

Protoplast generation in the nematode trapping fungi, *Arthrobotrys oviformis* and *Dactylaria parvispora*

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Abstract

A protocol for generating viable protoplasts from isolate of the nematophagous fungus *Arthrobotrys oviformis* is developed. The lytic enzyme Novozyme 234 is used in the study. Maximum protoplasts are released with 24 hours old *A. oviformis* mycelia after 35 minutes of treatment with the lytic enzyme Novozyme 234 at a concentration of 10 mg ml⁻¹. The nematophagous fungus *Dactylaria parvispora* however is found to be refractive to the process and protoplasts could not be generated under the range of conditions used in this study. The yield of protoplasts and the frequency of regeneration of *A. oviformis* is found to be highest when 0.6 molar Sodium Chloride is used as the osmotic stabilizer. This is the first report of an optimized method for generating viable protoplast from young mycelia of the nematophagous fungus *A. oviformis*.

Keywords: protoplast, nematophagous, fungus.

INTRODUCTION

Several decades have passed since the first reports appeared on isolation of protoplast from yeasts and filamentous fungi (Peberdy, 1979). These findings centered on the problem of cell wall dissolution and its rapid disintegration. Following reproducible success, the use of *Saccharomyces cerevisiae* protoplast has been of great assistance in studies related to organelles and in biosynthetic studies (Pasha *et al.*, 2007). Subsequently, generation of protoplasts became a subject of intense study in its own rights and allowed investigations for genetic modification of bacteria, fungi and plants (Zimmerman *et al.*, 1981; Gupta *et al.* 1997; Tucker and Orbach, 2007). Removing the cell wall followed by their fusion, uptake of nucleic acid and other such processes are available that were otherwise impossible with intact cells.

There has been long time effort in employing fungal isolates for control of nematodes that infest the intestine of dairy animals (Sanyal and Mukhopadhyaya, 2003). Chemical drugs have been in use for several decades in controlling these worms. However, issues related to drug resistance and drug residual effect in milk warranted exploration of eco-friendly and sustainable worm management programmes (Sanyal, 1998). Integrated Pest Management (IPM) with its multiple components, biological control being the most effective and stand alone one has been the technique of choice in recent times.

Significant effort and time has been invested in demonstrating the nematode-trapping efficacy of several isolates of fungi. This includes isolates of *A. oviformis* and *D. parvispora* investigated by us earlier (Nagee *et al.*, 2001). Although strain improvement by genetic methods finds place in the research agenda for this group of organisms and genes linked to nematode trapping activity have been investigated (Tunlid *et al.*, 1999; Genbank accession number AY207006 and

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Table 1: Rate of release of protoplasts from *A. oviformis* mycelia harvested at different time points.

<i>Osmotic stabilizer</i>	<i>Age of culture in hours</i>								
	12	14	16	18	20	22	24	26	28
0.4 M (NH₄)₂SO₄	0	0	4.31 (±0.11) x 10 ¹	1.99 (±0.42) x 10 ²	3.75 (±0.83) x 10 ⁵	1.01 (±0.34) x 10 ⁶	1.12 (±0.25) x 10 ⁶	8.34 (±0.32) x 10 ⁵	2.10 (±0.21) x 10 ⁴
0.6 M NaCl	0	0	8.21 (±0.33) x 10 ¹	5.22 (±0.21) x 10 ³	3.91 (±0.11) x 10 ⁵	1.00 (±0.31) x 10 ⁶	4.00 (±0.60) x 10 ⁶	1.77 (±0.33) x 10 ⁶	6.10 (±0.52) x 10 ⁴
0.7 M KCl	0	0	9.27 (±0.27) x 10 ⁰	6.21 (±0.55) x 10 ²	1.33 (±0.11) x 10 ⁵	5.34 (±0.77) x 10 ⁵	2.7 (±0.35) x 10 ⁶	1.99 (±0.41) x 10 ⁵	9.87 (±0.43) x 10 ³

AY126451), there is still vast scope for genetic improvement of these organisms in the laboratory and their subsequent exploitation in the field.

