes over embryos, b) female gametophyte strongly pigmented (30 days).

Figura 3. a) Desarrollo inicial de los esporofitos sobre las embriones, b) el gametofito femenino fuertemente pigmentado (30 días).

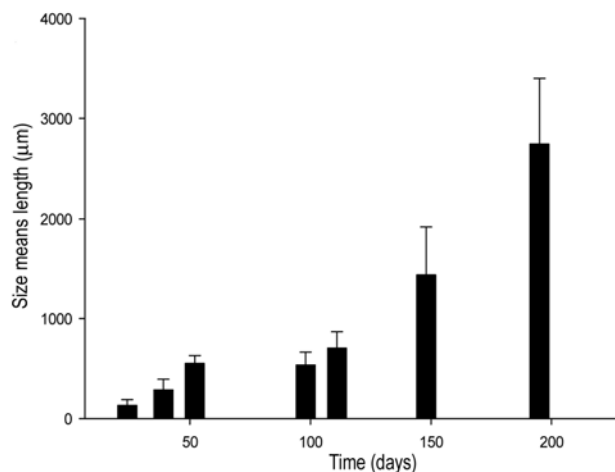


Figure 4. Growth of sporophytes during 195 days cultured in hatchery.

Figura 4. Crecimiento de esporofitos durante 195 días cultivados en hatchery.

sections reducing their size from 1.8 to 0.8 cm at the end of the experiment (Figs. 6 and 7).

At 87 days of cultivation the specimens reached an average length of 2.28 m with a maximum of 3 m. At the end of the study, the majority of the specimens had a holdfasts 6 cm in diameter, 3 m in length and a biomass of 2.7 kg; with a maximum of 7 kg wet weight per specimen (Figs. 8 and 9).

The results showed values of 91 g at the beginning of this stage with a 500% increase after 90 days, with average values of between 2.7 kg (Figs. 9 and 10).

DISCUSSION

These experiments utilized basic knowledge of the life cycle previously identified by North (1971), Alveal *et al.* (1982), and Braud *et al.* (1974). These authors achieved significant success transplanting the initial

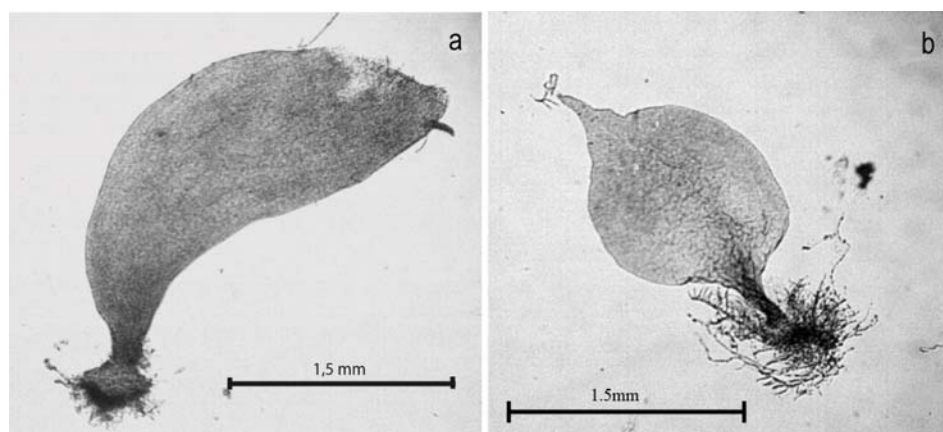


Figure 5. a) Initial sporophytes, b) the development of the holdfast disk (195 days).

Figura 5. a) Esporofitos iniciales, b) desarrollo del disco de fijación (195 días).

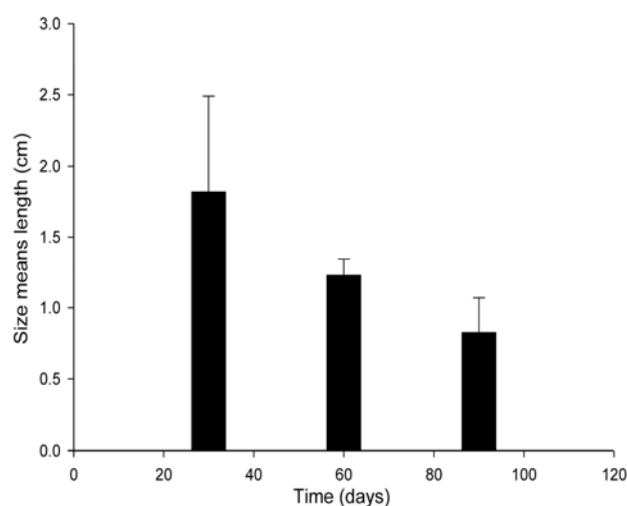


Figure 6. Variation in stipe size during the suspended culture phase.

