CIBTech Journal of Biotechnology ISSN: 2319–3859 (Online)
An Open Access, Online International Journal Available at http://www.cibtech.org/cjb.htm
2014 Vol. 3 (3) July-September, pp.1-7/Nair
Research Article

ISOLATION OF PROTOPLASTS FOR SUB-CELLULAR RACTIONATION AND REGENERATION AND FUSION

***Geetha G. Nair**
Department of Botany, RIE, Mysore-6
**Author for Correspondence*

ABSTRACT
An isolated protoplast is totipotent and is capable of regenerating a complete plant. The pinocytic property of protoplasts makes them an excellent material for studies on genetic engineering. Protonemal chloronema cells of Funaria hygrometrica have been used here for protoplast isolation as protonema is haploid, can be grown on a chemically defined medium and cloned. Both regeneration of protoplasts and sub-cellular fractionation have been worked out. Ultra-centrifugation followed by enzyme assay showed that intact protoplasts are obtained but get sandwiched between broken protoplasts. Loading of fractions on a second sucrose density gradient has been recommended.

INTRODUCTION
Somatic hybridization is the fusion of two somatic cells. Isolated and cultured protoplasts can now be fused with polyethylene glycol and Ca^{+2} in an alkaline medium (Evini *et al.*, 2006; Gupta, 2005). The culture of these fusion products has led to the formation of hybrid cells and gene recombination by non-sexual means. Isolated protoplasts have now become an important experimental system in physiological, biochemical and genetic work (Cove, 1979; Peberdy and Ferenczy, 1985; Galun, 1981; Cocking, 2000; Pati *et al.*, 2008).

The phenomenal chloronema cells of *Funaria hygrometrica* Hedw. were used here for protoplast isolation. Protonema is a developmental phase in the life cycle of a moss. The system was selected because protonema grows on a chemically defined medium; they are haploid and can be easily cloned. Moreover, a method for the isolation of pure, clonal and asexual cell lines-which grow as short multi-cellular chloronema filaments in liquid medium with low level of Calcium, has been developed. Also these cell suspensions can be easily pipetted and transferred (Bajaj, 1994; Thorpe, 1978).

MATERIALS AND METHODS
Equipments
Laminar air flow cabinet
Bench top centrifuge
Ultra centrifuge with SW 41 Rotor
Spectrophotometer
Refractometer
Fluorimeter
Binocular microscope
Gyratory shaker and water bath
Nylon filters or Nylon cloth
Millipore filters and membrane filters 0.45µ
Whatman Filter Paper No.1
Forceps
Conical test tubes, nitro-cellulose tubes
Pipettes, petri-dishes and beakers
Conical flasks of all sizes, conical flasks with side arms, filters and adapters
Capped bottles and vials
Syringes and needles (no.22)

Materials and Media

Protonemal chloronema cells of *Funaria hygrometrica*
MG2 and MMG media
2% Driselase enzyme solution in 15%w/v sucrose

