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24. Seaweed Biotechnology

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Marine macroalgae or seaweeds consist of a group of diverse plants taxonomically distinguished into Chlorophyta (green seaweeds), Phaeophyta (brown seaweeds) and Rhodophyta (red seaweeds). Generally found growing on rocks, pebbles or other plants in the intertidal or subtidal regions of the sea coast, seaweeds have been harvested for various purposes. A number of tropical seaweeds are eaten directly (sea vegetables), including green algae (*Ulva, Enteromorpha, Monostroma, Caulerpa*), brown algae (*Dictyota, cladosiphon*), and red algae (*Gracilaria, Euchema, Porphyra*) [1]. Brown algae served in the past as sources of iodine and bromine for the chemical industry [2]. In coastal areas throughout the world seaweeds such as *Sargassum* have been used as manure. In India, *Turbinaria* and *Hypnea* are used as manure for coconut plantations especially in coastal Tamilnadu and Kerala [3]. The major economic value of seaweeds is however associated with the polysaccharide that certain red and brown algal species contain. Algins, carrageenans and agars have all achieved commercial significance because of their food and industrial applications and are the basis of an estimated billion dollar global effort [4, 5]. While carrageenans and agars, from which agaroses are derived by purification, are obtained from different genera of red algae, algins are obtained from a number of brown algae and are present in all. A summary of sources, compositions, properties and more important applications of these polysaccharides is presented in Table 1 (modified from [1].

At present, most seaweeds are being harvested from naturally existing seaweed beds, resulting in over harvested populations and slow regeneration which does not meet the demand [7]. Several countries such as China, Japan, Korea, Chile, Vietnam and India have adopted aquaculture mehods including pond culture, on bottom culture, net culture and raft culture to augument production [8]

for the improvement and better utilisation of algal resources has been mooted [9]. The present review in an attempt to collate efforts made in this direction.

1. Seaweed Tissue Culture

In higher plant systems almost all of the existing technology in genetic engineering hinges on the availability of consistently reproducible protocols for the *in vitro* culture of cells, tissues or organs. A typical tissue culture cycle in higher plants, which are characterised by a high degree of organisation and differentiation into tissues and organs, consists of hormonal induction of dedifferentiation of cells of an excised plant part (explant) to form a callus, and the redifferentiation of cells of a callus to form a new plant. Higher plant cells are consequently considered to be totipotent [10], i.e., possessed of the genetic potential to direct the development of an entire plant from each cell of the organism.

Over the past couple of decades an increasing number of studies have dealt with seaweed tissue culture. Chen and Taylors (1978) [11] work with *Chondrus crispus* is one of the first such studies. Most subsequent research has been based on phycocolloid producing algae such as *Gelidium*, *Laminaria* and *Gracilaria* or edible species such as *Porphyra*, *Eucheuma* and *Undaria*. Because of their aquatic habitat and unique chemical composition, seaweeds present biotechnologists with problems which are quite different from those confronting workers on higher plants. Polne-Fuller and Gibor (1978) [12] identify four specific categories among the unique problems of algal tissue and cell cultivation as reproduced below:

A. Obtaining clean tissue that is free of other organisms

The surface of seaweeds are heavily infested by various microbial and larger epiphytes. Some of these organisms are embedded in cell walls and between the living cells. This present an unique problem in seaweeds since their meristematic cells are frequently located on the surface and will be damaged upon application of chemical cleaning.

B. Dissociating the tissue to viable cells and protoplasts

Algal cell walls are composed of macromolecules which are more complex than cellulose (the major molecule in higher plant cell walls), thus different enzymes are required from those used for dissociation of higher plants. Few such enzymes are available commercially.

C. Inducing divisions and regeneration of isolated cells and protoplasts to form complete plants

Algae do not generally respond to the recognized hormones which effect the development and growth of higher plants. Unfortunately, little in known about specific factors controlling algal growth and differentiation.

