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Genetic Identification and Mass Propagation of Economically Important Seaweeds

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<http://dx.doi.org/10.5772/62802>

Abstract

Seaweeds are a primary source of hydrocolloids, which can be processed into various food additives, cosmetics, and pharmaceuticals. The inability of current commercial seaweed farming projects to meet industrial demands is underscored by a plethora of challenges, which include the lack of high-quality germplasm with the desired cultural characteristics. This chapter describes the current trends in commercial seaweed production and the potential technological advances in production methods and genetic selection strategies, which can be applied to raise the productivity of seaweed farms. Molecular markers have become increasingly relevant to the selection of a diverse range of wild varieties for domestication, and this augurs well for strain identification. The development of high-density linkage maps based on molecular markers offers an avenue for the implementation of molecular breeding strategies based on quantitative trait loci (QTLs). Concurrently, productivity of existing varieties can be enhanced by the analysis of exogenous factors known to affect the growth and survival of tissue-cultured seedlings. The application of photobioreactors for tissue culture is another important development, which will be digressed upon. In addition to this, quality control which focuses on the comparison of chemical and physical qualities of the tissue-cultured and conventional cultivated seaweeds will become increasingly relevant to the development of industry standards for sustainable seaweed production to fulfill the increasing demands of seaweed-related industries.

Keywords: Acclimatization, Genetic marker, Marker-assisted selection (MAS), Photobioreactor, Quantitative trait loci (QTLs), Tissue culture

1. Seaweeds and their economic importance

Seaweeds are marine macroalgae generally found living in oceans and coastal areas throughout the world. The classification of seaweeds has been typically based on their phenotypical features, which include pigmentation and photosynthetic properties. Since the mid-nineteenth century, seaweeds have been empirically distinguished into three main divisions based on their color: red algae (phylum: Rhodophyta) consist of about 6000 species, brown algae (phylum: Ochrophyta) consist of about 1750 species, and green algae (phylum: Chlorophyta) consist of about 1200 species [1]. Until recently, a wide variety of seaweeds and their products have been studied for their industrial, culinary, and renewable energy applications, which include cosmetics, chemistry, paint, medicine, biofuel, etc. [2, 3]. Increasing global demand for seaweed resources and overexploitation of natural seaweeds have highlighted the need for sustainable seaweed cultivation to significantly increase captive production in mariculture systems.

Among all the seaweed-based products, hydrocolloids viz. carrageenans, agar, and alginates continue to be the principal extracts, which received commercial attention through their application in various industries [4, 5]. *Kappaphycus* and *Gracilaria* are the two most important red seaweeds in the world trade market, which have been reported to contribute significant amount (60–80%) of world's carrageenan and agar resources, respectively [3]. Seaweed hydrocolloids are mainly used in food-processing industries as is evident from their applications in health-care products such as carrageenan gel capsules and alginate micro-beads [4]. Apart from hydrocolloids industry, seaweed natural products are also a promising source of biologically active compounds with medical and pharmaceutical applications [6], and nutritional supplements for animals and plants [2, 7].

2. Current limitations of conventional seaweed breeding and alternative solutions

Emerging applications of hydrocolloids in food industry and other hydrocolloid-related industries have led to an enhancement of the economic values of red algae including *Kappaphycus*, *Eucheuma*, and *Gracilaria* species. Production of seaweed biomass has globally increased from 4 million wet tones in 1980 to 20 million wet tones in 2010 [8] with 95.5% of biomass produced from artificial cultivation in mariculture program [3, 9]. The economic viability of the seaweed industry is dependent on the production of high-quality germplasm with the desired traits viz. high growth rates, amenability to treatment with fertilizers and resistance to diseases for incorporation into breeding program. The high variation of morphological features in the wild [10, 11] and the lack of diagnostic morphological characters have resulted in the misidentification of cultivated seaweed strains, which in turn have contributed to a decline in productivity and quality of cultured seaweeds.

Current practices of seaweed cultivation are predominantly based on traditional methods, where seaweeds are exposed to environmental challenges and pathogens [12, 13]. Most of the

seaweeds are cultivated using seedlings produced by vegetative propagation from cultured germplasm. Through this practice, parasites or pathogens from the harvested seaweeds may be re-introduced and subsequently reduced the productivity of the farm. The other logistical problems faced by conventional seaweed farmers include the identification of appropriate sites for farming, labor intensive tasks such as inspection, disease, and seedling losses resulting from extreme weather conditions and water quality. In order to increase the productivity, modern biotechnology via tissue culture can be considered as one of the best options to overcome the conventional breeding challenges including shortage of raw material for planting and seedlings destruction by epiphytes, subsequently facilitate the propagation of high-quality seaweeds [9, 14, 15].

3. Application of molecular markers in seaweed breeding

The establishment of an effective seaweed breeding programs is founded on the selection of strains of seaweed with desirable cultural characteristics such as high growth rates, high carrageenan content, disease resistance, and accelerated growth in response to supplemental fertilizers. Phenotypical identification methods are currently the standards by which specific seaweed strains are selected. Although invaluable, morphological characterization can be time-consuming and requires a high level of expertise to discriminate key morphological features indicative of the seaweed species. In addition to this, the physical characteristics of seaweeds tend to be variable as they are directly influenced by environmental factors [16]. Most of these seaweeds cannot be distinguished on the basis of one or collection of specimen using morphological characters alone, and an exhaustive taxonomic study is essential before the variety can be identified. For example, high morphological plasticity within the Hawaiian *Eucheuma* seaweeds has led to the misidentification of three introduced eucheumatoid species [17]. A commercially important seaweed species is selected on the basis of the types of biopolymers that they synthesize, where the infrared spectroscopy of their gels has become a measurement to differentiate among genera and species [18]. Nowadays, morphological data have to be complemented with molecular data in order to characterize the desired species of seaweed. Molecular markers are an ideal tool for the classification of cultivated and wild seaweeds independent of their morphological appearance and growth stage. Their application can be extended to marker-assisted selection (MAS) and the development of isogenic strains for the application in current and future propagation programs. The development of molecular markers for germplasm will be of useful for species- and variety-specific identification, plant variety protection and interspecific and intergeneric crosses development for economically important seaweed species.

3.1. Genetic marker for identification of commercially important seaweed species

To date, the application of different genes for the genetic identification of seaweed species is widely carried out, where the targeted DNA regions are the nuclear, plastid, and mitochondrial DNA (mtDNA). Most molecular characterization targets seaweeds with economic value such as *Palmaria palmate* (Dulse), *Porphyra umbilicalis* (Nori), *Gracilaria changii*, *Kappaphycus alvarez-*

zii, *K. striatus*, *Euclidean denticulatum* (carrageenophytes), and many more [19–23]. Currently, large-scale DNA barcoding such as Red Algal Tree of Life Initiatives (RedToL) is analyzing phylogenetic relationship of 471 red seaweeds using two nuclear, four plastid, and two mitochondrial encoded gene markers [24]. China has also conducted a large-scale phylogenomic analysis of marine red algae revealing evolutionary lineages for rhodophytes [25, 26]. A good DNA barcoding locus should have adequate internal variability to enable differentiation at the species level and contain flanking regions that are conserved enough to study routine amplification across highly divergent taxa [27].