Methodology

Chloronema cells from the existing cell lines were grown in an MG2 medium which contained-
Ca $(No_3)_2$..4H2O - 50 mg 0.2ml/2l
KNO_3 .. 513mg 6ml/2l
$MgSO_4$ .. 250mg 4ml/2l
KCl - 250mg 4 ml/2l
KH_2PO_4 ..250 mg 4 ml/2l
Glucose - 10 gms (dissolve directly in 2l solution)
NaFeEDTA -43mg 2ml/2l
Heller's soln -10mg 4ml/2l
The flasks containing the MG2 medium with the inoculated cells were now placed under continuous light in a culture room at 20- 25 ◦C. When the cultures were fully grown the cells were filtered under pressure on nylon cloth without dehydrating the cells completely(This was done by using a Rotary vacuum pump and flasks with side arms ,filters and adapters) (Reinert and Bajaj, 1977; Rao and Prakash, 1995).
Next the filtered cells were weighed and a 2% enzyme solution prepared in a solution of sorbitol, and mannitol was added. Cells approximately 1gm/2ml of enzyme solution were added to the enzyme solution in a conical flask. These flasks were incubated for about 2 hours. Incubation of cells was also done in 2% driselase solution prepared in 15%w/v sucrose. The latter was found to be more ideal for isolation of protoplasts and gave a better yield (The flasks were kept in a radioactive water bath for incubation- this was because the protoplasts should not be shaken more than once per second-and this condition could only be fulfilled only in the radio-active water bath).
For regeneration $CaCl_2..2H_2O$ solution amounting to about $1/10^{th}$ of the volume of cells +enzyme solution in the flask was added to the flask immediately after incubation. The suspension in the flask was centrifuged (40 rpm) and washed with 15% w/v sucrose and recentrifuged (for 2 minutes as before). The protoplast pellet so got was resuspended in 2 ml of 15% sucrose. 0.25 ml of this suspension was plated on petri-dishes containing solid agar MMG medium. The whole process was carried out under sterile conditions in a laminar air flow cabinet. The 2% driselase solution was first filtered (to prevent clogging of millipore filters later) and then sterilized by millipore filtering. Cells were filtered not on nylon cloth but on filters under pressure without dehydrating them completely, as before and were then added to the sterile enzyme solution (approximately 1g/2ml) in conical flask. The flasks were incubated in a water bath with a shake of 1 per second at 24-25◦ degree Celsius for two hours. $CaCl_2$. $2H_2O$ was added as before immediately after incubation. The suspension of cells and protoplasts were centrifuged at 40 rpm for 2-3 minutes, the protoplast pellet was washed with 15% sucrose and centrifuged again .The pellet was resuspended again in 2 ml of 15% sucrose and 0.25 ml of the suspension was plated in petri-plates containing solid MMG medium.
This time the MMG medium was prepared without sorbitol as sorbitol used was not found to be good and the mannitol was doubled instead.
Sucrose---20 mg/ml- 10 gms/500ml
Glucose- 0.5 mg/ml---0.250 gms/500ml
Mannitol—27.3X2 mg/ml—27.3 gms/500ml
Casein hydrolysate – 0.25mg/ml—0.125 gm/500ml
α-NAA—2.5mg/ml---12.5mg/500 ml
Agar-1.5%---3.75 gm/250 ml
The plated petri dishes were kept under continuous light in the tissue culture room at 25-26◦ degree Celsius for 2 days.

For sub-cellular fractionation the protoplasts were lysed (immediately after incubation) by passing them through a syringe and needle (other methods like sonication were not used because this would damage and lyse the organelles too). A discontinuous sucrose density gradient was prepared with 60%, 50%, 40% and 30% w/v sucrose solutions from top to bottom of the ultra-centrifuging tube respectively. The lysed protoplasts were layered on top of the gradient. Ultra-centrifugation was done for about 2 hours. Definite volumes or fractions were collected in different test tubes by puncturing the nitro-cellulose tubes at the bottom. The different fractions were assayed for enzyme activity to locate thereby the various organelles in the fractions and separate them. The fractions were assayed for chlorophyll, for catalase- a marker enzyme for peroxisomes, for TPI- a marker enzyme of chloroplasts and for succinate dehydrogenase which is a marker enzyme for mitochondria (Griffith, 1979; Lehninger, 2009).

Assay for Chlorophyll: 0.1 ml of each fraction+ 0.5 ml of methanol was centrifuged for 5 minutes at 1000xg. The supernatant was decanted and the chlorophyll was re-extracted in another 0.5 ml of methanol. The two extracts were mixed with the help of a vortex and the O.D. read for 0.5 ml of extract in 1 cm path cuvette at 578 mμ. A blank was run with just methanol (Methods in Enzymology, 1957, 1967).

