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244

Isolation and culture of protoplasts from agar yielding seaweed *Gracilaria edulis* (Gmelin) Silva

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ABSTRACT

Viable protoplasts were isolated from cultured thallus of agar yeilding red seaweed *Gracilaria edulis* using an enzyme mixture of 3% ONOZUKA R-10 cellulase, 3% R-10 macerozyme, 1% agarase and 1% pectinase in a seaweed protoplast washing medium (SPW) containing seawater and 1 M mannitol. After repeated washing with sterile medium, the protoplasts were maintained in light and attempts were made on electrofusion of protoplasts.

Introduction

Protoplasts provide the starting point for many of the techniques of genetic manipulation of plants, in particular the induction of somaclonal (protoclonal) variation, somatic hybridization and transformation. Production of algal protoplasts is a relatively new field and lags far behind that of land plants (Berliner, 1981; Cheney *et. al.*, 1986). If production of viable protoplasts that can 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 and save the 25% of cultured material being used as seed stock (Chen *et. al.*, 1984).

Protoplasts have been produced from a number of species of multicellular marine algae and plants have been regenerated from protoplasts of Chlorophyta, Phaeophyta and Rhodophyta (Adamich and Hemmingsen, 1980; Berliner, 1981; Cheney *et. al.*, 1986; Polne - Fuller and Gibor, 1988). Isolation of protoplasts from agar yielding *Gracilaria tikvahiae* and *G. lameniformis* is reported for the first time in 1986 (Cheney *et. al.*, 1986). Subsequently isolation and culture of protoplasts from four species of *Gracilaria* have been reported by Bjork *et. al.* (1990). The present communication is the first report on the production of protoplasts from agar yielding red seaweed *Gracilaria edulis*, which is a part of an ongoing research project funded by the Department of Biotechnology, New Delhi.

Materials and Methods

Growing tips of *Gracilaria edulis* were cut and washed in sterilized sea water. These thallus bits were sterilized by dipping them in 0.1% $HgCl_2$ solution for 10 seconds and they were soon transferred to antibiotic

solutions of 0.5% each of Betadine and Clotrimazole for 5 miniutes with occasional stirring. The sterile bits were washed with excess sterilized seawater thrice under aseptic condintions and kept in sterile seawater at dark for overnight.

Viable protoplasts were isolated from the sterile pretreated thallus bits of *G. edulis* using an enzyme combination of 0.5 - 1.0% pectinase, 1-3% cellulase Onozuka R-10, 1-3% macerozyme R-10 and 1-2% agarase (Sigma) in seaweed protopast washing medium (SPW medium) at pH 6.0 (Table 1).

Table 1. *Composition of SPW medium stock (w/l)*

KNO_3	:	101:0mg
$CaCl_2$. 2 H_2O	:	1480 mg
Mg SO_4. $7H_2O$	:	246 mg
KH_2PO_4	:	27.2 mg
KI	:	0.16 mg
$CuSO_4.5H_2O$	:	0.02 mg
Mannitol	:	7-10%

The bits were chopped into small thin slices with a sterile surgical blade and transferred to sterile glass vials containing the enzyme mixture and gently agitated through a rotary shaker at 25-30 rpm. Release of protoplasts were monitored every hour through an inverted microscope (Ceti, Belgium). After 5-6 hours the liberated protoplasts were separated from the cell wall and other debris by sieving through a 60 μ bolting silk followed by centrifuging at 500 rpm for 5 minutes and the supernatant at 6000 rpm for another 8 minutes in a refrigerated centrifuge (Hettich, Germany) at 6-10° C. The protoplasts settled at the bottom were suspended in SPW medium with an osmoticum of 7-10% mannitol. These protoplasts were either attempted for fusion studies through cell fusion system (BTX 2000, USA) or for cuture trials in seaweed protoplast culture medium (TC-II medium, Chen, 1987).

Results and Discussion

In the beginning of this experiment, Onozuka R-10 cellulase, agarase and either R-10 macerozyme or pectinase were used in the presence of trypsin, papain or enzyme extracts of a blue - green alga, *Oscillatoria* sp., growing attached to *G. edulis* in culture (Kaladharan and Seetha, unpublished). Later the maximum degree of cell wall digestion and release of protoplasts were obtained using an enzyme mixture consisting of 3% of onozuka cellulase, 3% R-10 macerozyme, 1% agarase and 1% pectinase. From the third hour to sixth hour, maximum release of protoplasts occurred. The protoplasts assumed perfect spherical shape and viable protoplasts exhibited Brownian movement when observed under the microscope. The size of protoplasts ranged from 8-11μm in diameter probably released from outer cortex region (Fig. 1) and another set ranging from 20-25 μm in diameter probably from inner medullary cells (Fig. 2).

Yield of protoplasts varied considerably depending upon the age and morphology of the thallus. Hence growing meristematic tips of thalli were selected for production of protoplasts. Cheney *et. al.* (1986) used sporelings for obtaining maximum yield of viable and active protoplasts. Protoplasts maintained in TC-II medium (Table 2) were allowed to fuse electrostastically in a cell fusion system and are being monitored. In these preliminary attempts, spontaneous fusion of freshly released protoplasts of *G. edulis* could be observed in an instance. However, karyogamy could not be observed (Fig. 3).