Nuclear ribosomal regions, which include sequences of large subunit (28S or LSU), small subunit (18S or SSU), and the intergenic transcribed spacers (ITS1, ITS2), can be served as target sites for molecular markers because the ribosomal DNA (rDNA) genes contain both highly conserved and variable regions that can be used as diagnostic tools for certain organisms [28]. The small subunit (18S) and the large subunit (28S) regions are the most used regions for marker development as they are best suited for inference at high taxonomic levels [29]. However, ITS region is often targeted for intraspecific genetic studies in Chlorophyceae (*Codium fragile*) [30], Phaeophyceae (*Fucus serratus*, *F. evanescens*) [31], and Rhodophyceae (*Chondrus crispus*, *Ulva intestianlis*, *U. compressaa*) [32, 33] on account of its high rate of nucleotide substitution, permitting comparison between relatively diverged taxa [30, 33]. Hu et al. [19] had also used the ITS1 region to study the intraspecific relatedness among 59 *Porphyra yezoensis* (Nori) for selective breeding program of economically important nori crops.

mtDNA has a higher mutation rate that gives rise to variation in its DNA sequence [34]. mtDNA is usually used to analyze the phylogenetic relationships of groups within a species or individuals that are closely related [35]. The gene map of mtDNA of the red alga, *P. purpurea*, is available, where all the different genes have been successfully sequenced [36]. The mtDNA loci, which are generally targeted in seaweed identification, are cytochrome c oxidase subunit 1 (*cox1*), cytochrome oxidase subunit 2–3 intergenic spacer (*cox2–3* spacer) and cytochrome b (*cytb*) genes [17, 37–42]. Tan et al. [41] had evaluated the effectiveness of three mtDNA markers, *cox1*, *cox2*, and *cox2–3* spacer in barcoding the two commercial important carrageenophytes, *Kappaphycus* and *Euclidean* seaweeds, and has determined that the *cox2–3* spacer DNA marker is more suitable as a barcoding gene because its widespread use. Recently, Lim et al. [42] had found higher species diversity of *Kappaphycus* seaweeds in Southeast Asia (Malaysia, Indonesia, the Philippines, and Vietnam) using the mitochondrial *cox1* and *cox2–3* spacer.

Loci derived from the chloroplast genome (cpDNA) can be used for the identification of seaweed species due to the low frequency of structural changes and low sequence evolution rate of cpDNA [43]. The cpDNA loci that are routinely used for seaweed identification are the ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCo) gene, specifically the large subunit of the RuBisCo (*rbcL*) [41, 44–46] and the RuBisCo spacer [47–50]. In a study conducted by Geraldino et al. [46] molecular phylogeny of 23 specimens of red alga, *Hypnea flexicaulis* from three countries (Korea, Taiwan, and the Philippines), was successfully studied based on the plastid *rbcL* region. Guillemain et al. [48] utilized the RuBisCo spacer region to identify six species of Gracilariaceae: *G. gracilis*, *G. conferta*, *G. dura*, *G. multipartite*, *G. vermiculophylla*, and *G. longissima*, which exhibit a high degree of phenotypic similarity.

Identification of commercially important seaweeds based on standard DNA barcodes or single marker amplification has proven to be useful as the phenotypic plasticity of the species can confound traditional taxonomic approaches. **Table 1** showed the summary of nuclear, mitochondrial, and plastid DNA regions that are used for the identification of rhodophyta [51]. Molecular markers are still valuable, despite the increasing popularity of next generation sequencing technologies, where the identity of an unknown seaweed species can be acquired based on a simple polymerase chain reaction amplification and a single sequence read (two sequence reads if both strands are sequenced). Examples of DNA markers used to identify commercially important seaweeds are given in **Table 2** [46, 52–57].

DNA regions	Abbreviation	Size (bp)
Nuclear DNA		
Small subunit ribosomal DNA	<i>SSU</i>	~1800
Internal transcribed spacer ribosomal DNA	<i>ITS</i>	~650–1100
Large subunit ribosomal DNA	<i>LSU</i>	~2700
Plastid DNA		
Photosystem I P700 chlorophyll a apoprotein A1	<i>psaA</i>	~1600
Photosystem I P700 chlorophyll a apoprotein A2	<i>psaB</i>	1250
Photosystem II thylakoid membrane protein D1	<i>psbA</i>	~950
Plastid LSU (23S) domain V	<i>UPA</i>	~370
Ribulose-1,5-bisphosphate carboxylase large subunit	<i>rbcL</i>	~1350
Mitochondrial DNA		
Cytochrome c oxidase subunit 1 DNA barcode region	<i>COI-5P</i>	~664
Cytochrome c oxidase subunit 1 extended fragment	<i>COI</i>	~1232
Cytochrome b	<i>COB</i>	~940
Cytochrome oxidase subunit 2-3 intergenic spacer	<i>cox 2-3</i>	~350–400

Table 1. Summary of nuclear, mitochondrial and plastid DNA regions used for identification in Rhodophyta [51].

DNA markers	Primers	Primer sequences	References
Nuclear DNA			
SSU ribosomal DNA	Forward primer	5'-CAACCTGGTTGATCCTGCCAGT-3'	[52]
	Reverse primer	5'-TGATCCTTCTGCAGGTTACCTAC-3'	
ITS ribosomal DNA	Forward primer	5'-TCGTAACAAGGTTTCCGTAGG-3'	[53]
	Reverse primer	5'-TTCCTTCCGCTTATTGATATGC-3'	
Mitochondrial DNA			

DNA markers	Primers	Primer sequences	References
<i>cox1</i>	COXI43F	5'-TCAACAAATCATAAAGATATTGGWACT-3'	[46]
	COXI1549R	5'-AGGCATTTCTTCAAANGTATGATA-3'	
<i>cox2-3 spacer</i>	Cox2_for	5'-GTACCWTCTTTDRGRRKDAATGTGATGC-3'	[54]
	Cox3_rev	5'-GGATCTACWAGATGRAAWGGATGTC-3'	
Plastid DNA			
<i>rbcL</i>	F7	5'-AACTCTGTAGTAGAACGNACAAG-3'	[55, 56]
	R753	5'-GCTCTTTCATACATATCTTCC-3'	
RuBisCo spacer	Forward primer	5'-TGTGGACCTCTACAAACAGC-3'	[57]
	Reverse primer	5'-CCCCATAGTTCCCAAT-3'	

Ambiguous nucleotide codes according to IUPAC: K=G/T, R=A/G, W=A/T, D=A/G/T.

Table 2. Examples of DNA markers used for seaweed identification.