The present study reports rapid release of protoplasts from *A. oviformis* following treatment with Novozyme 234 cell wall degrading enzyme. This is followed by their subsequent regeneration and self-fusion in the presence of Polyethylene Glycol (molecular weight 6000). The aim of this work is to establish a protocol for protoplast generation and fusion technology. This will facilitate a basis for introducing advantageous genetic alterations and establishing their selection that is otherwise not feasible technically by classical molecular biology techniques.

MATERIALS AND METHODS

Organisms

A. oviformis and *D. parvispora* used in this study were isolated (Nagee *et al.*, 2001) and maintained in Potato Dextrose Agar (Hi Media Private Limited, India) slants amended with tetracycline (35 µg/ml) and stored at 6°C.

Chemicals and enzymes

Novozyme 234 was obtained from Sigma Aldrich (USA). Other chemicals used in this study were of Analar grade. Pre-mixed dehydrated media were used from Hi Media Private Limited, India.

Culture conditions

Fungal spores were harvested from pure cultures of *A. oviformis* and *D. parvispora*, freshly grown on Potato Dextrose solid medium by adding 2 ml of sterile phosphate buffer (pH-5.8). The spore suspension was then vortexed for 30 seconds and 250 µl was immediately dispensed to inoculate 9 different Czapek Dox liquid medium-containing flasks and incubated for 12–28 hours at 28°C on a rotary shaker at 180 revolutions minute⁻¹. Periodic harvesting of mycelia was done from each of the flasks after 12, 14, 16, 18, 20, 22, 24, 26 and 28 hours respectively.

Experimental design and formation of protoplast

Harvested mycelia were collected aseptically by filtration through G1 filter and washed with cold 0.2 molar phosphate buffer pH 5.8. 100mg of these wet mycelia harvested from each of the flasks were then added to tubes divided into 5 sets, each comprising of 6 tubes. The five sets were labeled as [(NH₄)₂SO₄, KCl, NaCl, Sucrose and MgCl₂ respectively and the six tubes of a set were labeled as 0.3M, 0.4M, 0.5M, 0.6M, 0.7M and 0.8M respectively with the name of the set prefixed to it. (Example: KCl 0.3M, KCl 0.4M, KCl 0.5M, KCl 0.6M, KCl 0.7M, KCl, 0.8M). All tubes contained 0.2 molar phosphate buffer, 10 mg ml⁻¹ of Novozyme 234. Each set of tubes contained the respective osmotic stabilizer [(NH₄)₂SO₄, KCl, NaCl, sucrose or MgCl₂] at a concentration of 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 molar concentration respectively. The tubes were incubated at room temperature with gentle

shaking. Protoplast yield were determined using a Neubauer haemocytometer.

Protoplast regeneration

The reaction mixtures were filtered through a G (porosity-2) sintered glass funnel and centrifuged at 900 revolutions minute⁻¹. The pellet suspended in stabilizer solution, was diluted and subsequently plated on to regeneration medium. Regeneration as carried out in Czapek Dox agar medium. Osmotic stabilizers were added as additional supplements along with Triton-X 100, which served to restrict the colony formation. The soft agar overlay (0.5%) on the top had similar composition.

Table 2: Protoplast yield and regeneration frequency of 24 hours old *A. oviformis* mycelia treated with Novozyme 234 for 35 minutes in presence of varying concentrations of different osmotic stabilizers.

Osmotic stabilizer	Protoplast yield/ml of reaction mixture ^a	Regeneration frequency (%) ^b
0.4 M (NH ₄) ₂ SO ₄	1.12 (±0.25) x 10 ⁶	2.87 ± 0.70
0.6 M NaCl	4.0 (±0.60) x 10 ⁶	8.1 ± 0.55
0.7 M KCl	2.7 (±0.35) x 10 ⁶	4.1 ± 0.40

^a Mean ± S.D. expressed, n = 6

^b Mean ± S.D. expressed, n = 3

Protoplast regeneration

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Protoplast fusion

For self-fusion of the protoplasts, the mycelia protoplast were separated from mycelial debris as described above and washed with 0.7 molar NaCl. 1 x 10⁷ protoplasts were collected through centrifugation and suspended in 1 ml of pre-warmed (30°C) solution comprising of 30% (w/v)

Polyethylene glycol, molecular weight 6000 (Sigma Aldrich, USA) in 0.02 molar CaCl₂. 2H₂O, 0.06 molar glycine and adjusted to pH 7.5 with 0.01 molar NaOH. After incubation for 35 minutes at 30°C, 100 µl of the suspension was observed under a phase contrast microscope and photographed using an automatic Nikon-make microscope camera.

