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Preparation, fusion and regeneration of protoplast of *Volvariella volvacea*

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The experimental findings revealed that the combination of Novozyme 234 + cellulase + chitinase + pectinase enzymes is the best for the release of protoplasts from 4 days old mycelia of *Volvariella volvacea* under sodium chloride osmotic stabiliser and phosphate buffer (pH 6.0). The regeneration of protoplast was also best in phosphate buffer (pH6.0). In another experiment UV- mutants were produced and several asp^- and adn^- mutants are developed. In the progeny analysis it was found that 36 out of 38 asp^- mutant monosporous cultures and 45 out of 49 adn^- monosporous cultures retain their parent mutant phenotype.

Key words: *V.volvacea,* release of protoplasts, regeneration of protoplasts, UV-mutants

INTRODUCTION

Volvariella volvacea is cultivated as the fifth most important edible mushroom in the world. It is commonly known as Paddy straw mushroom or Chinese mushroom which is also known for its unique aroma and texture. Roy *et al*. (2014) have reported that the nutritional value of these mushrooms depend on the type of the agricultural waste used for its cultivation. Mukhopadhyay *et al*. (2006) have reported that nucleic acid content changes during fruit body production in *V.volvacea*. Enzymes play vital role in the development of mushrooms their nutritive value and flavours. Cellulolytic enzymes play a significant role in natural biodegradation process of agro-industrial wastes on which *V. volvacea* is cultivated (Jonathan *et al*, 2010; Jonathan and Aldeoys, 2011). *V. volvacea* is one of the easiest mushrooms to cultivate but its yield of fruiting bodies is low and unstable. But primary homothallism makes conventional breeding programme difficult. For this inconvenience protoplast fusion is advised as the most effective method of carrying out gametic crosses in laboratory.

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The effectiveness of the technology of protoplast solely depends on the optimum conditions for the successful regeneration of protoplast. Several research studies have been reported on the optimum conditions for the preparation and regeneration of protoplasts in several species of Basidiomycotina. Several studies have also been done on the edible mushrooms *viz*. *Agrocybe, Pleurotus, Schizophyllum* and *Lentinus*, but very few studies on the regeneration of protoplasts of *V. volvacea* only.

The present investigation aims to conduct a thorough experiment on the optimization of conditions for fusion and regeneration of protoplasts of *V. volvacea*

MATERIALS AND METHODS

The general procedure for the preparation, fusion and regeneration of protoplasts of *V. volvacea* was based on the works of Mukhopadhyay *et al* (2006).

Protoplast culture

The vegetative mycelium of *Volvariella volvacea* was grown in a liquid medium (pH 6.8) containing (w/v) glucose 20 g, yeast extract 2 g and soy tone

1.5 g at 32^{0}C in a rotary shaker (200 cycles per minute) for 4 days.

After 4 days the mycelia were harvested and washed three times with distilled water and then thrice with osmotic stabiliser solution (0.6M NaCl in 0.01M phosphate buffer; pH 6.0). A Novozyme 234 solution (5 mg/ml) was prepared in osmotic stabilizer solution and the enzyme solution was sterilized with the help of filter. A quantity of freshly harvested 4-days old mycelia (equivalent to about 20 mg dry wt.) previously washed with water and osmotic stabilizer, was incubated in 20 ml culture tubes with 3 ml of Novozyme 234 solution for 18 h at 32^{0}C (Santiago,1982 a,b).
The released protoplasts were separated from the undigested and fragmented mycelia by filtering through G-2 sintered funnel and then centrifuged at 2000 g for 20 minutes. The sedimented protoplasts were suspended in osmotic stabilizer solution and filtered through G-3 sintered funnel. The filtrate was again centrifuged at 2000 g for 20 minutes. The protoplast pellet was suspended in osmotic stabilizer solution and checked carefully under microscope to ensure the absence of any mycelial fragments. The number of protoplasts were counted with a haemocytometer.

The regeneration medium was prepared by adding glucose 10 g (w/v) and yeast extract 500 mg (w/v) per litre in osmotic stabilizer solution and supplemented with agar 20 g (w/v). An aliquot of 0.1ml of protoplast suspension containing about 60-70 protoplasts was spread out on regeneration medium in Petriplate and incubated for 72 h at 32^{0}C. Regeneration frequency was measured at the ratio of the number of colonies developed to the number of protoplasts added per plate of diluted protoplast preparations.