O.D. at 578 mu = mg chlorophyll per 0.5 ml of extract. Chlorophyll in mg/ml of fraction was calculated.
7.4

Table 1: Results of Assay for Chlorophyll

Fraction No.	Optical density	mg.Chl/0.5 ml extract	mg Chl in 1.1 ml extract in 0.1 ml of fraction	mg. Chl in 1 ml of fraction
Bulk	+0.000			
1	+0.055	0.005905	0.0129	0.129mg/ml
2	+0.078	0.01024	0.0232	0.232
3.	+0.055	0.01149	0.0253	0.253
4.	+0.032	0.004324	0.0095	0.095
5.	+0.076	0.01027	0.0226	0.226
6.	+0.025	0.003378	0.0074	0.074
7.	+0.091	0.01229	0.0270	0.0270
8.	+0.053	0.007163	0.0158	0.158
9.	+0.045	0.009638	0.0212	0.212
10.	+0.092	0.01244	0.0324	0.324
11.	+0.038	0.005136	0.0113	0.113
12.	+0.031	0.004190	0.0092	0.092
13.	+0.024	0.003243	0.0071	0.071

Assay for Catalase: Catalase decomposes H_2O_2 into water and oxygen and activity was measured by finding the decrease in O.D. of H_2O_2 in 240 mμ region.
3 ml of 1:200 H_2O_2 solution plus 100 ml of each fraction-O.D. was read for this aliquot after 20, 60 and 120 seconds respectively. Blank was run with H_2O_2 solution and activity measured as O.D/min/ml.

Table 2: Results of assay for Catalase

Fraction no.	O.D.	Δt (mins)	ΔO.D/Δt for 100 ml	Activity O.D./min/1000ml
1	0.002	1.67	0.001198/min/100 ml	0.01198/min/ml=0.012min/ml
3	0.006	1.67	0.003593/min/100ml	0.03593=**0.036/min/ml**
4.	0.005	1.67	0.002994/min/100ml	0.02994=0.030/min/ml
5.	0.004	1.67	0.002395/min/100ml	0.02395=0.024/min/ml
6.	0.004	1.67	0.002395/min/100ml	0.02395=0.024/min/m
7.	0.009	1.67	0.005389/min/100 ml	0.05389=**0.054/min/ml**
8.	0.004	1.67	0.002395/min/100ml	0.02395=**0.054/min/ml**
9.	0.010	1.67	0.005988/min/100ml	0.05988μ=**0.060/min/ml**
11.	0.007	1.67	0.004192/min/100ml	0.04192=**0.042/min/ml**

Assay for Triose Phosphate Isomerase
Reagents required for the assay
1) KCN 1.9 mg/2ml- neutralize with dilute HCl and make up to 10 ml
2) $CaCl_2$ 0.8 M-10 ml
3) Dichlorophenol indophenol (DCIP)-3 mg/40 ml
4) Phenazone methosulphate (pms) - 10 mg/ml- to be prepared fresh each time
5) Succinate 0.5M-10 ml (1.35gm/10 ml)
6) 0.5M PO_4 buffer pH=7.4 dilute to 0.1 M
In the spectrophotometric method several levels of PMS are coupled with 2-6 dichlorophenol indophenols for V max by following the reduction of DCIP at 600 nm
React ion mixture or assay will contain
1ml—PO_4 Buffer
0.1ml Ca C l_2
0.3 ml DCIP
0.1ml Succinate
0.1ml KCN
1.25 ml water= 2.85 ml +0.05 ml PMS (50ʌ) +0.1 ml (100ʌ) extract or fraction was read at 600 nm with water or blank. Activity was calculated as O.D./min/ml.
Reaction mixture in TRA buffer (50 Mm pH 7.4 + 10 mM $MgCl_{2)}$
NADH-50ʌ/10 ml
Gly-3-P- 20 μl/reaction
α-glycerophosphate DH-1μl/ml
Take 1 ml of reaction mixture in a cuvette, add say 50 ʌ of each fraction and start reaction by Glyceraldehyde -3- phosphate. This was done in a fluorimeter by measuring the rate of formation of DPN^+ and activity was calculated as nm/min/ml (Methods in Enzymology, 1957).