RESULTS

A. oviformis belong to class Deuteromycetes of kingdom Mycota. We have earlier demonstrated their ability to trap live nematodes (Nagee *et al.*, 2001). In recent studies conducted in our laboratory, cryo-preserved spores from *A. oviformis* are shown to have significant potential for affecting biological control of animal nematodes in areas of intense animal activity. Ability to introduce foreign genes within an economically important filamentous fungus such as *A. oviformis* depends upon the availability of a viable protocol for generating protoplasts, which is an important stage in the process of introducing foreign genes within such an organism.

Through the technique of differential display-reverse transcriptase (DD-RT) PCR using nematode-induced and un-induced cultures, we had earlier identified a region of exon-II of the serine protease gene indicating the role of this gene in nematophagous activity of *A. oviformis* and *D. parvispora* (Genbank accession number AY207006 and AY126451 respectively). However, appropriate exploitation of this finding to generate a superior strain requires a gene delivery system in these organisms. To our knowledge, as of today, there are no reports on protocol for release of protoplasts in *A. oviformis* except for a citation for *A. oligospora* (Tunlid *et al.*, 1999).

The lytic enzyme mixture produced by *T. harzianum* and marketed as Novozyme 234 (Sigma Aldrich, USA) is well characterized and widely used for release of protoplasts from filamentous fungi (Hamlyn *et al.*, 1981; Kolar *et al.*, 1985). In this study, its effect on generation of protoplasts from *A. oviformis* and *D. parvispora* is investigated.

In case of *A. oviformis*, protoplast are generated from mycelia harvested at different time points after inoculation of liquid media with fungal

spores. It is observed that no protoplasts are generated from mycelia harvested after 14 hours of inoculation. Thereafter, there is a gradual rise in number of protoplasts formed from mycelia harvested between 14-24 hours with a peak value at 24 hours for all three osmotic stabilizers followed by a decline in the next 4 hours (Table 1).

Walls from cultures in the early and mid-exponential phase of growth are more susceptible to lytic enzymes than those derived from old cultures of a fungus. It is found that protoplast yield from *Aspergillus flavus* is highest when culture in the exponential phase of growth are used. This has earlier been confirmed for other species (Peberdy, 1979). Possibly, 24 hours old mycelia of *A. oviformis* represent an appropriate phase of growth where the mycelial walls are most susceptible to lytic enzymes.

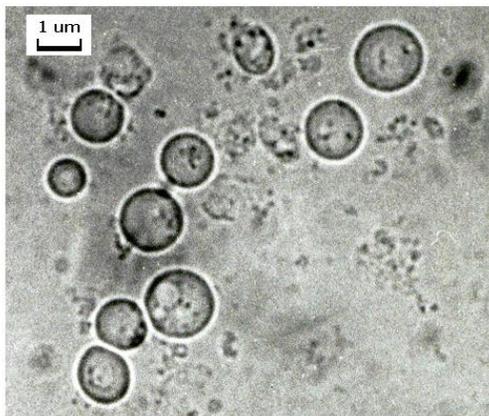


Figure 1: Formation of protoplast in *A. oviformis* following treatment with Novozyme 234.

The protoplasts are released from *A. oviformis* after 35 minutes of incubation with the lytic enzyme (Figure 1). On the other hand, *D. parvispora* is found to be refractive to protoplast release even after incubation of the mycelia (harvested at different time points and ranging from 12 to 28 hours) with the lytic enzyme for more than 120 minutes.