Table 1 Seaweed Polysaccharides

yaccharide	Important raw material (genera)	Composition	Important properties	Selected Applications
(Mixtures of e and ectin)	*Gelidium, Gracilaria Gelidiella Pterocladia Gracilariopsis Porphyra, Hypnea*	Agarose = alternating 1,4 linked α-D galactose and 3, 6-anhydro α-L-galactose backbone (agarobiose) substituted with varying percentages of methoxyl ester sulfate and ketal pyruvate groups Agaropectin = alternating D-galactose and L-galactose units. D-galactose can be substituted by D-galactose-4-sulphate, by 4,6-0-(1 carboxy ethylidene)-D-galactose in certain terminal chain positions or even by D-galactose 2,6-disulphate, while part of L-galactose can be replaced by 3, 6 anhydro-L-galactose	Agaroses = * Gel aqueous solutions at low concentrations * Form ion dependent thermoreversible gels * Controllable electroendosimosis (EEO) * Minimal non-specific protein reactivity * Significant degree of hysteresis Agars = * Gel aqueous solutions at low concentration * Form thermoreversible gels * Relatively inest * Significant degree of hysteresis * Retain moisture * Resist hydrolysis by terrestrial microorganisms	Matrices for: * Electrophoresis * Immunoassays * Microbial and cell culture * Chromatography * Immobilized Systems * Baking icings * Jelly candies * Canned meats * Dental impression media * Laxatives * Microbial culture matrix * Raw material for agarose
'Alginates	*Macrocystis Laminaria Sargassum Turbinaria*	1,4-linked α-L-guluronic acid and β-D-mannuronic acid subunits in G.G, M.M. and M.G. domains	* Ammonium and alkali metal salts are soluble in water, whereas free alginic acid and alkaline earth and Group III salts are insoluble and can form gels * Bind water	* Frozen foods to maintain structure on thawing * Baking icings * Salad dressings * Tabletting agent * Dental impression media * Textile sizing * Matrices for immobilized systems
eenans		*kappa* and *iota* Alternating 1,3-linked α-D-galactose and 1,4 linked 3,6-anhydro-β-D-galactose backbone (carrabiose) substitiuted with varying percentages of ester sulfate	* Thicken aquous systems * Suspend solids * Bind moisture * Stabilize emulsions * Control flow and texture properties of food systems * High protein reactivity—strong interactions with milk proteins	* Frozen dessert stabilizers * Chocolate milk stabilisers * Texturizers for low-fat foods * Low calorie jellies * Toothpaste binders * Air freshners * Personal care products * Pet foods
	Euchenna (*cottonii*), *Kappaphycus* (*alvarezii*), *Gigartina* (*radula*)	4-sulfated on the galactose subunits (~ 25% ester sulfate)	* Form strong rigid gels with potassium and calcium ions * Exhibit synergy with locust bean and konjac gums.	
	Euchema (*spinosum*)	4-sulfated on the galactose subunits and 2-sulfated on the 3,6-anhydrozalactose subunits (~ 32% ester sulfate)	* Form elastic aqueous gels with calcium ions * Exhibit synergy with locust bean gum and starch * Suspend particulates	
la-	*Chondrus* (*crispus*) *Gigartina* (*radula*)	Alternating 2-sulfated 1,3-linked α-D-glactose and 2,6-disulfated 1,4-linked β-D galactose backbone (minimal 3,6-anhydro-β-D-galactose) (~ 35% ester sulfate)	* Non gelling aqueous system viscosifier	

1.1 Callus induction and plant regeneration in seaweed tissue culture

Callus formation is defined as the induction of a disorganised growth of cells (or dedifferentiation) in a differentiated tissue as a result of wounding [13]. The structural and cellular organization of seaweeds differ significantly from higher plants. The plant body or thallus in macro algae has evolved three main types of cellular organization *viz* filamentous, pseudo parenchymatous and parenchymatous. Although these three levels of organisation can be found among members of all three division, most tissure culture work has been carried out with pseudoparenchmatous and parenchymatous thalli (for review, see [14]). The ease with which callus can be induced from an explant, or cultured upon excision from the explants, seems to vary among members of three divisions. Calluses rarely developed from mature sections of intact tissue of Chlorophycean members but developed from the tissue sections in frequencies of 2–10% (Laminariales) or 3–27% (Fucales) in Phaeophycean and 0.1–3% in Rhodophycean members [15]. The potential for callus development in seaweeds in sometimes influenced by thallus thickness [14]: in the red and brown algae, development of callus from intact explants has been mostly described in cases where the thallus is made up of several cells, while in green seaweeds such as *Enteromorpha* and *Ulva*, the thallus is only one to two cell layer. In red seaweeds, callus may arise from cortical cells (e.g. *Gracilaria verrucosa* [16]), but more often arises from the medullary cells. No mention in made in literature of the potential for callus induction in explants from different areas of the thallus, and regeneration of calli into new plants has rarely been reported in red seaweeds [14], with the exception of *Laurencia* sp. [17]. Unlike calli from higher plants, that induced in red algae mostly cease growth upon excision from the explant [14].

In brown seaweeds too, the medullary cells are associated with callus growth [18]. However unlike calli induced in red seaweeds, in some brown seaweeds, calli have been reported to resume growth upon excision from the explant (e.g. *Sargassum muticum* [19]). Also, differences have been observed within different parts of the thallus to develop a callus (e.g. blade > stipe > rhizoid) [20], possibly related to the nutritional status of the cells involved [14].