3.2. Quantitative trait loci (QTLs) for economically important traits

Quantitative trait locus (QTL) analysis is a statistical method that links two types of information, phenotypic data (trait measurements) and genotypic data (usually molecular markers), in an attempt to explain the genetic basis of variation in complex traits [58]. A wide range of agronomic traits in crop plants, including plant productivity and stress tolerance, are complex traits controlled by QTLs [59]. Identification of these QTLs will facilitate the development of novel varieties of seaweeds via conventional breeding approaches as well as genetic engineering. A (QTL) is a region of DNA (the locus) that correlates with variation in a phenotype, which is designated as the “Quantitative Trait”. The QTL contains the genes that encode for the phenotype is tightly linked to the trait over successive generations. Traits of agronomic importance may be controlled by a single gene or in most cases by multiple genes. For example,

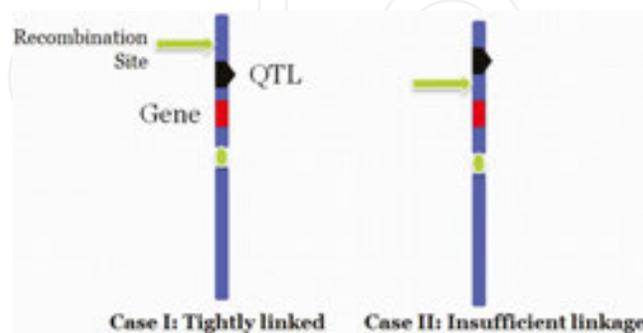


Figure 1. The gene encoding the quantitative trait is designated as a quantitative trait locus (QTL). The genetic markers for this trait can be located with the gene itself; however, in most cases, breeders will utilize the regions flanking the locus to design the markers. This is to ensure that the QTL can be tracked over successive breeding cycles and the markers are tightly linked to the QTL.

if we bred a disease tolerant seaweed with a seaweed that yields a high amount of carrageenan, we can generally assume that the resultant hybrid will be a high yielding, disease tolerant variety. This is a generalized assumption based on the hypothesis that the traits are discrete (**Figure 1**). Markers are generally selected based on regions that flank the quantitative trait (**Figure 2**) and these can be validated over successive breeding cycles.

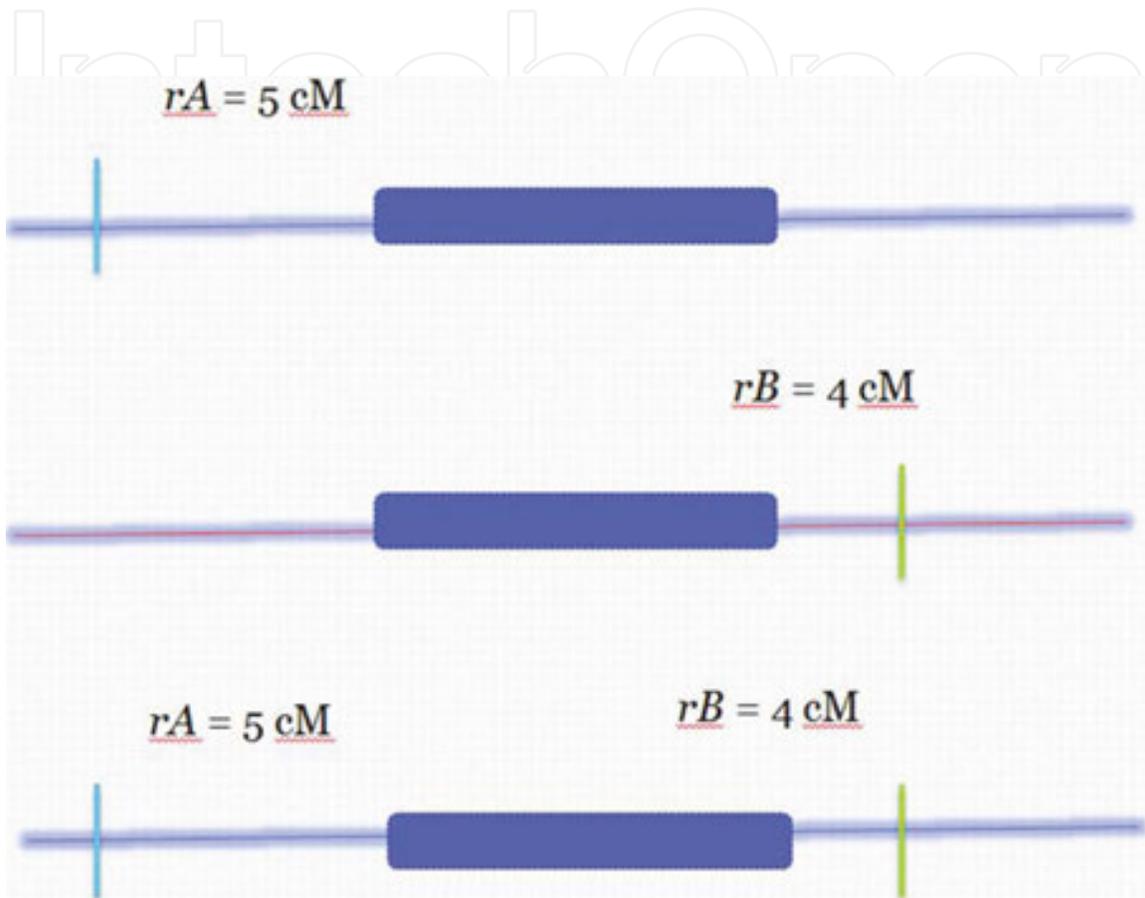


Figure 2. The ideal set of markers for QTL mapping is located on either side of the QTL and at a map distance of 4–10 cM on either side of the QTL. This ensures reliability over several cycles of recombination due to tight linkage.

However, it should be noted that the majority of traits are not discrete. Analysis of hybrids reveals that segregating populations consist of individuals with continuous traits. In the case of seaweeds, one example of this class of QTL can be the diversity of pigmentation observed in variants of *E. denticulatum* and *K. alvarezii*. This can be attributed to the process of indeterminate hybridization in the wild. Continuous traits cannot be analyzed in the same manner as discontinuous traits because a multiplicity of interactions between genes and their regulatory elements determines their phenotypic presentation. Commercial cultivators of seaweeds screen wild populations and select variants with traits such as rapid growth, resistance to diseases, adaptation to different salinity levels, carrageenan yield and type, response to treatment with fertilizers and ability to overcome parasites. All of these traits are controlled by multiple genes. For example, a rapid growth rate will be determined by the genetic proclivity to fix and utilize dissolved carbon dioxide at a higher rate as compared to slow

growing varieties. Tolerance to diseases and parasites may be the result of the ability to secrete antagonistic principles that inhibit pathogens. Traits controlled by multiple genes segregate independently according to Mendel's Laws, and their expression as phenotypes will be affected by environmental factors.

Commercial breeders adopt a range of approaches in order to integrate diverse traits in order to produce an elite strain with all the desired agronomic traits. This approach entails the selection of wild genotypes purely on the basis of their phenotypic characters. This is an ideal strategy to adopt in the case of seaweeds, where no established elite strains exist. Marker-assisted gene pyramiding aims to produce individuals with superior economic traits according to the optimal breeding scheme, which involves selecting a series of favorite target alleles after cross of base populations and pyramiding them into a single genotype [60]. The strategy for pyramiding is depicted in **Figure 3**.

