PROTOPLASTS - A POWERFUL TOOL IN GENETIC MANIPULATION OF COMMERCIAL SEAWEEDS

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INTRODUCTION

Protoplast is an important tool for parasexual modification of genetic content of plant cells (Vasil and Vasil, 1980). Production of algal protoplasts and their fusion are relatively new fields and lag far behind that of terrestrial plants (Berliner 1981, 1983; Cheney *et al.*, 1986). To date, protoplasts have been isolated from several algae, most of which are blue green algae and green algae (Adamich and Hemmingsen, 1980). Protoplasts have been obtained from some marine brown algae (Kloareg and Quantrano, 1987). In the Rhodophyta, viable protoplasts have been isolated from only two marine genera *Porphyra* and *Gracilaria*. Protoplasts have been isolated and subsequently regenerated from four *Porphyra* species - *P. yezoenzis* (Saga and Sakei 1984; Fujita and Migita 1985), *P. suborbiculata* (Tang 1982; Mizukami *et al.*, 1995), *P. perforata* (Polne-Fuller and Gibor 1984; Saga *et al.*, 1986) and *P. nereocystis* (Waaland *et al.*, 1990). Cheney *et al.*, (1986) reported the successful isolation of viable protoplasts from *Gracilaria tikvahiae* and *Gracilariopsis lemaneiformis*, but their efforts to regenerate whole plants were unsuccessful.

NEED FOR GENETICALLY MANIPULATED SEAWEEDS

In India seaweed resources are commercially exploited for the production of phycocolloids such as agar and sodium alginate. Seaweed industries in India depend entirely on *Gracilaria edulis, G. verucosa* and *Gelidiella acerosa* for agar and species of *Sargassum* and *Turbinaria* for sodium alginate from the natural beds along the coasts of Tamil Nadu and the Gulf of Kutch. The Indian species of agar yielding *Gracilaria* are inherently poor yielders of agar (10-12% dry weight) with poor quality (gel strength of 100-150 g/cm^2), though they are foliose type and can grow luxuriantly. However, species of *Gelidium* and *Gelidiella* are high yielding resources with better gel strength, but they are crustose type and slow growing seaweeds making their exploitation very difficult. Carrageenan is another important phycocolloid obtained from *Hypnea valentiae* and *H. musciformis* in India and the *Eucheuma* species from the Philippines. The Indian species of Carrageenophytes yield 20% carrageenan, whereas *Eucheuma* species can yield 40%. The phycocolloid industry in India would become commercially attractive if a genetically

manipulated seaweed with fast growth, high biomass production coupled with better yield and quality of product could be achieved through protoplast isolation and somatic hybridization techniques.

Isolation of protoplasts

Plant cells differ from animal cells in two respects : they have a cellulose cell wall and are held together by their middle lamellae. Isolated protoplasts are living cells from which the walls have been removed. Protoplasts are isolated either mechanically through plasmolysis or enzymatically treating them with cell wall degrading enzymes. Techniques for the removal of the cell wall using cell wall degrading enzymes were developed in the 1960s (Cocking, 1960) and these made possible the production of large number of healthy protoplasts from many plant tissues. Since then, protoplasts have become recognized as powerful research tool in physiology, biochemistry and more recently in molecular biology and genetic engineering. Cells are separated from tissue samples by a sonicator and then treated with a mixture of pectinase, cellulase and macerozyme with reciprocal shaking (40-50 strokes/min). Protoplasts are isolated at 22° C - 28° C.

Commercial preparations of cell wall lysing enzymes extracted from fungi as well as sea snail gut enzymes are available in the Market (Table 1). Agar solublising enzymes extracted from marine species of *Oscilatoria,* an epiphyte on *Gracilaria edulis* is tried and success is being evaluated in isolation of viable protoplasts from *G. edulis* (Kaladharan and Seetha, Pers. Commun.) Protoplast preparation almost always contain cellular debris in the form of undigested cuticle, vascular elements, cell walls, tissue fragments, etc. which must be removed rapidly and adequately. Much of the debris can be removed by filtration through miracloth or stainless or nylon filters followed by washing on millipore filters or by centrifugation at low speed (<500 rpm).