The available information makes it difficult to explain why some calli can grow independently of the explant while others cannot. In higher plant systems induction and maintenance of a callus, as well as regeneration of plants from a callus, is mediated by hormones or plant growth regulators [21]. Although there have been reports suggesting the involvement of plant growth regulators [22, 23] in seaweeds, their presence has not yet been unequivocally established. While seaweeds are not known to respond to most hormones used in higher plant tissue culture, increased growth in *Dictyota dichotoma* cultures [24], or increased callus formation on explants of *Laurencia* sp. [25], have been reported upon addition of a water soluble extract of the respective alga. There is also sufficient evidence to confirm the hypothesis [26] that under non-axenic conditions surface microorganisms are able to supply growth promoting substances to their "hosts". As early as 1953, Ericsson and Lewis [27] demonstrated the transfer of vitamins from bacteria to algae. Chandramohan in 1971 [28] showed that bacteria living on the surface of *Enteromorpha intestinalis* could synthesize the growth regulator indole acetic acid (IAA) from tryptophan. A clear case of bacteria contributing to the 'normal' development of seaweeds was documented by Provasoli and Pintner [29] and Tatawaki *et al.* [30], where *Ulva* and *Monostroma* developed abnormally in axenic cultures grown in artificial seawater medium. The plants regained normal morphology upon the addition of the bacteria which were isolated from the plant. It has been speculated that the symbiotic association between bacteria and algal host may induce the release of oligosaccharins from the plant cell walls [14]. Oligosaccharins are often released in higher plants as a result of fungal or bacterial attack and appear to be involved in defense as well as growth regulation. [31].

Another notable feature of seaweed tissue culture has been that the conditions under which callus has been obtained are quite variable and, in gerneral, the occurrence of a callus cannot be attributed to any specific set of experimental conditions [14]. This suggests that internal factors inherent to the explants are more important than the culture conditions employed.

Aguirre-Lipperheide *et al.* [14] in their review, opine that the apparent success in obtaining callus using tissue culture techniques in seaweeds should be interpreted cautiously. Two points to be considered should be the percentage of callus formed and the size of the calli. When compared to callus cultures of higher plants, seaweed calli are generally slow growing and small in size (~1–3 mm) and the reaction to wounding often ceases after some weeks. Also, the occurrence of calli in some seaweeds is sporadic and the percentages often very low (< 2%) Consequently the suitability of callus derived from explants as a basis for cell suspension cultures, (for example, for secondary metabolite production) is debatable [14]. It has been demonstrated that under appropriate conditions, seaweed polysaccharides can be produced by callus culture, including agar from *Pterocladia capillacea* [32], but whether this would ever be a cost effective production method for polysaccharides is doubtful.

1.2 Protoplast culture and somatic hybridisation in seaweeds

Protoplasts (plant cells devoid of their cell walls) have been isolated and cultured in all the three classes of seaweeds. In the green seaweeds, the development of callus has seldom been reported, but regeneration into a whole new plant has been reported in many cases. In the brown and red seaweeds regeneration of protoplasts into callus and into new plants is recorded in several species including commercially important seaweeds such as *Porphyra* [19, 32], *Gracilaria* spp. [9, 33] and *Chondrus crispus* [34] (for review, see [14]). It thus appears that this technique is becoming well established in seaweeds. It is possible that this behaviour might be connected with the fact that a protoplast represents the lowest possible unit of eukaryotic cell organization, and thus it is easier to reprogram growth from this point than in the case of a mass of walled cells with no clear definition of structure. At present, protoplasts are more suitable for establishing

cell suspension culture than callus derived from explants, and may have some potential in secondary metabolite production, for example, cultured protoplasts of *Kappaphycus alvarezii* have been reported [35] to secrete carrageenan fragments. It has also been suggested that if generation of viable protoplasts that form cell walls and divide could be achieved reproducibly and on a large scale, the protoplasts could be used as seed stock for macroalgal culture [6].

The fusion of cells or protoplasts permits the recombination of cytoplasms and genomes of widely differing origins. During the last decade, the fusion of terrestrial plant protoplasts has been routinely achieved through either chemical means or by electrical impulses. Heteroplasmic fusions have been reported in the green seaweeds, such as, *Ulva* spp. [36], *Enteromorpha* spp. [37] and *Monostroma* spp. [38], and in the red alga *Porphyra* spp. [39-44]. Fusion has been brought about either by the use of polyethylene glycol [39-41] or by the electrofusion technique [42-44]. Fusion efficiencies were found to depend on the concentration and nature of reagents used to adjust the osmotic pressure of the medium [43].

2. Gene Mapping and Sequencing in Seaweeds

A prerequisite for the genetic manipulation of an organism is an adequate understanding of genome structure, sequence and gene expression. In seaweeds, the presence of anionic polysaccharides which have similar properties to nucleic acids (they are precipitated by ethanol at about the same pH as nucleic acids) have complicated the isolation of DNA and RNA. Modified extraction protocols to obtain DNA sufficiently pure for molecular applications such as restriction endonuclease digestion, southern blot hybridizations and amplification via the polymerase chain reaction (PCR), include CsCl-gradient ultracentrifugation [45], treatment with CTAB [46], hydroxyapatite binding [47] or purification on sepharose columns [48]. Since the viscous polysaccharides are released by grinding in liquid nitrogen, extraction by softening cell walls using lithium chloride in a procedure that does not require grinding of tissues has also been reported [49].