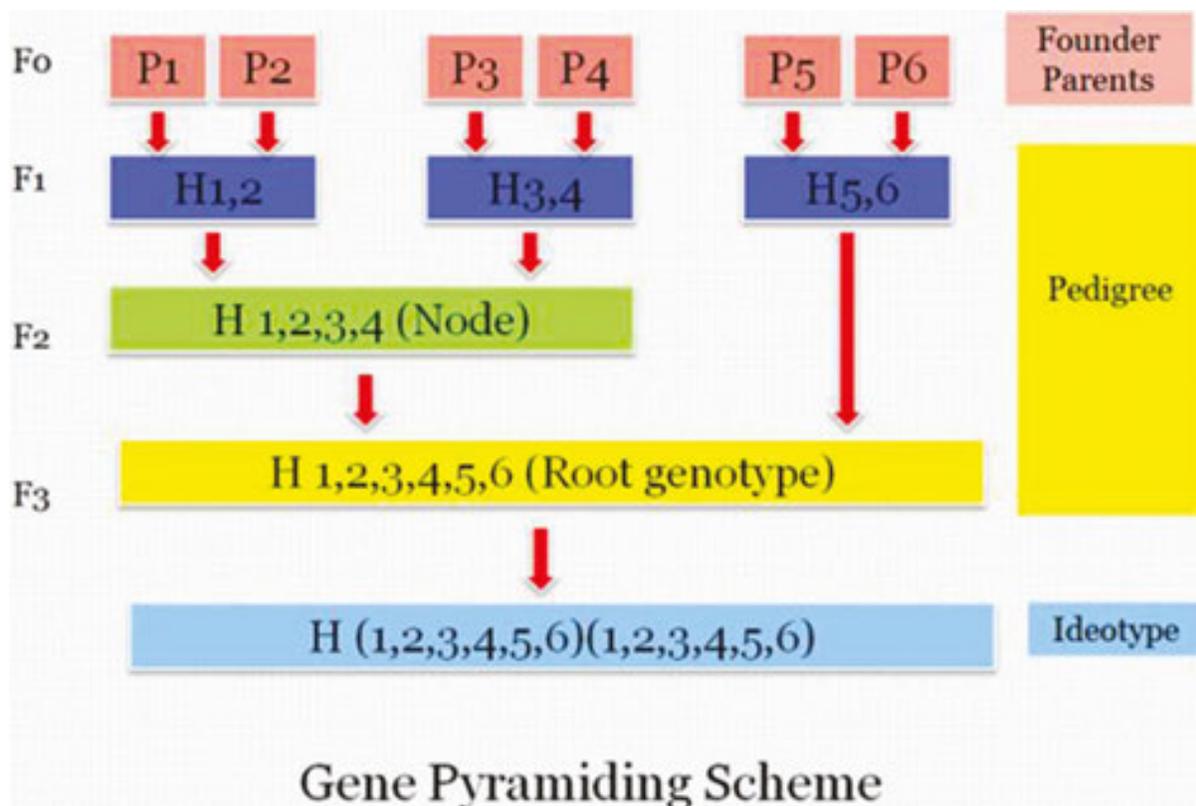


Figure 3. Marker-assisted pyramiding of ideal agronomic traits in seaweeds. In this scheme, six varieties with the desired traits are merged into one ideotype over successive breeding cycles. The loss or integration of QTLs can be assessed at every generation using polymerase chain reaction (PCR)-based molecular markers.

An alternative approach which can be undertaken when a grower has an elite strain, which needs to be supplemented with additional traits is backcrossing with the parental genotype over successive generations. This approach (**Figure 4**) mitigates the likelihood of genes encoding for undesired traits from the wild type from manifesting in the hybrid phenotype.

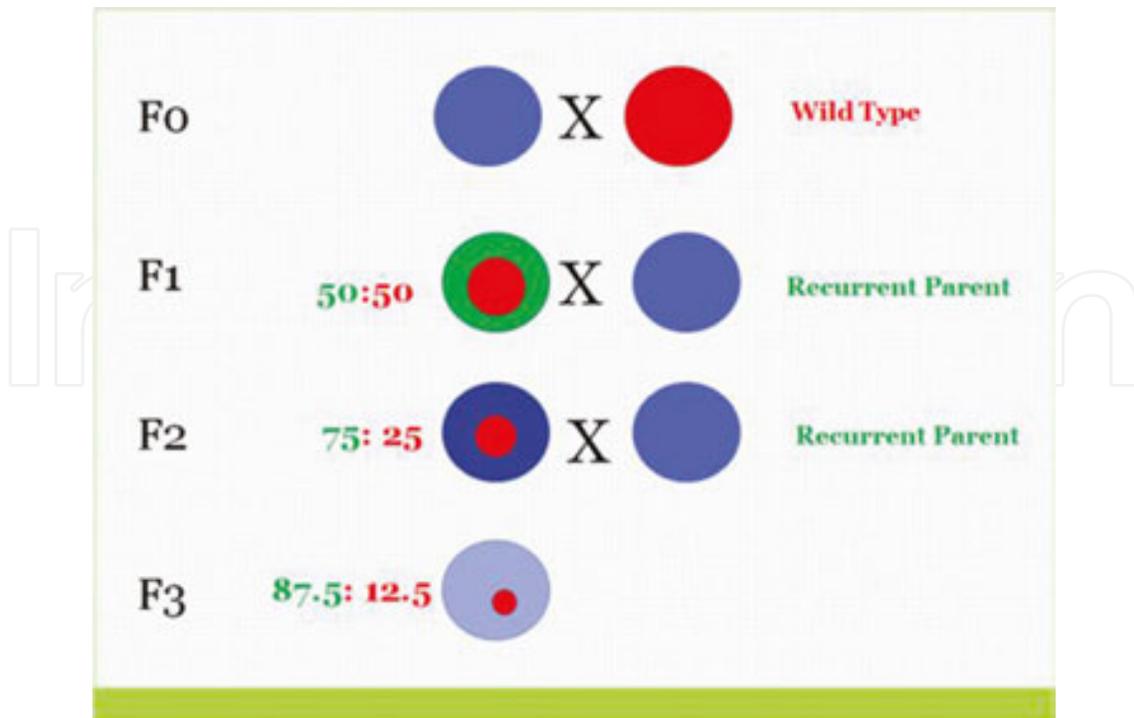


Figure 4. Marker-assisted backcrossing using molecular markers can be applied in circumstances, where it is necessary to improve the elite strain.

The first step in developing a linkage map using QTLs involves information pertaining to phenotypes and their association with specific genotypes [61]. Ideally, molecular breeding should commence with the collection of all the available phenotypes from the wild. The second step will be the identification of specific traits, which are associated with each phenotype; once this has been established, the third step will involve the elucidation of genomic information and its conversion into suitable molecular markers. This can be done using available genomic information or on the basis of expressed sequence tags (ESTs) [62–64]. Seaweeds, unlike terrestrial plants, have unusual breeding cycles [65] and the mechanism need to be established prior to commencing a defined breeding program. New insights into the draft genomes of *Chondrus crispus* [66], *Ectocarpus siliculosus* (Dillwyn) [67] will provide a strong basis for linkage mapping in related species of seaweeds.