Table 1. ***Composition of enzyme solution for cell wall degradation (Chen, 1987)***

Composition	Concentration
Sea snail enzyme mixture	2.0 g
Onozuka R-10 cellulase	1.5 g
R-10 pectinase	0.5 g
Sorbitol	9.2 g
$CaCl_2 . 2H_2O$	80 mg
$NaH_2PO_4 . H_2O$	10 mg
$Ca(H_2PO_4)_2 . H_2O$	10 mg
Distilled water	100 ml
pH	7

Techniques for the isolation and culture of protoplasts from seaweeds have been developed only recently and the amount of background research in this field is limited. The first report of protoplast isolation was from the green alga *Enteromorpha intestinalis* (Millner *et al.*, 1979). Since then protoplasts have been isolated from two genera of red seaweeds *viz. Porphyra* and *Gracilaria,* two other genera of green seaweeds *viz. Ulva* and *Monostroma* and six other genera of brown seaweeds *viz. Dictyota, Laminaria, Macrocystis, Undaria, Sargassum* and *Sphacelaria.*

Characteristics of protoplasts

The isolated protoplasts assume a spherical shape and acquire some properties common to animal cells. They show budding and can take up even virus particles and chloroplasts by endocytosis. A unique feature

of the seaweed protoplast is its ability to synthesis a new cell wall around it *in vitro.* This reformed cell undergoes divisions to produce a clump of cells (Nagata and Takebe, 1970; Takebe and Nagata, 1973). It is also possible to induce differentiation of organs in this tissue and obtain entire plants by suitable experimental manipulation (Vasil and Vasil, 1980). Plants derived from protoplasts are useful for the study of the phenomenon of somaclonal variation. The removal of cell wall permits modification of the genetic content through protoplast fusion or by gene transfer. As protoplasts can be regenerated into whole plants, one of their most powerful application is as vehicles for the transfer of foreign genes. One basic prerequisite for potential use of protoplasts in such studies, is the ability to isolate them readily in large numbers and to culture them *in vitro* to form cell colonies as well as whole plants. The period for which the protoplasts remain naked is most important, because it is during this time that they can be specially subjected to experimentation.

Culture of protoplasts

Protoplasts are cultured *in vitro* as suspension and drop cultures. Protoplasts are suspended in a liquid medium at a density of about 10^5/ml and cultured in very shallow layers (soft agarose two-layer medium, Mizukami *et al.*, 1995) in 25 ml Erlenmeyer flasks, with or without shaking. Protoplasts suspension at a density of 10^4 or 10^5/ml are placed in 50µl drops in plastic petri dishes, sealed with parafilm and incubated. Inorder to provide adequate humidity in the cultures, drops of water are placed in the middle of the petri dish and monitored regularly with the aid of an inverted microscope. Low light intensities are favourable (50 µE/m²/S) from the source of white fluorescent tubes at desired photoperiod. The medium commonly used for culture of seaweed protoplasts is given in Table 2. Sorbitol or mannitol are used to maintain the osmoticum of protoplasts.

Table 2. ***Composition of Tc-11 culture medium for seaweed protoplasts (Chen, 1982).***

Composition	Concentration (μM)
$NaNO_3$	0.5
NH_4NO_3	0.25
$Na_2SiO_3 . 9H_2O$	0.2
Na_2 EDTA	10
FeEDTA	10
$FeCl_3 . 6H_2O$	1
MgSO4 . $7H_2O$	2
$Na_2MnO_4 . 2H_2O$	5
H_3BO_3	5
$NaH_2PO_4 . H_2O$	20
$CaCl_2 . 2H_2O$	0.5
KCL	2.0
Phenylacetic acid	0.1
Pyridoxine-HCl	0.1
p-Hydroxyphenylacetic acid	0.2
V-3 ə	2.0 ml
Pl-5X*	2.0 ml
Kinetin	1.0
α-NAA	1.0
IAA	2.0
Seawater	1000 ml

ə V-3: Thiamine - HCl 0.5 mg, Nicotinic acid 0.1 mg, Ca-pantothenate 0.1 mg, Biotin 1 µg, Folic acid 2 mg, Thymine 5 mg, Cobalamine 1 µg, Inositol 5 mg, Cyanocobalamin 1µg.

**Pl-%x:$MnCl_2$ 7 µM, $ZnCl_2$ 8 x 10^{-5} µM, $CoCl_2$ 2 x 10^{-2} µM, $CuCl_2H_2O$ 2 x 10^{-5} µM.*

Pl 5X and V-3, 2 ml of stock solution into 1000 ml-given required amounts.

The pH of TC-11 medium adjusted to 7.5.

Fusion of protoplasts (Somatic hybridization)

Genetic manipulation of seaweeds can be achieved by fusion of protoplasts either by chemically using poly ethylene glycol (PEG), poly-L-ornithine (PLO), etc. or induced electrically through cell fusion system (Fig. 1). Some cations (Ca++) are also found to increase fusion frequencies. It is therefore possible to produce new intraspecific, interspecific and even intergeneric hybrid cells and to regenerate these into whole thallus (Kao and Michayluk, 1974). This technique offers the possibility of transferring genes responsible for disease resistance, improving the yield and quality of colloids or stress tolerance to the new hybrid strains when this can not be accomplished by sexual crossing. Protoplast fusion also results in new chloroplastic and mitochondrial gene combinations (Cybridization), so that the cytoplasmic characters can be manipulated and modified. New and unique cytoplasmic entities can therefore be created for experimental investigation and possible eventual exploitation in varietal improvement in seaweeds. Although isolation and culture of protoplasts have been taken up in many seaweeds, regeneration has been achieved in *Enteromorpha, Ulva, Monostroma* and *Sphacelaria,* but not in any of the complex red or brown seaweeds except *Porphyra* (Table 3).

