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Marine macroalgal nursery: A model for sustainable production of seedlings for large scale farming

Vishal Gupta[a,*], Nitin Trivedi[b], Silvano Simoni[c], C.R.K. Reddy[d,*]

[a] Biological Oceanography Division, CSIR-National Institute of Oceanography, Goa 403004, India
[b] DBT-ICT Centre for Energy Biosciences, Institute of Chemical Technology, Mumbai 400019, India
[c] Department of Chemical Engineering Materials and Environment, University of Rome, Rome, Italy
[d] Division of Biotechnology and Phycology, CSIR-Central Salt and Marine Chemicals Research Institute, Bhavnagar, Gujarat 364002, India

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ABSTRACT

Innovative technologies in seaweed aquaculture are the need of the hour for sustaining the emerging global market demands. The supply of seedlings for year-round sea farming has remained as a major challenge for large-scale production of seaweeds. This study describes a simple innovative model integrating the designing of a prototype with an effective protocol for seedling production and their propagation for the continuous production of seedlings from seaweeds. The novelty of this model includes its design enabling maximum seedlings production and demonstration of reusability of enzyme employed for protoplast (seedlings) production for five times without compromising over the protoplast yield and viability. The laboratory test trials conducted to assess the effectivity of this model in the production of seedlings from protoplasts with *Ulva* yielded seedling biomass of 0.25 kg fresh wt. (> 200 folds of initial biomass) in 44 days culture period over 0.33 m^2 surface area. The present prototype is simple, scalable and facilitates a consistent production of a large number of quality seedlings from relatively a small sample size. A concept layout for scale-up production of seedlings in an outdoor facility is also proposed based on prototype tested in this study. The findings reported in this study collectively not only promote the concept for establishing the macroalgal nursery for large-scale production of quality seedlings suitable for extraction of pharmaceutical and nutraceutical grade products but also provide an option for rapid restoration of species, if found under threat.

1. Introduction

Marine macroalgae (seaweeds) farming represents an important component of aquaculture production and accounts for 27.3 million tonnes fresh weight (20% of total aquaculture production) in 2014 [1] with an annual market value over USD 6 billion [2]. The continuous exploration of seaweed resources for newer products in food, phycocolloids, fine chemicals, cosmetics, and agro-industry has given rise to their overwhelming demand worldwide [3]. Industrial dependency over wild stocks for biomass harvest is surely unsustainable and is often being seen as a cause of market volatilization and a threat to local biodiversity. Recently, there has been a dramatic increase in the wholesale price of agar to 35–45 USD/kg due to short supply of raw material, *Gelidium* to global agar industry [4]. The fundamental challenge, therefore, is managing the seaweed biomass supply chain for growing market demands.

To suffice the seaweed biomass demand, there has been a continuous effort to increase the crop productivity through technological interventions together with new initiatives to expand the farming activity in newer regions beyond Asian countries, more particularly in Europe and America [4,5]. Despite the growing demands for production of seaweed biomass, there have not been radical developments in seaweed aquaculture technology and lags far behind that of agriculture and animal aquaculture in terms of output and transformation in production practices [6,7]. In seaweed farming, primarily either vegetative fragments or reproductive cells (spores or gametes) are used as a source of seedlings (propagules) for propagation in the sea [8–14]. The vegetative propagation has undoubtedly been successful for those seaweed species particularly with higher proliferation potentials of vegetative fragments as in the case of *Kappaphycus*, *Gracilaria*, *Gelidiella*, *Gelidium* etc. [15–21]. However, a considerable harvest of the crop (one-fourth of total harvest) is consumed as seed material for subsequent farming activity [14]. Also, repeated use of seedlings from the same genotype results in loss of vigour, a decline in production, susceptibility to a variety of diseases and pests [22–27]. Another critical risk factor involved in farming of seaweed in open sea is the threat of being complete

* Corresponding authors.
E-mail address: crk@csmcri.res.in (C.R.K. Reddy).

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loss of crop due to abrupt changes in climatic factors, for example, complete loss of *Undaria pinnatifida* along the Sanriku Coast, Japan in March 2011 by tsunami, and *Porphyra yezoensis* at Jiangsu Province, China during 2009–2010 due to sudden rise in seawater temperatures [28].

In contrast, spores have been used as a source of seeding material for farming of those seaweed species (*Porphyra*, *Pyropia*, *Ulva*, *Monostroma*, *Saccharina*, *Undaria* etc.) found with greater reproductive potentials [29–35]. Spores are seeded on artificial substrata and cultured in land-based seedling rearing facility under controlled conditions till attaining suitable plantlet size needed for transplantation [36–39]. Following the seeding operations, the ropes with growing plantlets were out-planted in the open sea for field cultivation [11,38,39]. This kind of practice could continuously supply the feedstock for cultivation but the success largely determined by reproductive potentials of species and thus is considered as a major limiting factor. The deep insights in the reproductive biology of seaweeds have led to develop a combination of various abiotic factors which could induce the reproductive maturity and subsequent sporulation independent of natural life cycle [12,34]. However, it requires systematic experimentation for extended periods to optimize the conditions that induce maturity and sporulation in a given species. Also, the set of conditions optimized for sporulation is highly species specific, varies with the age and physiological background of plant and thus is not reproducible [12,40]. Therefore, it is highly desirable to develop alternative sources and method for seedling production that would be sustainable, scalable, yield a large number of quality plantlets at ease and is totally independent of spores and vegetative fragments.