Current research work on QTL mapping in seaweeds has focused on commercially cultivated species, which are *Laminaria japonica* [68] in which QTLs for frond length and width were identified using AFLP mapping. Linkage maps for hybrid populations of *Saccharina japonica* X *S. lonigissima* have been developed, and the authors have concluded that the map is not sufficiently dense in order to identify quantitative traits and establish linkage [69]; however, this represents the first step in development of a putative linkage map, which has the potential for transferability to closely related species. Genetic analysis of the marine red algae (*P. yezoensis*) has provided a basis for the identification of genes related to photosynthesis and the fixation of chromogens [70]. Interestingly, the similarity between seaweed genomes has been highlighted in *Laminaria digitata*, in which case a majority of molecular markers could be

applied in *Saccharina japonica* [71]. The first attempt at linking genetic markers with economic traits has been made in the case of the red alga *Pyropia haitanensis* using sequence-related amplified polymorphism (SRAP) markers [72], and specific genes related to stress tolerance have been identified in the model seaweed *Ectocarpus siliculosus* [73] by profiling the gene expression of enzymes that counteract oxidative stress. The mapping of QTLs in seaweeds is currently in its infancy. Unlike commercially cultivate crops such as rice and wheat, the culture of diverse kinds of seaweeds is confined to small geographic regions. The cost of development of QTLs needs to be offset with the returns on investment, which can be realized when the molecular markers have been validated and applied for breeding. The seaweed farming and breeding community urgently needs to identify a universal model seaweed, which can be characterized at the genomic level before a fairly accurate QTL map can be developed.

3.3. Marker-assisted selection (MAS)

Marker-assisted selection is defined as an indirect selection method of an individual with desired traits in a breeding program based on DNA markers [74]. The important of MAS in a seaweed breeding program is to obtain basic genetic knowledge of the chosen commercially important seaweed. Some desired seaweed traits, such as crop yield or phycocolloid content, may be controlled by one gene or a group of genes. Therefore, it is beneficial to develop markers for a range of commercially important seaweed species to provide the foundation needed for MAS in the seaweed breeding program [75].

In seaweed farming, specifically for the phycocolloid industry, the desired traits of seaweed would be disease resistance, suitable carrageenan content, high productivity, and yield. These desired characteristic or traits can be genetically simple, where only one gene is involved. However, most economically important crops tend to have traits that are genetically complex, where it is controlled by many genes (QTL) and the environment [75]. For example, Babu et al. [76] had detected a total of 47 QTLs for drought resistance traits from various plant water stress indicators to increase production and yield of rice in rainfed agriculture ecosystems. To date, there are no reports in the literature of the application of MAS in seaweed breeding program. Recently, Maili et al. [77] had successfully developed eight out of 112 single loci DNA markers to discriminate between varieties of *K. alvarezii*, *K. striatus*, and *E. denticulatum* seaweeds, where the markers could be applied in MAS and hybrid development. In future, application of DNA markers in MAS could be used as a tool that can help seaweed breeders to select more efficiently for desirable traits for the improvement in the culturing method of seaweed.

4. Mass propagation of seaweed seedlings via tissue culture

Repeated vegetative propagation applied in conventional seaweed cultivation was found decreasing genetic variability of seaweeds and subsequently contribute to the decreased in growth rates and yields and increased susceptibility to diseases [78]. Micro-propagation via tissue culture technology has been proposed as an alternative method compared to conven-

tional breeding to resolve the seedling shortage problem and increase the productivity of seaweed raw materials. Micro-propagation is a versatile tool to produce high number of uniform specimens from selected strains with desirable characteristics and increase seed stock production in shorter period of time [79]. However, challenges including lack of optimized protocols to obtain axenic cultures and regeneration of explants have limited the widespread use of tissue culture technology in commercial seaweed production. The efficacy of seaweed tissue culture is depends on the effective manipulation of endogenous (age, source, developmental stage, and physiological state of explants) and exogenous (media composition, light, salinity, pH, and temperature) factors [80, 81]. Current researches have been strategized to improve the culture conditions for mass production of high-quality laboratory seedlings to enhance the overall productivity of seaweed cultivation [3, 9, 14, 15].

4.1. Preparation of axenic cultures

Explants have to be sterilized in order to obtain the axenic cultures for mass propagation in tissue culture [82]. Seaweed samples collected from the wild are associated with a significant level of biological contamination, which is likely to be commensal or symbiotic; therefore, it is necessary to surface sterilize the explants with general disinfectants as well as targeted antibiotics prior to cultivation. Povidone iodine and alcohol are common disinfectants used for surface sterilization as they have a localized activity compared to the narrow spectrum antibiotics with their functionality limited to specific classes of microbes [83]. Surface sterilization of seaweeds is difficult as they lack of thick protective surface, and therefore, sodium hypochlorite and similar agents can easily damage the tissues especially newly regenerated thallus [84]. Prolonged exposure of explants to excessive disinfectants (e.g., more than 5 min in 2% betadine and more than 72 h in 5% antibiotic mixture) was reported causing patches of damaged surface on thallus and explants [85].

4.2. Media composition

Culture media commonly used for rapid propagation of rhodophyte are reported to be Provasoli's enriched seawater (PES) [86], seawater supplemented with von Stosch (VS) solution [87], and seawater enriched with half strength "f medium" (F/2) [88]. The selection of culture media for seaweed propagation is highly dependent on the nutrient level, ambient water, and cultured species. The optimized culture medium for economically important *K. alvarezii* was discovered to be seawater enriched with 50% of PES solution, whereas enrichments with 50% of VS and 50% of F/2 solutions were found not effective for *K. alvarezii* cultures [15]. Besides, *G. changii* cultured in 25% of PES enriched seawater was revealed propagating well and demonstrated promising growth rate [80]. Although some rhodophytes have been reported to grow well in VS and F/2 media [89, 90], the difference in media used may be due to the source of different explants or different genotype of explants. PES medium has low concentration of nutrients, whereas F/2 and VS media have a higher concentration of salts which may interfere with the growth of *K. alvarezii*. The F/2 medium is literally formulated for growing coastal marine algae especially diatoms [88], while the VS medium is developed for culturing and investigating the life cycle of the freshwater red algae *Bangia atropurpurea* [91].