Table 3: Results of Assay for TPI

Fraction no.	Amount of each fraction used ʌ or ml	Rate in div/2 min	Activity(nm/min/ml)
2	50 ʌ	6 Div/2 mins 3 div/min	3x0.15x20=9 nm/min/ml
3	50 ʌ	3.5 Div/2 mins 1.75 div/min	1.75x3=5.25 nm/min/ml
4	50 ʌ	15.5 Div/2 mins 7.75 div/min	**7.75x3=23.25 nm/min/ml**
5	50 ʌ	1.5 Div/2 mins 0.75 div/min	0.75x3=2.25 nm/min/ml
6	50 ʌ	15.5 Div/2 mins 7.15 div/min	**7.75x3=23.25 nm/min/ml**
7	50 ʌ	13.5 Div/2 mins 6.75 div/min	**6.75x3=20.25 nm/min/ml**
8	50 ʌ	11.5 Div/2 mins 5.75 div/min	5.75x3=17.25 nm/min/ml
9	50 ʌ	3.5 Div/2 mins 1.75 div/min	1.75x3=5.25 nm/min/ml
10	90 ʌ	15 Div/2 mins 7.5 div/min	7.5x0.15x100/9=12.5 nm/min/ml
11	50 ʌ	5.5 Div/2 mins 2.75 div/min	2.75x3==8.25 nm/min/ml
12	50 ʌ	11 Div/2 mins 5.5 div/min	5.5x3=16.5 nm/min/ml
13	**50 ʌ**	**20 Div/2 mins** **10 div/min**	**10x3=30 nm/min/ml**

Assay for Succinate Dehydrogenase: SDH catalyses the oxidation of succinate by a number of artificial electron acceptors. Here in this spectrophotometric method, several levels of PMS are coupled with 2.6 - dichlorophenol indophenols for Vmaximum by following the reduction of DCIP at 600nm (Methods in Enzymology, 1957). Activity was calculated as O.D./min/ml. Blank was run with water.

Table 4: Results of Assay for SDH

Fraction no.	Optical density 10 20 120 secs			Δ O.D.	Δt mins	ΔO.D./Δt for 100 µl	Activity O.D./min/ml
2	+0.512	+0.512	+0.512	0.000	1.83	0	0
3	**+0.576**	**+0.576**	**+0.576**	**0.000**	**1.83**	**0**	**0**
4	+0.576	+0.573	+0.592	0.004	1.83	0.002186	**0.02186/0.022**
5	+0.531	+0.531	+0.530	0.001	1.83	0.0005464	0.005464/0.005
6	+0.610	+0.607	+0.604	0.006	1.83	0.003278	0.03278/0.033
7	+0.651	+0.644	+0.641	0.010	1.83	0.005464	**0.05464/0.055**
8	+0.574	+0.592	+0.590	0.004	1.83	0.002186	0.02186/0.022
9	+0.580	+0.577	+0.575	0.005	1.83	0.002732	0.02732/0.028
10	-0.532	-0.536	-0.540	-0.008	1.83	0.004371	**0.04371/0.044**
11	-0.553	-0.557	-0.561	-0.008	1.83	0.004371	**0.04371/0.044**
12	-0.473	-0.488	-0.478	-0.025	1.83	0.01366	0.1366/0.137
13	**-0.503**	**-0.532**	**-0.537**	**-0.036**	**1.83**	**0.01767**	**0.1967/0.200**

Refractive indices of the various fractions were found out with the help of a refractometer.