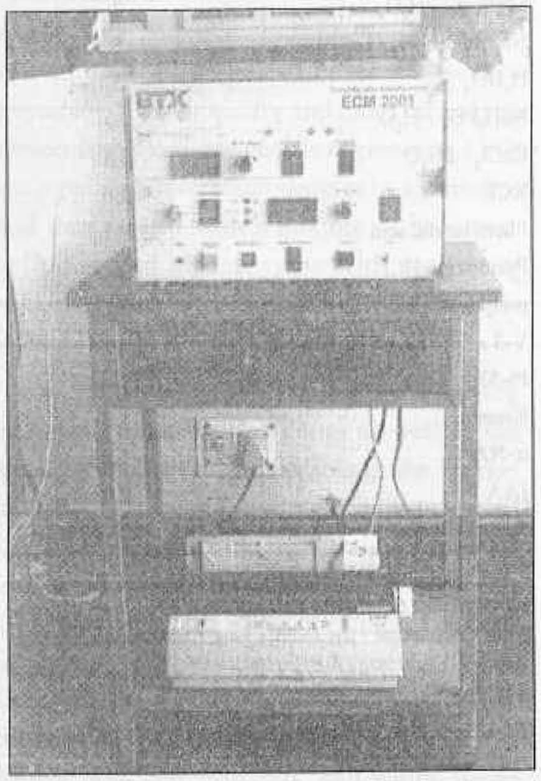

Fig. 1. ***Cell fusion system btx ecm 2001 model***

Conclusion

Seaweeds are the prime source of phycocolloids such as agar, carrageenan and algin which have important commerical, food and medicinal uses. Although research on seaweed protoplasts lags very far behind that of land crops, steady and intensive works by a handful of workers during the current decade have rendered spectacular advances in regeneration of protoplasts and even interspecific somatic hybrids. Besides strain improvement through protoplast fusion and somatic hybridization, the expertise on culture of seaweed protoplasts can enable to understand and eventually manipulate the biosynthesis of commercially important cell wall polysaccharides *in vitro* so as to achieve better yield and superior quality.

Table 3. ***Isolation and culture of protoplasts from seaweeds***

Species	Isolation condition	Results	Reference
Enteromorpha intestinalis	4%driselase, 1.2M sorbitol 0.4%pectinase, pH 6.0	85-90% viable	Milner *et al.*, 1979
Porphyra suborbiculata	enzyme extract from *Turbo coronatus* and cellulase R10 2%	cell wall regeneration and cell division	Tang, 1982
Ulva linza & *Monostroma angicava*	Cellulase R10 4% & pectolyase 2% in 2M gluscose	Plant regeneration	
E. linza, *M.zostericola* & *U.pertusa*	Cellulase R10 1M Mannitol pH 6	Plant regeneration	
Laminaria japonica & *P. yezoensis*	crude extract of *Strongylocentrotus intermedius* in 1.2M Mannitol, 0.1M Tris, pH 7	Viable	Saga & Sakai, 1984
P. perforata	crude extract of *Haliotis* sp., 0.6M Sorbitol in seawater, pH 6.0	Plant regeneration	Polne-fuller & Gibor, 1984
Gracilaria tikvahiae & *G. lameneiformis*	Cellulase R10 3% macerozyme R10 3% agarase 1% & spectolyase 5%	cell wall regeneration and cell division	Cheney *et al.*, 1986
Macrocystis pyrifera & *Sargassum muticum*	extract of *Haliotis* sp. macerozyme 1%, pectinase 1% and cellulase R10 2% in 1.0M sorbitol, pH 6.0	good yield of protoplasts, no regeneration	Saga *et al.*, 1986
E. intestinalis & *U. angustata*	Cellulase 3% & acerozyme 1%	Plant regeneration from callus formed	
P. leucosticta	extract of *Littorina littorina*, cellulase R10 1.5% & pectinase 0.5% in 0.5M sorbitol	cell division, plant regeneration in TC 11 medium	Chen, 1987
Sphacelaria sp.	cellulysin 2%, pectolyase Lyase Y23 0.5% Alginate lyases 0.25UA/ml 0.8 mannitol, pH 5.8	Plant regeneration from apical cell protoplasts	
L. saccharina & *L. digitata*	cellulase 2%, mannuronate lyase 0.5-5 UAml, Guluronate lyase 1-2 UA/ml BSAO 0.4%, pH 6.5	viability > 80% wall regeneration	

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