The auxotroph were produced with UV-irradiation. An aliquot of 10 ml protoplast suspension (1.2 X 10^6 protoplasts per ml) was spread in a Petridish (90 mm diameter) and irradiated with UV-irradiation for 15 minutes at 32^{0}C in the dark. The UV-irradiation was provided with 15W Philips germicidal lamp emitting 254 nm at a dose rate of 1J/m^2 per second. After the exposure period the irradiated protoplasts were kept for 30 minutes in the dark to allow the full expression of the damage induced by UV-irradiation. The irradiated protoplasts were then diluted with osmotic stabilizer solution and plated for regeneration on solid medium. Simultaneously non-irradiated controls were also prepared (Mukherjee and Sengupta, 1986,1999).

All the regenerated colonies were transferred to complete medium (CM) of Raper and Miles (1958) and modified minimal medium (MM) of Yoo *et al* (1988). The MM contains (g/l) dextrose 20; K_2HPO_4 1; $MgSO_4$, $7H_2O$ 0.5; KH_2PO_4 0.46; agar (Difco) 15. The MM was supplemented with different amino acids (25μg/ml), nucleic acid bases (20 μg/ml) or vitamins (1 μg/ml) to find out the nature of the auxotroph.

For fusion of protoplasts, 1ml of protoplast suspension (about10^7/ml) from two auxotroph strains (adenine requiring and aspartate requiring) were mixed in 8 ml of fusogen (PEG-Ca^{++}) for 20 minutes at 32^0 C and fusants were collected by centrifugation in 2 ml of 0.6M mannitol. The collected fusants protoplasts were regenerated as described earlier. After 7-10 days of incubation visible colonies were isolated and transferred to PDA medium for further growth. All colonies that expressed normal mycelial growth rate were selected for further study.

RAPD analysis of the isolated DNA

Individual genomic DNA isolated from each of the four different growth stages of the material was used as the template in the PCR amplification. The isolated DNA was denatured for 5 minutes at 94^{0}C and immediately cooled in ice for 3 minutes. After this 2 ng of the denatured DNA was added to a reaction tube containing 24 ìl of the following mixture: 10X taq buffer containing 20 mg Tris-HCl (pH 8.4), 10 mM KCl, 3 mM $MgCl_2$ and 0.2 mg/ml gelatin; 0.1 μM primer1, primer2 and primer3 (Genei Laboratory); 60 μM each of dATP, dCTP, dGTP and dTTP and 15 units of taq DNA polymerase. A drop of mineral oil was added to the 25μl reaction mixture to minimise evaporation from the reaction tubes. The reaction tubes containing the mixtures were placed in a DNA Thermal Cycler of Perkin Elmer. The DNA amplification was performrd with an initial cycle of denaturation (2 min. at 94^{0}C); followed by 35 cycles of denaturation (2 min. at 94^{0}C); annealing

(2 min. at 40^{0}C) and extension (1.30 min. at 72^{0}C). Lastly a final incubation was done at 72^{0}C for 10 minutes.

After amplification each reaction mixture was mixed well with 100ìl of chloroform and the aqueous phase was separated from the mineral oil- chloroform phase.

To assay the amplification products the method of agarose gel electrophoresis was used. A 1.5% agarose gel containing 98mM Tris (pH8.0), 89mM H_2BO_3, 2mM EDTA and 1.34mM ethidium bromide was used for the assay. 2ìl of loading and tracking dye (30% glycerol and 0.25% bromophenol blue in distilled water) were added to the amplification reaction mixture and 10ìl of the amplified sample was loaded onto the gel. Electrophoresis was conducted at a constant 80 V. After the electrophoresis the amplified products were viewed and photographed using a transilluminator.

RESULT AND DISCUSSION

Isolation of protoplasts and preparation of hybrid fusants

For preparation of protoplast-fusant hybrids, protoplasts were isolated from the actively growing mycelium of *Volvariella volvacea* by the action of lytic enzymes following the methods described in the Materials and Methods section.

The experimental data obtained are given in Tables 1-4.