4.3. Plant growth regulators

The addition of plant growth regulators and their role in seaweed tissue culture have been extensively reviewed [92–94]. Cellular competence to plant hormones in cultivated seaweeds is significant only if the cells possess ability to perceive, transduce, and respond to the hormonal signal [95]. The common plant hormones used in seaweed tissue culture are auxins (indole-3-acetic acid, 2,4-dichlorophenoxyacetic acid), cytokinins (benzyladenine, isopentenyladenine, kinetin), and gibberellins (gibberellic acid). The presence of phytohormones in tissue culture medium is known to be able to stimulate tissue elongation and contributes to the overall plant growth [15]. Generally, auxins are used to increase protein synthesis, induce morphogenesis, and elicit changes in genetic expression of explants [96], while cytokinins are used to stimulate cell division, enhance metabolic activities, and affect cell differentiation in seaweed tissue cultures [97]. Combination of auxins (α -naphthalenacetic acid and phenylacetic acid) and cytokinins (N^6 -(2-isopentenyl) adenine and 6-benzylaminopurine) has been reported to induce the highest callus growth in *K. alvarezii* [98], whereas indole-3-acetic acid and 6-benzylaminopurine in their combination have been revealed to stimulate the regeneration process of *K. alvarezii* [15, 79].

4.4. Organic fertilizers and biostimulant

The organic requirements for axenic seaweed culture are remained unclear although additions of organic complexes (coconut milk, yeast, and algal extracts) to increase the growth rates of seaweed tissue have been reported [99]. Three commercially available formulated fertilizers and biostimulant in global market for seaweed cultivation are Acadian marine plant extract powder (AMPEP), Gofar600 (GF), and natural seaweed extract (NSE). AMPEP is extracted from *Ascophyllum nodosum* [100], while GF and NSE are the mixture extracts of brown seaweeds including *A. nodosum*, *Sargassum* sp., and *Laminaria* sp. in different ratios of concentration [101]. The use of AMPEP was first reported to successfully induce the regeneration of young plants from different varieties of *Kappaphycus* seaweed [100]. Other studies have also highlighted the positive influence of AMPEP application on the growth and health of *K. alvarezii* cultures both in vitro and in the field [101–104]. Brown seaweed extracts as contained in AMPEP have been discovered to potentially activate the natural defense system of *K. alvarezii* against pathogens and ameliorate the negative impacts of exposure to oxidative bursts, which may result in bleaching of thallus [105].

5. Exogenous factors affecting seaweed tissue culture

Studies on optimizing the growth of economically important seaweeds, especially *Kappaphycus* and *Gracilaria* spp., in tissue culture conditions can help to mass propagate these viable species for continuous, steady, and defined production, while circumventing the barriers of seasonality and environmental vagaries in seedlings production. Several protocols for callus induction and thallus regeneration of wide variety of seaweeds are available in the literatures [3, 85, 106]. A number of studies have also reported direct regeneration of micro-propagules

from the explants of red algae for maintenance and clonal propagation of maricultural stock [15, 78, 98]. Apart from media composition and supplementation of phytohormones and fertilizers, the abiotic factors determined to have significant effect on the growth of seaweed tissue cultures are reported to be light intensity, aeration activity, salinity, and pH [15, 83]. The daily growth rate (DGR) of seaweeds in tissue culture optimization was measured and calculated as $DGR = [(W_t/W_0)^{1/t} - 1] \times 100\%$, where W_0 is the initial fresh weight, and W_t is the final fresh weight of the seedlings after t days of culture [107].

5.1. Light intensity

Light source is one of the most important parameters to be optimized in seaweed cultivation. The intensity, wavelength, and spectral quality of light, all influence the photosynthetic productivity of algae. Different strains or varieties of seaweed may exhibit different optimum growth range and tolerance to different light resources. *K. alvarezii* strains from Sabah, Malaysia, have been discovered to achieve optimum growth under photon flux density of 75 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ with DGR of $4.3 \pm 0.5\% \text{ day}^{-1}$ [15]. Various forms of *K. alvarezii* and *E. denticulatum* from the Philippines have been reported grew under irradiances of 25–160 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ with optimum growth in 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ [98], while two different strains of *E. denticulatum* and *K. striatus* from Southern Japan have been revealed attained highest growth rate under light irradiance of 145 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ [108]. Light intensity in the range of 5–100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ is commonly used for tissue culture of *K. alvarezii* and other phycocolloid yielding seaweeds [85, 109]. Explants were found responded to higher light intensity by producing more buds and subsequently leading to an increase in biomass. However, further increase of light intensity was found to be a detriment to the growth of seaweeds as this might be due to the effect of photoinhibition [83]. Nitrogen concentration in seaweed tissues was reported influenced by light intensity, where the increase of light intensity beyond a critical level has resulted in the decreased of crude protein content in seaweeds [110].

5.2. Aeration activity

Aeration is an ideal method of energy transfer; whereby, atmospheric carbon dioxide is diffused into the culture medium. Continuous aeration is an important process to provide enough carbon dioxide for carbon fixation in seaweed metabolism. Meanwhile, carbon source can be provided in the organic form such as glycerol yet the addition of glycerol in the culture medium was found to reduce the morphogenetic capacity and totipotency of the explants [111]. Continuous aeration (at 30.0 L h⁻¹) was found to significantly enhance the growth of *K. alvarezii* with an optimum DGR of $4.2 \pm 0.3\% \text{ day}^{-1}$ [15]. Growth of *Gelidium pulchellum*, another red algae, was reported to achieve maximum rate under continuous light with the support of aeration activity [112]. Mixing of the culture can be accomplished by different mechanisms such as aeration, sparging, pumping, mechanical agitation, or a combination of these depending on the scale and type of cultivation systems [15, 113]. Agitation by mixing is considered as the key parameter for the equal distribution of nutrients and cells in the liquid phase [113], and maintenance of their uniform concentration to increase the mass transfer rate [114]. Growth of cultures is normally enhanced under aerated conditions provided the other culture

requirements (light, photoperiod, salinity, pH, temperature) are remained constant [115, 116]. Appropriate aeration activity may increase thallus exposure to light, eliminate nutrient diffusion barriers, improve gas exchange, and facilitate heat transfer to avoid thermal stratification [15].

5.3. Salinity

Salinity is reported to be one of the factors affecting the growth [15, 80] and exerting strong influences on the photosynthetic capacity [117, 118] of the cultured seaweeds. Prolonged exposure to low salinity may induce stress that led to reduced photosynthetic efficiency, inhibited cell division, and subsequently result in stunted growth and declined in growth rate [80]. *K. alvarezii* explants cultivated in the salinity range of 25–35 ppt have been reported to achieve higher DGR of 4.2 ± 0.4 to $4.7 \pm 0.5\%$ day⁻¹ as compared to those cultivated in salinities of 20 and 40 ppt with DGR of 2.6 ± 0.3 and $2.2 \pm 0.4\%$ day⁻¹, respectively [15]. While *Gracilaria* seaweeds have been found growing well in a wide geographical range with salinities from 15 to 60 ppt, their optimal growth performance was still reported in salinities around 30 ppt [119]. *K. alvarezii*, *E. denticulatum*, and *G. changii* explants were observed to be unhealthy in the exposure to hyposaline conditions (below 20 ppt) with the formation of ice-ice whitening and bleaching throughout the branches leading to fragmentation and completely damage of the branches [80, 120]. Extremely low salinity may cause oxidative stress in which peroxide may be accumulated in the explants and lead to loss of thallus rigidity as observed in *Gracilaria corticata* under exposure to 15 ppt [121]. Moreover, *K. alvarezii* explants treated in hypersaline conditions (above 40 ppt) have also been reported to exhibit lower growth rate as did those treated in hyposaline conditions [15], where the growth metabolism may be sacrificed near the salinity tolerance limits to carry out osmoregulation for survival in a short period of time [122].