Figura 6. Variación del tamaño del estipe durante la fase de cultivo suspendido.

stages of *M. pyrifera* in France, and subsequent development in the sea resulted in plants of more than 15 m in length.

The culturing of *M. pyrifera* done by different authors in Chile followed the methodology of Merrill & Guilligan (1991), who inoculated ropes with gametophytes and sporophytes of *Nereocystis* which were then developed in the sea. However, prior to this, Braud *et al.* (1974) and Gutiérrez *et al.* (2006), using a similar method, also achieved the development of adult specimens in the sea.

Celis & Alveal (2003), and the present study, installed microscopic phases of *M. pyrifera* on ropes and grew them in 2000 L tanks with commercial



Figure 7. Sporophytes on the ropes after the inter phase stage (30 days on the sea).

Figura 7. Esporofitos en cuerdas después de la etapa de interfase (30 días en el mar).

nutrients and controlling temperature, pH, salinity, water flow and illumination. In this way, the production of laminar biomass in the sea was made significant by avoiding the generation of stipes, and thereby, demonstrating a system clearly distinct from that used by Westermeier *et al.* (2007, 2010, 2011), Braud *et al.* (1974), and Gutiérrez *et al.* (2006), who used smaller capacity containers in the laboratory and with the development of stipes in the sea. Among the species of the genera *Macrocystis*, *Durvillaea*, and *Lessonia* in continental Chile, *M. pyrifera* and *Lessonia trabeculata*, have given positive results during the culturing process (Edding & Tala, 2003; Westermeier *et al.*, 2006, 2011).

The massive use of inoculated ropes combining the manual process of fixing the primary ropes as

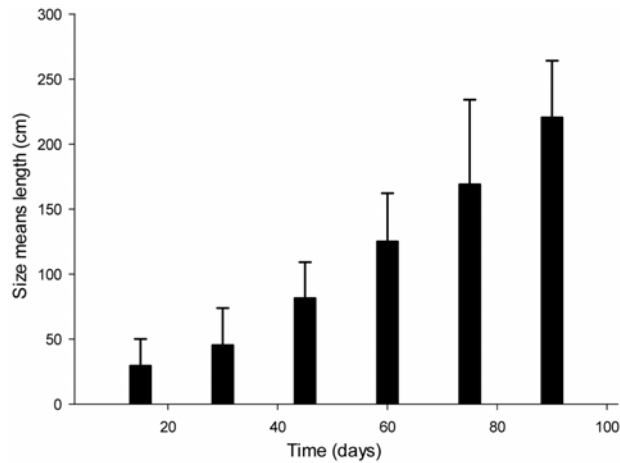


Figure 8. Growth of sporophytes in the suspended culture (90 days).

Figura 8. Crecimiento de los esporofitos en cultivo suspendido (90 días).

indicated by Gutiérrez *et al.* (2006) and Westermeier *et al.* (2007), anchoring and harvesting, without any doubt, is labor intensive. With this system of cultivation, there is direct development of the lamina on the surface, which would undoubtedly facilitate the harvest and the possibility of new generations from the post-harvest remnants and natural re-inoculations on the artificial substrates. The surface crops system prevents the processes of pruning by decapod crustaceans which remove the initial stipes when the crops are installed at the bottom of the sea.

This means of culturing demonstrated an outstanding increase in the biomass of the lamina in systems placed on the sea surface and a decrease in the size of the stipe during the suspended culture, generating, as a result, energy conservation for the plants in order to produce greater lamina biomass as well as the formation of holdfasts of only 6 cm in diameter in contrast to the size achieved by Westermeier *et al.* (2011) using crossed specimens of *M. pyrifera* and *M. integrifolia* (*M. pyrifera*). This differed significantly from the results obtained by Gutiérrez *et al.* (2006) where the weight of the stipes was equivalent to that of the sample lamina cultivated at 2 m depth during seven months which included swells and waves that caused important losses to thalli 30-50 cm in length.