In Table 1, the data revealed that of the four lytic enzymes used, the combination of all the four lytic enzymes was the best for the release of protoplasts (54.42 X 10^7/ml).This was followed by the Novozyme 234 + cellulose + chitinase (32.12 X 10^7/ml), Novozyme 234, 6mg/ml (29.04 X 10^7/ml), Novozyme 234, 4mg/ml (28.49 X 10^7/ml), Novozyme 234 + chitinase (28.42 X 10^7/ml), Novozyme 234 + pectinase (26.64 X 10^7/ml), Novozyme 234 + cellulase (24.54 X 10^7/ml), Novozyme 234, 2mg/ml (18.86 X 10^7/ml), pectinase (11.86 X 10^7/ml), chitinase (11.44 X 10^7/ml) and cellulase (10.64 X 10^7/ml).

Table 1: Effect of lytic enzymes on yield of protoplast from mycelium of *Volvariella volvacea*

Lytic Enzymes	Unit of lytic enzyme/ml	No. of protoplasts/ml X 10^7
Novozyme 234	2mg	18.86±0.36
	4mg	28.42±0.46
	6mg	29.04±0.54
Cellulase	6U	10.64±0.44
Chitinase	6U	11.44±0.48
Pectinase	4mg	11.86±0.58
Novozyme234 + cellulase	4mg + 6U	24.54±1.64
Novozyme 234 + chitinase	4mg + 6U	28.42±1.86
Novozyme 234 + Pectinase	4mg + 4mg	26.64±1.62
Novozyme 234 + Cellulase + Chitinase	4mg + 6U + 6U	32.12±1.49
Novozyme 234 + Cellulase + Chitinase + Pectinase	4mg + 6U + 6U + 4mg	54.42±1.82

Lytic enzymes were prepared in 0.5 M mannitol

The data in Table 2 revealed that 4 days old mycelium was best for the release of protoplasts (30.32 X 10^7/ml), which was followed by 3 days old (22.48 X 10^7/ml), 5 days old (21.82 X 10^7/ml),6 days old (18.24 X 10^7/ml), 2 days old (17.42 X 10^7/ml), 7 days old (16.48 X 10^7/ml), 9 days old (13.48 X 10^7/ml),1 day old (10.64 X 10^7/ml) and 10 days old (10.48 X 10^7/ml) mycelium

Table 2: Effect of age of the mycelium on yield of protoplasts from mycelium of *Volvariella volvacea*

Age of mycelium (day)	No. of protoplasts per ml X 10^7
1	10.64±0.48
2	17.42±1.06
3	22.48±1.32
4	30.32±1.84
5	21.82±1.36
6	18.24±1.12
7	16.48±0.86
8	14.36±0.64
9	13.48±0.56
10	10.64±0.48

Novozyme 234 was applied as 4mg/ml in 0.5M mannitol

In order to find out the best osmotic stabiliser for the release of protoplasts, five osmotic stabilisers, namely, sodium chloride, potassium chloride, magnesium sulphate, mannitol and sorbitol were used. The experimental data obtained are given in Table- 3.

The data in Table-3 revealed that sodium chloride was the best osmotic stabiliser (82.16 X10^7/ml) which was followed by magnesium sulphate (64.38 X10^7/ml), mannitol (25.86 X10^7/ml), sorbitol (21.48 X10^7/ml) and potassium chloride (18.42 X10^7/ml).

Table 3: Effect of osmotic stabiliser on yield of protoplasts from mycelium of *Volvariella volvacea*

Name of osmotic stabiliser (0.06M)	No. of protoplasts per ml X 10^7
Sodium Chloride	82.16±0.48
Potassium Chloride	18.42±0.64
Magnesium Sulphate	64.38±2.16
Mannitol	25.86±1.86
Sorbitol	21.48±1.74

In order to find out the effect of buffer and pH on the release of protoplasts, four buffers, namely, succinate, phosphate, maleate, and acetate were used. The range of pH used was 4.0 to 7.5. The experimental data obtained are given in the Table 4.

The data in Table 4 revealed that phosphate was the best buffer for the release of protoplasts which was followed by maleate, succinate and acetate. In the phosphate buffer, pH 6,0 was optimum (98.40 X 10^7/ml), followed by pH 6.5 (52.18 X 10^7/ml), pH 5.5 (46.82 X 10^7/ml) and pH 7.0 (26.16 X 10^7/ml). In maleate buffer, pH 6.0 was optimum (68.38 X 10^7/ml), followed by pH 5.5 (48.24 X 10^7/ml), pH 6.5 (44.54 X 10^7/ml) and pH 7.0 (16.12 X 10^7/ml). In succinate buffer, pH 6.0 was optimum (38.44 X 10^7/ml) followed by pH 5.5 (31.42 X 10^7/ml), pH 5,0 (27.16 X 10^7/ml), pH 4.5 (17,48 X 10^7/ml) and pH 4.0 (7.84 X 10^7/ml). In acetate buffer pH 5.5 was the best (26.12 X 10^7/ml) followed by pH 5.0 (18.36 X 10^7/ml), pH 4.5 (14.48 X 10 /ml) and pH 4.0 (6.12 X 10^7/ml).