Seaweed protplasts besides being used as seed stock for large scale mariculture, the report that protoplasts of *Kappaphycus alvarezii* secretes carrageenan fragments in culture (Zablackis *et. al.*, 1993) open up new

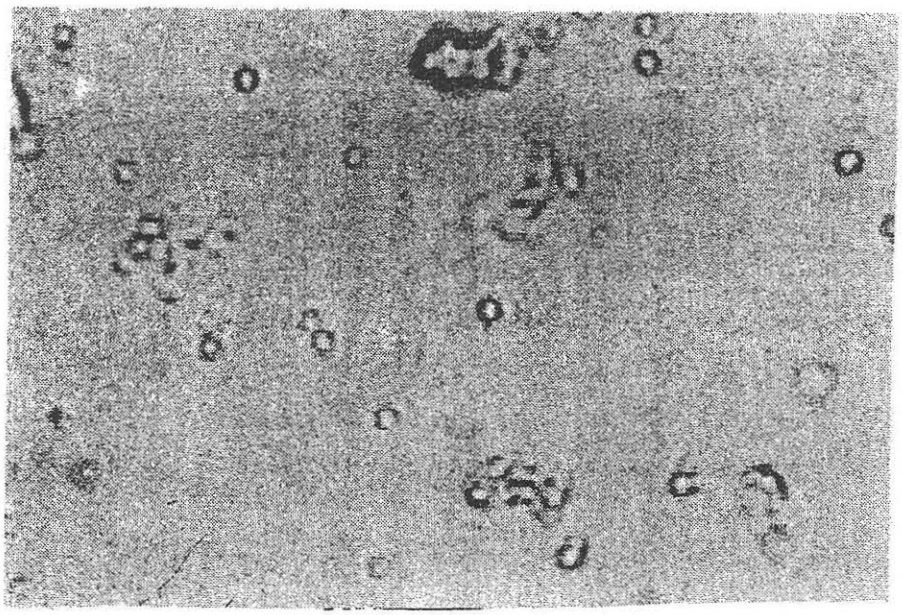

Fig. 1. *Protoplasts of* G. edulis *released from cortical cells.*

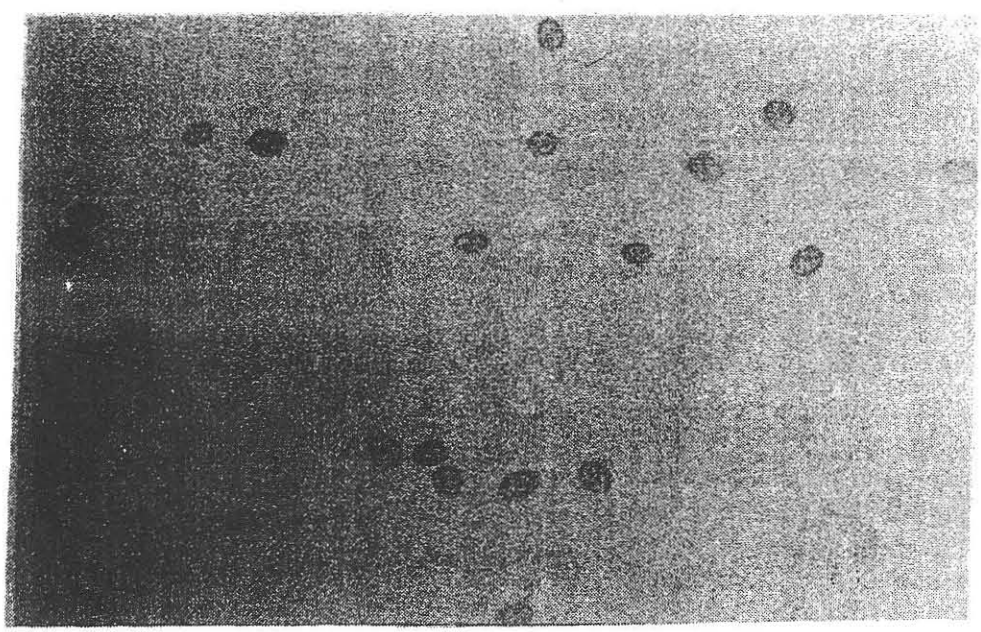

Fig. 2. *Protoplasts of* G. edulis *released from the medullary cells.*

Fig. 3. *Spontaneous fusion (Plasmogamy) of protoplasts.*

Table 2. *Composition of TC-II Medium*

$NaNo_3$	0.50mM	Alpha NAA	1 µM
NH_4NO_3	0.25mM	Seawater	1000 ml
$Na_2SiO_3.9H_2O$	0.20mM	filter sterlize with pore size	0.2 µm
Na_2EDTA	10 µM	pH	7.5
FeEDTA	10 µM	#V-3	
$FeCl_3.6H_2O$	1 µM	Thiamine HydroChloride	0.5 mg
$MgSO_4.7H_2O$	2 µM	Nicotinic acid	0.1 mg
$Na_2MoO_4.2H_2O$	5 µM	Ca-pantothenate	0.1 mg
H_3BO_3	5 µM	Biotin	1.0 µg
$NaH_2PO_4.H_2O$	20 µM	Folic acid	2.0 µg
$CaCl_2.2H_2O$	0.5 µM	Thymine	5.0 µg
KCI	2 µM	Cobalamine	1.0 µg
Phenyl acetic acid	0.1 µM	Inositol	5.0 µg
Pyridoxine	0.1 µM	Cyanocobalamine	1.0 µg
p-hydroxy phenyl acetic acid	0.2 µM	* PI-5x	
# V-3	2.0 ml	$MnCl_2$	7.0 µM
*PI-5X	2.0 ml	$ZnCl_2$	3x10 µM
IAA	2 µM	$CuCl_2.H_2O$	2x10 µM
Kinetin	1 µM	$Co\ Cl_2$	2x10 µM

possibilities for understanding and manipulating the metabolism of phycocolloid biosynthesis and can pave way for invitro production of phycocolloids.

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