5.4. pH

The ordinary seawater is slightly alkaline (pH ~8) with bicarbonate ions (HCO₃⁻) constituted about 91% of total dissolve inorganic carbon (DIC), followed by 8% of carbonate ions (CO₃²⁻), and 1% of dissolved CO₂ [123]. Alterations in seawater pH may vary the equilibrium of carbonate system and change the concentration of inorganic carbon species [124], subsequently affect the growth of seaweeds which depend on the supply of inorganic carbon for photosynthesis. The pH range for normal growth of most seaweed cultures was reported to be 7–9 with optimum growth in between 8.2 and 8.7 [125]. *K. alvarezii* explants were discovered to attain higher growth rates when cultured in the alkaline conditions (pH 7.5 and 9.5) with respective DGR of 5.5 ± 0.7 and $4.7 \pm 0.6\%$ day⁻¹ as compared to the acidic condition (pH 5.5) with DGR of $1.2 \pm 0.4\%$ day⁻¹ [15]. The increased of hydrogen ions (H⁺) concentration and decreased of photosynthetic carbon source (HCO₃⁻) under acidified condition may severely limit the photosynthesis process of explants and reduced their growth rate [124]. Proteins are the primary effector molecules potentially influenced by environmental conditions and associated with the response to various abiotic stresses. Enzymes involved in biological activities are generally respond immediately to the changed of pH and achieve their highest performance under the optimum pH range [125]. Low growth rate of *K. alvarezii* explants in acidic conditions

may also due to the denaturation of proteins beyond the tolerance limit, which in turn hinders the cellular physiological and biological processes of seaweeds [15]. However, better growth of purple *K. alvarezii* morphotype in slightly acidic condition (pH 6.7) was reported indicating different *Kappaphycus* varieties may respond differently to pH conditions [104].

6. Optimal growth of seaweed micro-propagules in tissue culture and photobioreactor

In order to maximize the growth of micro-propagules and enhance the productivity of seaweed propagation, incorporation of optimized parameters in their combination in tissue culture system (**Figure 5a**) and application of photobioreactor with optimal growth condition (**Figure 5b**) are highly recommended. Maximum DGRs of *K. alvarezii* and *G. changii* have been reported to achieve $5.5 \pm 0.7\% \text{ day}^{-1}$ [15] and $6.6 \pm 1.5\% \text{ day}^{-1}$ [80], respectively, under optimized tissue culture condition. The growth of *K. alvarezii* micro-propagules has further been increased to $6.5 \pm 0.2\% \text{ day}^{-1}$ in a customized airlift photobioreactor with incorporation of optimized growth parameters [15]. These growth rate achievements were found significantly higher than the earlier reports of 3–4% day^{-1} for the growth of *K. alvarezii* in tissue culture [126] and 1–1.5% day^{-1} for the growth of *G. dura* in vertical polythene-tube-column culture [9].

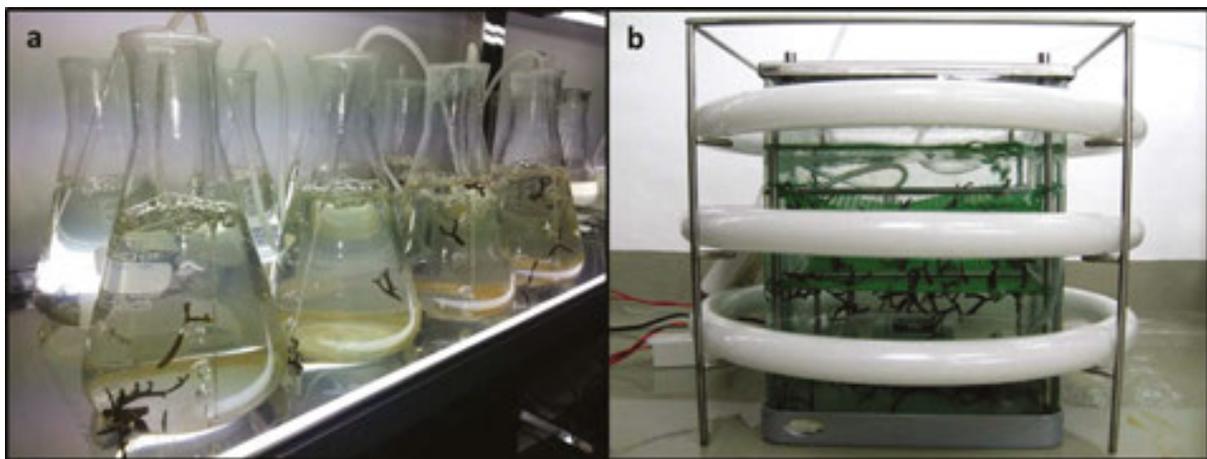


Figure 5. (a) Tissue culture and (b) photobioreactor cultivation of *K. alvarezii* under optimal growth conditions.

Although macroalgal tissue culture is underdeveloped relative to that of land plants, there are more than 85 species of seaweeds from which tissue culture aspects including successful callus formation, plant regeneration, somatic embryogenesis, and thallus development have been reported [92, 127]. Exploitation of seaweed organogenetic potential for the isolation of superior clones has been initiated since the late twentieth century to improve the performance of cultivated species including *Chondrus*, *Gigartina*, *Gracilaria*, and *Kappaphycus* [127]. Studies on optimizing the growth of the commercially important seaweeds in tissue culture can help to mass propagate these viable species and open up new opportunities to produce and recover

seaweed products from cell and tissue aggregates in photobioreactor [15]. The development of bioprocess engineering including bioreactor design and identification of strategies for secondary metabolite production can expedite the production of valuable compounds from seaweeds [128] and subsequently derive the maximum benefits from photobioreactor-grown cultures for various industrial applications.

7. Acclimatization of tissue-cultured seedlings prior to farming

While the studies of seaweed tissue culture and micro-propagation have been reported from various literatures, information about acclimatization and successful out-planting of tissue-cultured seedlings are still limited to date. Acclimatization to ex vitro conditions (nursery or glasshouse) is necessary to provide a buffer condition to the seaweed cultures for suitable adaptation before their exposure to the complex open sea environment [9]. Direct planting out of tissue-cultured seaweeds without going through the acclimatization phase may cause stress and shock to the seedlings due to sudden changes in environmental conditions [101]. Therefore, effective acclimatization process is considered to be a key element to enhance the survival rate of tissue-cultured seaweeds after they have been out-planted to the open sea.