The released protoplasts were regenerated in glucose-yeast extract- agar medium by using phosphate and maleate buffer at pH 6.0 and the data are presented in Table 5.

The data in Table 5 revealed that 0.01M phosphate buffer (pH6.0) was the best for regeneration of protoplasts with regenerated percentage of 6.36 than that of 0.01M maleate buffer (pH6.0) with regenerated percentage of 2.12.

In other experiment the aspartate and adenine mutants of *Volvariella volvacea* were produced following the treatment of UV radiation. For this a suspension of protoplasts (8ml) was subjected to UV radiation in a Petridish (90mm diameter) at 30^{0}C in dark under mild agitation. The source of UV

Table 4: Effect of buffer and pH on yield of protoplasts from mycelium of *Volvariella volvacea*

Buffer	pH	No. of protoplasts per ml X10^7
Succinate	4	7.84±0.84
	4.5	17.48±0.86
	5	27.16±1.12
	5.5	31.42±1.64
	6	38.44±1.82
Phosphate	5.5	46.82±1.86
	6	98.40±4.24
	6.5	52.18±2.86
	7	26.16±1.74
	7.5	Nil
Mal eate	5.5	48.24±1.14
	6	68.38±2.84
	6.5	44.54±1.82
	7	16.12±1.02
Acetate	3.5	Nil
	4	6.12±0.86
	4.5	14.48±1.14
	5	18.36±1.24
	5.5	26.12±1.36

Table 5: Regeneration of protoplasts from mycelium of *Volvariella volvacea*

Buffer of the lytic enzyme	Regeneration (%) + SE in solid glucose yeast extract medium
0.01M phosphate (pH 6.0)	6.36±0.65
0.01m maleate (pH6.0)	2.12 ±0.43

radiation was a Philips germicidal lamp (15W) at 254 nm at a dose rate of 1J /m^2 per second. After the UV treatment the protoplast suspension was kept on dark for 30 minutes for allowing the full expression of the damage caused by UV radiation. Then these were diluted with osmotic stabiliser solution and plated on regeneration solid medium for growth. Similarly untreated control set of protoplasts was also regenerated. These were then grown on the complete medium.

From these several aspartate and adenine mutants were screened out and these were stored at 15^{0}c for further use. These isolates were then cultivated and from the basidiocarps, monosporus isolates of mutants were obtained following the method of Chang and Li (1991). For the analysis of progeny,

Table 6: Data (average of three replications) showing the growth of aspartate$^-$ mutant protoplast strain of *Volvariella volvacea* in MM supplemented with different concentrations of aspartate

Concentration of Aspertate (µg/ml)	Growth (cm/48h)	
	Aspertate mutant protoplast strain	Parental protoplast strain
10	03±0.11	10±0.16
20	07±0.12	12±0.18
30	12±0.11	15±0.19
40	18±0.14	18±0.18
50	24±0.16	23±0.12
60	29±0.18	28±0.16
70	34±0.19	32±0.18
80	37±0.20	35±0.18
90	40±0.32	38±0.24
100	45±0.32	40±0.30

Table 7:Data (average of three replications) showing the growth of Adenine$^-$ mutant protoplast strain of *Volvariella volvacea* in MM supplemented with different concentrations of adenine

Concentration of Adenine (µg/ml)	Growth (cm/24h)	
	Adenine mutant protoplast strain	Parent protoplast strain
2	04±0.10	14±0.14
4	08±0.12	15±0.15
6	17±0.14	27±0.16
8	26±0.20	29±0.22
10	34±0.24	32±0.28
12	36±0.26	34±0.32
14	39±0.28	36±0.34
16	42±0.32	38±0. 40
18	43±0.40	40±0.42
20	45±0.42	42±0.44

the monosporous isolate was grown on mushroom minimal medium (MM) of Yoo *et al* (1988) supplemented with 100 ìg/ml of aspartate and adenine.