Protoplasts represent the homogenous cell population devoid of cell walls and are capable of regenerating into de novo plants [41]. There has been significant progress made in isolation and regeneration of protoplasts from diverse seaweeds [42]. The earlier studies have provided the proof of concept of using protoplast for seedling production in *Monostroma* and *Porphyra* [43–46]. The advantages of employing protoplasts as seed material include the production of a large number of plantlets from relatively a small fraction of donor biomass, homogenous population, bypasses optimization of stressors combination, rapid and free from any fouling contaminants. However, their potentials have rarely been exploited for developing land-based nursery for plantlets rearing.

This study describes a simple, scalable, integrated model for the production of a large number of protoplasts-based plantlets from *Ulva*. The species *Ulva* are emerging as potential biofuel crops with demonstrated ethanol productivity of about 10% on dry biomass basis [47]. The commercial scale production of biofuel from seaweed resources is not at near sight due to lack of regular supply of biomass of desired scales. The sustainability in seaweed farming can greatly be achieved by combining the seedling production facility with farming activity.

The model, therefore, demonstrated in this study allows continuous production of seed-stock for sea farming. Also, it offers the scope for producing industrial grade feedstock suitable for extracting sensitive and high-value ingredients for nutraceutical and pharmaceutical applications which are otherwise hard to gain from feedstock harvested from open sea cultivation.

2. Materials and methods

2.1. Sample collection

Thalli of *U. lactuca* were collected from Okha (N 22° 27.04′; E 69° 03.58), Gujarat, along the west coast of India and brought to the laboratory under cool conditions. The species was identified based on different morpho-anatomic characters as well as rbcL gene amplification established in author laboratory. During life cycle study, the collected *Ulva* species was found to be sporophytic based on its natural release of quadriflagellate spores. Selected thalli were thoroughly rinsed with autoclaved seawater (ASW) to remove dirt and epiphytes. The unialgal culture of this alga was established by growing it in sterile enriched seawater medium [48] with GeO_2 (10 mg L^{-1}) for a week under white fluorescent LED lamps of irradiance intensity about 150 μmol photon $m^{-2} s^{-1}$ with a 12:12 h light:dark photoperiod. During this period, the culture media was changed every two days.

2.2. Isolation and purification of protoplasts

The algal thalli were made axenic and protoplasts isolation was carried out following the protocol described [46] for green seaweeds. Briefly, algal thalli was chopped into small pieces (< 1 mm) and rinsed two to three times in ASW to remove debris. Chopped tissues were then transferred to a Petri dish (60 mm × 15 mm) containing 5 mL polysaccharide degrading enzyme mixture [46] and incubated at 25 ± 1 °C on a rotary shaker (80 rpm) in dark for 3 h. During incubation period, periodic microscopic observations were made to ascertain protoplast release. After incubation, the contents of the Petri dish were gently filtered through a nylon mesh of pore size 30 μm to remove cellular debris and undigested fragments. The solution thus filtered was centrifuged at 120 ×*g* for 5 min. Protoplasts get settled as green pallet and the supernatant was collected. The pellet was resuspended in ASW and the protoplast yield was estimated using a hemocytometer. The viability of isolated protoplasts was determined by counting those which actually regenerated in the laboratory culture. For enzyme re-usability, the supernatant collected after centrifugation was utilized as an enzyme mix for treatment of chopped thalli for protoplast isolation. The protoplasts yields and viability were measured after every re-use of enzyme mix. Enzyme mix was re-used till no protoplasts were obtained.

2.3. Biomass production after using protoplasts as seed culture

To investigate the biomass yields, protoplasts were isolated from 100 mg algal tissue of *U. lactuca*. The isolated protoplasts were suspended in 10 Petri-dishes each with 90 mm size containing 15 mL seawater and cultured in dark for 24 h at 25 °C. Thereafter culture was continued under cool white fluorescent LED light at photon flux intensity of 150 μmol $m^{-2} s^{-1}$. The culture was continued till small germlings were obtained. Small germlings were scrapped from the surface and then transferred to aerated flat bottom round flask with enriched seawater medium volume of 400 mL. The biomass was allowed to grow for 20 days. During these culture periods medium was changed after every one week. Biomass productivity was calculated based on the final weight obtained. The daily growth rate of the biomass was calculated following the equation: DGR (%) = $\{(W_T/W_0)^{1/T} - 1\} \times 100$ where W_T is weight of biomass after time T and W_0 is the initial weight.

2.4. Bench-scale model for Ulva seedling production

To develop a bench-scale model for *Ulva* seedling generation, two types of acrylic tanks horizontal tank (size 55 × 30 × 20 cm l × b × h) and vertical tank (size 55 × 30 × 55 cm l × b × h) were constructed. The tanks were designed in such a way that the horizontal tank can accommodate one acrylic sheet of size 53 × 28 cm (l × b) while the vertical tank was made with slots to accommodate multiple sheets. The horizontal tank was used for initial seeding of protoplasts and vertical tank was used for subsequent culture and propagation of protoplast-derived germlings. Protoplasts were isolated from 100 mg tissue of *U. lactuca* as described above. The isolated protoplasts were homogenously spread over the one side of the sheet placed in horizontal tank. The seeded plate was incubated undisturbed for 2 days. At third day, again protoplasts were isolated from 100 mg of tissue and seeded on the other side of the sheet. Sheet was kept undisturbed for 2 days and on 5th day sheet was transferred and placed vertically in the vertical tank for further regeneration and development of protoplasts. The vertical tank

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