Transferring of micro-propagated *K. alvarezii* seedlings from an in vitro flask culture to a partially in vitro tank culture (**Figure 6a**) prior to their acclimatization to outdoor nursery (**Figure 6b**) has been recommended to improve their survival capability and growth performance. Investigation of factors or parameters affecting the DGR of micro-propagated *K. alvarezii* during their acclimatization in outdoor nursery has also been carried out and reported [101]. Through the observation, *K. alvarezii* seedlings were found to achieve optimum growth with DGR of $7.14 \pm 0.30\% \text{ day}^{-1}$ when acclimatized in seawater enriched with mixed-algae fertilizer as formulated in NSE, under daily replenishment of seawater and culture density of 0.40 g L^{-1} . The acclimatization protocol was found to promote faster and healthier growth to

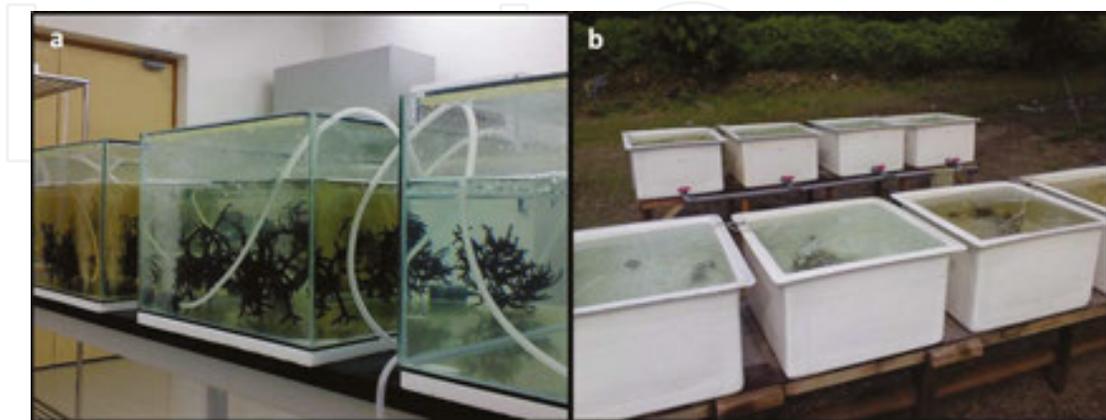


Figure 6. Acclimatization of micro-propagated *K. alvarezii* seedlings in (a) partially in vitro tank culture and (b) outdoor nursery.

K. alvarezii seedlings with DGR of $3.91 \pm 0.16\%$ day⁻¹ and $83.33 \pm 5.77\%$ of survival after they have been out-planted to the open sea.

Furthermore, acclimatization of *G. dura* in outdoor tank culture with continuous aeration and daily replenishment of 2/3 seawater without supplementation of nutrients has been suggested to improve the growth performance of the species prior to their transplantation to open sea [9]. The growth rate of *G. dura* seedlings during their acclimatization phase was reported to achieve $2.25 \pm 0.14\%$ day⁻¹ as comparable to other outdoor cultures of *Gracilaria* species [129]. Apart from that, the used of perforated polythene bags covered in nylon net bags has been recommended for out-planting of acclimatized seedlings to avoid loss of biomass, which may result from grazing and drifting [9]. The application of floating rafts accompanied with net conveying has also been discovered to ease the seeding, maintenance, and harvesting processes of seaweed farming in open sea [130].

8. Quality assessment of tissue-cultured seaweeds

Comparison of the quality between tissue-cultured and conventional cultivated *K. alvarezii* (**Figure 7**) has been reported especially on their growth rate and carrageenan properties [131]. After 60 days of post-cultivation from their first introduction as seedlings in open sea, tissue-cultured *K. alvarezii* has been reported to achieve higher growth rate ($6.3 \pm 0.1\%$ day⁻¹) as compared to conventional cultivated seaweeds ($3.4 \pm 0.3\%$ day⁻¹). No epiphytes have been discovered on the tissue-cultured *K. alvarezii*, while the presence of epiphytes and symptoms of “ice-ice” disease were observed on the conventional cultivated seaweeds [101]. From the analyses of their semi-refined carrageenan properties, tissue-cultured *K. alvarezii* was found to produce higher carrageenan yield with significantly better quality in viscosity, gel strength, and sulfate content (**Table 3**) in contrast to conventional cultivated seaweeds [131].

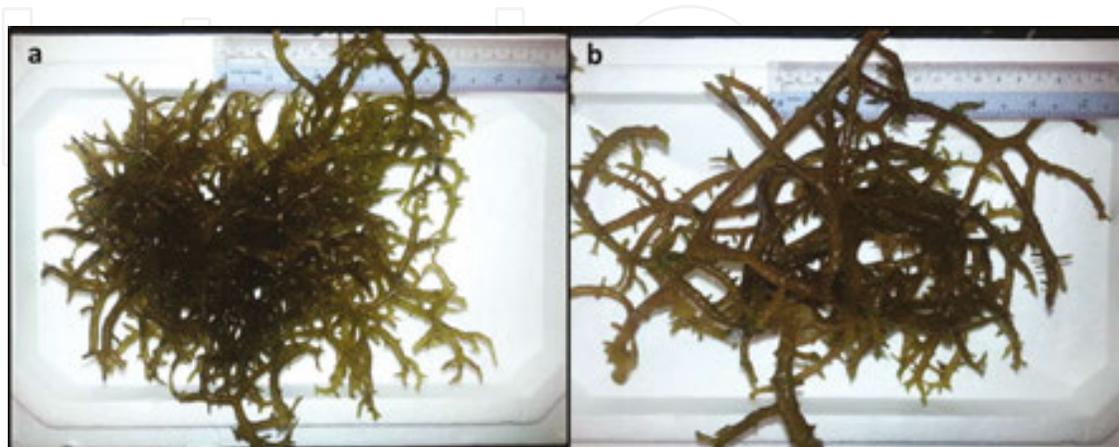


Figure 7. Comparison of growth rate and quality between (a) tissue-cultured and (b) conventional cultivated *K. alvarezii*.

Carrageenan properties	Tissue cultured <i>K. alvarezii</i>	Conventional cultivated <i>K. alvarezii</i>
Yield (%)	67.3 ± 16.4	51.5 ± 21.0
Viscosity (cP)	1280.0 ± 25.0	87.8 ± 20.9
Gel strength (g cm ⁻²)	703.5 ± 14.1	288.3 ± 19.3
Sulphate content (%)	34.2 ± 10.9	7.5 ± 6.7

Table 3. Semi-refined carrageenan properties of tissue cultured and conventional cultivated *Kappaphycus alvarezii* [131].

In terms of other chemical composition, tissue-cultured *K. alvarezii* has been revealed to produce significantly higher total lipids and mineral elements including calcium, magnesium, beryllium, cobalt, copper, lithium, manganese, and zinc against the conventional cultivated seaweeds [132]. Research finding has suggested tissue-cultured seaweeds to be a better food source for consumption and other seaweed-related industries. Bioprocess technology for the production of high-value chemicals such as food additives and biomedicinals from cell and tissue cultures of different macroalgae has been proposed and developed using specially designed photobioreactor [128, 133]. Additionally, new approaches in understanding seaweed physiology, biochemistry, and molecular biology has been anticipated to contribute new insights into human nutrition and enable genetic engineering of favorable agronomic traits to improve the quality and the overall productivity of commercially important seaweeds [134].

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