The experimental data are presented in Tables 6, 7, 8 and 9.

The data in Table 6 and Figure1 revealed that the growth of the aspartate mutant was more in MM medium supplemented with different concentration of aspartate than that of parental protoplast strain. The data in Table 7 and Figure 2 also revealed that the growth of adenine mutant protoplast strain

Table 8: Progeny analysis of Asp$^-$ mutant protoplast strain

No. of monosporus isolates tested	Progeny analysis of Asp$^-$mutant protoplast strain			
	Asp$^-$ no.	Percentage	Asp$^+$ no.	Percentage
12	11	91.67	1	8.33
9	9	100	0	0
10	10	100	0	0
7	6	85.71	1	14.29

was more in MM medium supplemented with different concentrations of adenine than that of parental strain.

Table 9:Progeny analysis of Aden$^-$ mutant protoplast strain

No. of monosporus isolates tested	Marker protoplast strain			
	Aden$^-$	Percentage	Aden$^+$	Percentage
16	15	93.75	1	6.25
18	16	88.89	2	11.11
15	14	93.34	1	6.66

The progeny analysis of Asp$^-$ mutant protoplast strain (Table 8) revealed that 36 out of 38 monosporous cultures retained their parent mutant phenotype, whereas Adn$^-$ mutant protoplast strain (Table 9) revealed that 45 out of the 49 monosporous cultures retained their parent mutant phenotype.

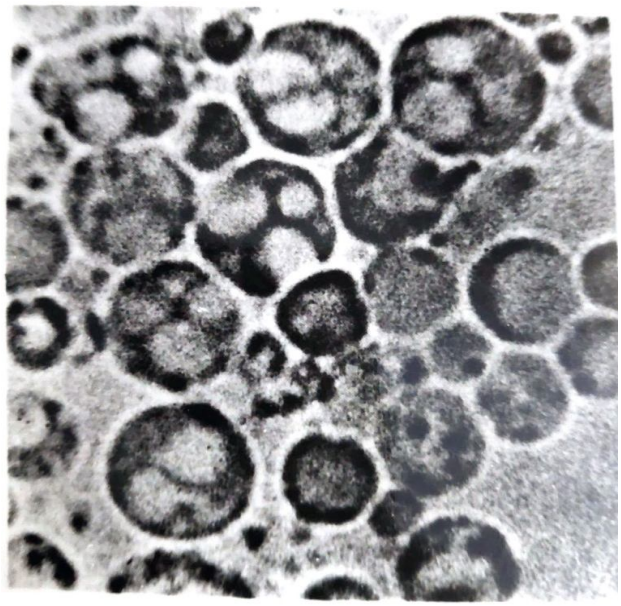

Fig. 1 : released round protoplasts with vacuoles from 4-day old vegetative mycelium of *Volvariella volvacea*

The data on the release of protoplasts and their regeneration revealed that although Novozyme 234+ cellulase + chitinase and pectinase mixture enzyme solution is the best for release of

protoplasts but Novozyme 234 released protoplasts are the best for regeneration purposes (Fig.2). The data also revealed that four days incubation is best for the release of protoplasts (Fig.1). Sodium chloride as osmotic stabiliser and phosphate buffer (pH6.0) are the best for release of protoplasts. The regeneration percentage of protoplast is very low (6.36%) in *V.volvacea*. Similar observations are reported by several scientists on other fungi. The data also reveal that about 94.345 (average) monosporus isolates of Asp^- mutant protoplast strains and about 91.96% (average) monosporus isolates of Adn^- mutant protoplast strain of *V.volvacea* retained their parent phenotype characters. Similar observations have been reported by earlier scientists on *V.volvacea* and other fungi.

Fig. 2 : Regenerated hyphae from the released protoplasts of *Volvariella volvacea*

It is known that *V.volvacea* is a primary homothallic species, i.e. self-fertile without any crossing. It is observed that there is a great variation among the monosporus isolates of *V.volvacea*. In order to find out the genetics of sexuality in this fungus the release of protoplasts and their regeneration have been done by scientists earlier.

In fungi the application of protoplast technology is well established. In *V. volvacea*, as the mushroom is a primary homothallic species, the development of genetic strains for better crop and protein value is not possible by sexual crossing. As such the protoplast mutants with markers (Asp^- and Adn^-) could be efficiently utilised for the development of protoplast fusants hybrids to counteract the above mentioned difficulty.

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