Table 5: Refractive indices of the fractions taken for study

Fraction no.	Concentration	Refractive index (µ)
1	62.4%	1.4475
2	62.6%	1.448
3	60.2%	1.4425
4	25.6%	1.432
5	53.1%	1.4275
6	51.6%	1.4235
7	47%	1.4135
8	43.6%	1.407
9	41.2%	1.402
10	36.4%	1.3425
11	33.4%	1.3875
12	27.4%	1.3765
13	18.8%	1.3015

RESULTS AND DISCUSSION

No good regeneration was obtained due to certain technical difficulties. Most of the protoplasts were killed during plating. Also, α-naphthol acetic acid had not been added in the solid medium. After ultra-centrifugation 4 bands of chlorophyll were obtained. This was peculiar because only one single band of chlorophyll is obtained normally. Therefore, a few of the bands obtained could be due to broken protoplasts. Also, a survey of the tables the fractions gives 4 chlorophyll peaks and only 3 TPI peaks. Two of the TPI peaks showing higher peak activity (i.e. fractions 4&6) run in-between the chlorophyll peaks (fractions 2,3, 9 and 10) suggesting that the intact protoplasts are getting sandwiched in between broken chloroplasts.

Succinate dehydrogenase does not show much activity and gives small peaks in fractions 4, 7 and 10. Catalase gives multiple peaks like chlorophyll and seems to be running along with chlorophyll. A clear catalase peak is seen in fraction 7 but the peaks of catalase and chlorophyll are overlapping.

Supernatant seems to be containing all the enzymes (i.e. fraction no.13 which shows activity in all the assays except one). A much better separation could have been obtained if fractions 5-8, and 9, where all the enzymes show high activity were collected and loaded on a second discontinuous density gradient and centrifuged.

REFERENCES

Bajaj YPS (1994). Biotechnology in Agriculture and Forestry 27. *Somatic Hybridisation in Crop Improvement* (Springer –Verlag) Berlin **I**.

Cocking EC (2000). Turning Point Article. Plant Protoplasts, *In Vitro Cellular & Developmental Biology - Plant* **36** 77-82.

Cove DJ (1979). The uses of isolated protoplasts in plant genetics. *Heredity* **43** 295-314.

Evini M, Rajkumar K and Balaji P (2006). Isolation,Regeneration and PEG-induced Fusion of Protoplasts of *Pleurotus pulmonarius* and *Pleurotus florida*. *Mycobiology* (published online).

Galun E (1981). Plant Protoplasts as Physiological Tools. *Annual Review of Plant Physiology* **32** 237-266.

Griffith OM (1979). *Techniques of Preparative, Zonal and Continuous Flow Ultra-centrifugation*, 3rd edition. Beckman Instruments, Inc., Spinco Div.: Palo Alto, CA.

Gupta PK (2005). *Plant Breeding, Plant Propagation and Plant Biotechnology* (Rastogi Publications) Meerut.

Lehninger Albert L (2009). *Biochemistry-The Molecular Basis of Cell Structure and Function* (Kalyani Publishers).

Methods in Enzymology (1957). *Preparation and Assay of Substrates,* edited by Nathan Kaplan (Academic Press) **3** 3-1154.

Methods in Enzymology (1967). *Oxidation and Phosphorylation,* edited by Ronald W Esta brook and Maynard E Pullman) (Academic Press).

Pati PK, Sharma M and Ahuja PS (2008). Protoplast Isolation and Culture, In Plant Biotechnology: Methods in Tissue Culture and Gene Transfer. *Tissue Culture and Transfer,* edited by Keshavachandran R and Peter KV (Universities Press (India) Pvt. Ltd.) India **11** 115-132.

Peberdy JF and Ferenczy L (1985). Fungal protoplasts. Applications in Biochemistry and Genetics. *Mycology Series,* Marcel Dekker, New York **6** 354.

Rao KS and Prakash AH (1995). A simple method for the isolation of protoplasts. *Journal of Biosciences* **20**(5) 644-655.

Reinert J and Bajaj PS (1977). *Applied and Fundamental aspects of Plant Cell, Tissue and Organ Culture* (Springer Verlag) Berlin.

Thorpe Trevor A (1978). Frontiers of Plant Cell Tissue Culture. The International Association for Plant Tissue Culture, Canada.