Isolation and Regeneration of Protoplasts from *Ectocarpus*

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Protocol

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This article describes how to obtain isolated cells with no surrounding cell wall by enzymatic digestion of *Ectocarpus* filaments. The resultant protoplasts are totipotent and regenerate to produce individual algae under appropriate culture conditions. The yield of protoplasts and their capacity to regenerate are highly dependent on the *Ectocarpus* strain used, the stage of the life cycle, and the culture conditions. The highest yields are obtained with young gametophyte filaments cultivated at low density. The naked, wall-less cells produced by this protocol can be used for several applications, including studies of cell wall regeneration, investigation of the role of the cell wall in determining cell fate, and as a source of naked cells for the development of methods for introducing diverse molecules into the cell.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPES: Please see the end of this article for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Antibiotic mix <R>

The antibiotic mix is prepared in Provasoli-enriched seawater (PES) <R>.

Chelation medium <R>

Digestion medium <R>

Ectocarpus filaments (see **How to Cultivate *Ectocarpus*** [Coelho et al. 2012a])

Regeneration medium <R>

Equipment

Centrifuge

Cell strainers (40 µm) (BD Falcon)

Forceps (fine; dipped in ethanol and allowed to dry under the hood)

Microscope

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Neubauer hemocytometer
Orbital incubator
Pasteur pipettes
Petri dishes (plastic; 90-mm)

METHOD

1. Weigh the algal material (~500 mg fresh weight) after removing any excess seawater.
2. Place the *Ectocarpus* filaments in a 90-mm plastic Petri dish and separate the filaments with forceps.
3. Add 15 mL of chelation medium and incubate in the dark for 20 min at 13°C.

   Treatment of the filaments with the calcium chelator EGTA before enzymatic digestion increases the protoplast yield.
4. Replace the chelation medium with 15 mL of digestion medium and incubate in the dark at 13°C with gentle agitation for up to 6 h.
5. Check the material regularly under a microscope. When the cell wall has been digested (see Fig. 1), pass through a 40-µm filter to remove the undigested filament material.
6. Wash the protoplasts twice by centrifuging at 50–100*g* for 15 min at 4°C and resuspend them in 30 mL of regeneration medium. Count the number of protoplasts obtained using a Neubauer hemocytometer.
7. To regenerate the cell walls, cultivate the protoplasts at very low density in 30 mL of regeneration medium in the dark at 13°C. After 2 d, start very slowly to reduce the osmolarity by adding ~3 mL of PES containing antibiotic mix. After a further day, transfer the regenerating protoplasts to low light, and continue to reduce the osmolarity by adding ~3 mL of PES containing antibiotic mix until the regeneration medium is completely replaced. Finally, after a further day, the protoplasts (which at this point have regenerated their cell wall) can be transferred to light conditions of 20 µmol photons m^{-2} s^{-1}, that is, the standard culture conditions described in **How to Cultivate *Ectocarpus*** (Coelho et al. 2012a).

RELATED INFORMATION

For further details regarding the biology of *Ectocarpus* and the development of genomic and genetic tools for this organism, see ***Ectocarpus*: A Model Organism for the Brown Algae** (Coelho et al. 2012b).

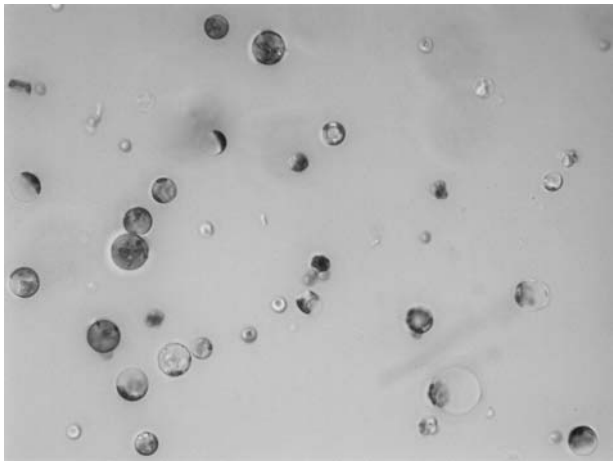

FIGURE 1. Protoplasts of *Ectocarpus* after incubation in digestion medium for 5 h.

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RECIPES

Antibiotic Mix

Reagent	Quantity (for 100 mL)
Penicillin G	1 g
Streptomycin	0.5 g
Chloramphenicol	0.1 g

Dissolve the penicillin G and streptomycin in 90 mL of water, then add the chloramphenicol dissolved in 10 mL of 100% ethanol. Filter through a 0.22-µm membrane and store in 5-mL aliquots at −20°C. Add 5 mL of the antibiotic mix to 1 L of Provasoli-enriched seawater (PES).

Chelation Medium

Reagent	Quantity (for 1 L)	Final concentration
NaCl (MW = 58.44)	40.9 g	700 mM
$MgCl_2 \cdot 6H_2O$ (MW = 203.3)	6.1 g	30 mM
$MgSO_4 \cdot 7H_2O$ (MW = 246.48)	7.39 g	30 mM
KCl (MW = 74.55)	1.49 g	20 mM
EGTA (MW = 380.35)	7.6 g	20 mM

Adjust pH to 5.5 using HCl. Autoclave or filter through a 0.2-µm membrane. Store at 4°C.

Digestion Medium

Reagent	Quantity (for 1 L)	Final concentration
NaCl (MW = 58.44)	23.4 g	400 mM
$MgCl_2 \cdot 6H_2O$ (MW = 203.3)	26.4 g	130 mM
$MgSO_4 \cdot 7H_2O$ (MW = 246.48)	5.4 g	22 mM
KCl (MW = 74.55)	11.9 g	160 mM
$CaCl_2$ (MW = 147.02)	0.3 g	2 mM
2-(*N*-morpholino)ethanesulfonic acid (MW = 195.2)	1.95 g	10 mM

Adjust the pH to 6.5. Filter through a 0.2-µm filter and store at 4°C. Just before use, add 1% (w/v) cellulose (Sigma) and 3 U/mL alginate lyase (Boyen et al. 1990).

Provasoli-Enriched Seawater (PES)

Reagent	Quantity (for 1 L)
Natural seawater	1 L
Provasoli solution	20 mL

If possible, seawater should be collected by boat at some distance from the coast. Filter the seawater using a 5-µm mesh. Aliquot into Nalgene bottles (in glass bottles a precipitate can form), autoclave, and store at 13°C. Autoclave the filtered seawater and the Provasoli solution separately to avoid precipitation. This recipe is based on Starr and Zeikus (1993); we use half-strength PES (i.e., 10 mL of Provasoli solution per 1 L of autoclaved seawater).

Regeneration Medium

Reagent	Quantity (for 1 L)	Final concentration
$MgCl_2 \cdot 6H_2O$ (MW = 203.3)	30.5 g	150 mM
KCl (MW = 74.55)	7.5 g	100 mM
$NaHCO_3$ (MW = 84.01)	0.34 g	4 mM
KNO_3 (MW = 101.11)	0.202 g	2 mM
NaH_2PO_4 (MW = 119.98)	0.012 g	100 μM

Adjust the volume with natural seawater.

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