Reports of mapping and sequencing of seaweed genomes are sparse. In *Gracilaria tenuistipitata* the chloroplast ribosomal-protein encoding genes have been located, cloned and characterised [50]. A 1365 bp region around this gene was also sequenced and the gene order found to be identical to that detected in the chloroplast DNA of liverwort, tobacco and maize. The plastid gene for the rp 122 protein in *G. tenuistipitata* has also been isolated and sequenced [51].

It has been proposed that nucleic acid analysis including restriction analysis and detection of DNA sequence homologies could help in furthering our understanding of speciation, phylogenetic and evolutionary biogeography within the algae [52–54], and in examination of heterosis [55]. In phylogenetic analysis, targets for sequence homology determination among the seaweeds have been the 5S ribosomal RNA [56], nuclear small subunit rRNA genes [53], the plastid *rbcL* gene coding for the larger subunit of ribulose-1, 5-bisphosphate carboxylase/oxygenase (RUBISCO) [57], nuclear genes encoding cytosolic and chloroplast

glyceraldehyde-3-phosphate dehydrogenases (GADPH) [58], gene amplification products [59], the β-tubulin gene [60], subunit 3 of cytochrome *C* oxidase [61], and small subunit rRNA (SSUrRNA) from the mitochondria. [62] Restriction analysis of plastid DNA fragments has been used to determine relatedness in red algal species [45]. In one study [63], this technique was used to prove that two macroalgae previously thought to be distinct species were actually identical. Nuclear DNA reassociation kinetics has also been used to determine inter- and intraspecific variations in selected agarophytes and carrageenophytes [64].

3. Genetic Engineering of Seaweeds

Recombinant DNA technology has been effectively used for the improvement of crop plants due to the availability of suitable plasmids and expression vectors, such as the Ti plasmid of *Agrobacterium tumefaciens* [65], or viral vectors such as Cauliflower Mosaic Virus [66], as well as methods of direct DNA delivery to effect transformation such as electroporation, use of PEG, etc. [67], or using microprojectiles [68]. One of the difficulties inherent in the application of recombinant DNA technology to seaweeds has been the development of appropriate selection markers, vectors and efficient transformation systems.

Of twenty one red algal genera studied [45], five were found to contain circular ds DNA plasmids. Some of these have been isolated and studied, and one 3.5 kbp plasmid from *Gracilaria lemaneformis* was sequenced to reveal two potential open reading frames. In this species, plasmids are present in a high copy number per cell and may provide useful vectors for algal transformation. The DNA sequence and structural organization of the GC2 plasmid from the agarophyte *Gracilaria chilensis* has been determined [47]. This 3827 bp circular plasmid has one major open reading frame that generates a transcript and could encode a 411 amino acid polypeptide.

It has also been suggested that recombinant viruses particularly the large ds DNA viruses that are known to infect eukaryotic algae can be used as transformation vectors for marine macroalgae [69].

4. Commercial Implication of Seaweed Biotechnology

The majority of commercial polysaccharide products have existed for years with the same specifications and in general, companies are reluctant to alter those long accepted extracts and blends, and the raw materials from which they are recovered [6]. Also, since many seaweed polysaccharides are destined for human consumption, they are therefore strictly regulated. For instance, in several countries including USA, only carrageenans from specific seaweeds meet food ingredient regulation, and this discourages the quest for new species as sources of raw material. Consequently, the immediate impact of biotechnology in commercial cultivation of seaweeds could possibly be the following:

(1) In the generation of sufficient amounts of selected strains of marine macroalgae for cultivation: Currently about 25% of cultivated material is used

for seeding the next crop. If consistently reproducible methods of generation of plants from protoplasts are developed, these could be used as seedstock [6].

(2) In the use of DNA sequences or molecular probes to prove equivalence of raw material: Because the species acceptable as sources have been identified by name in the regulation, alternative, perhaps more abundant or easily cultivated, species are often excluded, even though polysaccharide extracts from them may be essentially identical [6]. A combination of molecular biology, refined analytical methods such as NMR spectroscopy and traditional morphological taxonomy will possibly produce a more appropriate list of acceptable raw materials [70].

(3) In the development of engineered polysaccharides in the non-consumables markets: Genetically altered algae will present the same regulatory problems that surround genetically engineered crop plants whose products are targetted for human consumption. However since various polysaccharides are required for industrial purposes, a cost-effective alternative may be obtained from genetically engineered seaweeds.

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