Inoculation and growth trials of *M. pyrifera* on hard substrates or submerged rocky platforms have not been conducted. However, in tanks, the handling of the early stages can be controlled and verified through microscopic observations (Westermeier & Möller, 1990); Gutiérrez *et al.* (2006) as well as Macchiavello

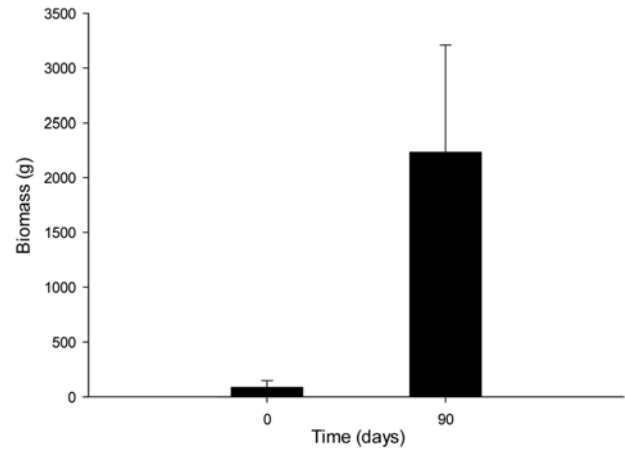


Figure 9. Biomass variations during 90 days of culture at the sea.

Figura 9. Variación de biomasa durante 90 días de cultivo en el mar.



Figure 10. Adult sporophytes at the end of the study.

Figura 10. Esporofitos adultos al final del estudio.

et al. (2010) obtained good yields: 13-22 kg m⁻¹ and 24 kg m⁻¹, measured in linear meters of rope respectively, which were close to the 21 kg m⁻¹ achieved by Celis & Alveal (2003). The increase in size to 175 cm at 120-150 days, obtained by Macchiavello *et al.* (2010) on vertical lines in the sea, shows that an annual harvest is possible using the tested methods, even though the size reached was much less than the 14 m reported by Westermeier *et al.* (2006). Macchiavello *et al.* (2010) projected an annual production of more than 100 ton per hectare per year.

The method used, in this study, to manage the developmental conditions of the initial gametophytes and sporophytes of *M. pyrifera* contrasts with those

utilized by Gutiérrez *et al.* (2006) who selected the spores and later inoculated the substrates by placing them in a cylinder. This study took advantage of the ability to control factors such as salinity, pH, nutrients, water flow and illumination, made possible by the use of 2000 L tanks with sea water filtered through 0.45 μm to permit the inoculation, development and subsequent mass production of *M. pyrifera* directly on ropes at the surface. The development of the first stages of the *M. integrifolia* and *M. pyrifera* forms in barrels by Westermeier *et al.* (2007) proved to be successful. Likewise, Macchiavello *et al.* (2010) had success using the addition of GeO_2 to limit the presence of diatoms, one of the things that most strongly interfere during tank culturing. The inoculation in large volume tanks limits the usefulness of expensive reagents like germanium dioxide (GeO_2) and laboratory nutrients. For this reason, the use of agricultural nutrients was given high priority in this study.

One of the critical aspects of the initial phase of culturing in tanks is taking into account the constant care needed to maintain the pH. This should fluctuate, ideally, between 8-8.2. Likewise, the development of opportunists like algae from the Chlorophyta group or diatoms, and occasionally tunicates and bivalves, especially in the final stages of development, should be avoided. Culturing on the surface helps avoiding the effects of interfering species. For this reason, care and cleaning processes should be done on a permanent basis especially when the thalli of *M. pyrifera* are small (<2 cm) (Celis & Alveal, 2003). Increases in fouling influence the floating ability of the substrates in the sea. However, in this culture it was detected that shading from plants with large, abundant fronds impacted their neighbors by impeding them to grow with a homogeneous rate.

None of the previous studies on culturing of brown algae in Chile discussed the more productive systems of commercial culture, especially in terms of the installation in the sea. The substrates should be manageable in the laboratory, onboard boats, and in the sea.

For the model organism *Macrocystis*, the type of massive culture proposed will have to answer several concerns. With this alga the culture systems utilized are not natural like those used with *Gracilaria*, which have proven to be successful. For *Macrocystis* the inoculation system, the use of ropes, the anchoring system, adjustments and the harvesting system, the repositioning of biomass, the reuse of substrates, culturing on the sea floor or on the surface, all of them give an idea of the complexity of the total system. In addition there, is the labor and the time necessary to

recover the investment which can only be correctly determined through pilot and pre-pilot investigations.

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