It must be noted that it is desirable to obtain rapid release of protoplasts for different reasons. When studies pertaining to biological enzymes, metabolic pathways are to be undertaken, prolonged incubation of mycelia with lytic enzyme may alter and decrease the resting cell activity. Further, a shorter contact of the lytic enzyme with the cell wall may increase the

regeneration efficiency (Lakshmi and Chandra, 1993).

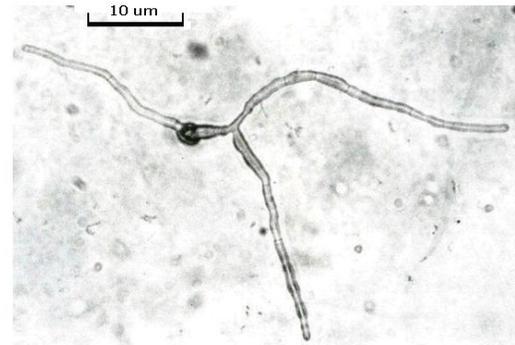


Figure 2: Regeneration of protoplast into mycelial structure in presence of 0.6 molar NaCl as the osmotic stabilizing agent.

While the commercially available lytic enzyme Novozyme 234 is found to release protoplast effectively within 35 minutes of incubation with freshly grown mycelia in *A. oviformis*, it is possible that a combination of lytic enzymes such as Novozyme 234 and Cellulase may further decrease the time required for the release of protoplasts.

Among a range of osmotic stabilizers of varying concentrations used in this study, the best results are obtained at concentrations of 0.4M, 0.6M and 0.7M for $(\text{NH}_4)_2\text{SO}_4$, NaCl and KCl respectively, amongst which, the highest value is obtained from 0.6M NaCl (Table 2). The regeneration frequency of the protoplasts is also found to be highest when the age of the culture prior to treatment with the lytic enzyme is 24 hours (Table 2; Figure 2). One of the reasons might be that 24 hours old mycelia represent a genetically and physiologically mature stage, that is reached gradually from the time of inoculation. On the other hand, after 24 hours th mycelia start getting anucleated which is reflected in their ability to regenerate effectively.

A stabilizer is essential for imparting osmotic protection to the new-born protoplasts after the cell wall is removed by enzymatic treatment. An extensive range of inorganic salts, sugars and sugar alcohols have been successfully used for this purpose. However, inorganic salts have been found to be more effective as osmotic stabilizer for filamentous fungi and sugar and sugar alcohols are more effective for yeasts. The cause and purpose of the use of osmotic stabilizers is not entirely for osmotic protection but is also

related to other factors such as cation-binding to the hyphal walls (Peberdy, 1979).

Regeneration efficacy of the protoplasts of *A. oviformis* does not vary with time after which it was harvested after inoculation. Literature supports the fact that uniform regeneration and reversion is not a general property of all protoplasts (Jarl *et al.*, 2004). This frequency can be variable even for any single species. Studies with *Fusarium culmorum* show that reversion frequency is influenced by carbon source in the regeneration medium and reversion value of 5-82% is obtained varying this component (Peberdy, 1979). One general reason for low reversion frequency of protoplast is however the absence of nucleus. Further, protoplast originating from distal end of the hyphal organization is known to lack the capacity of regeneration, which may account for poor regeneration frequency.

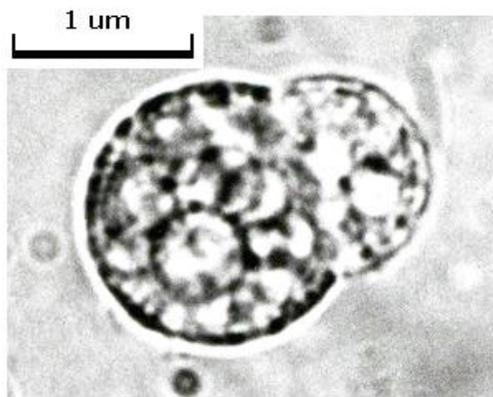


Figure 3: Two protoplasts of *A. oviformis* fusing in the presence of 30% polyethylene glycol (molecular weight 6000) as the fusogenic agent. Granular cytoplasmic material is visible under higher magnification.

During self-fusion of the protoplasts in *A. oviformis*, immediately after the addition of polyethylene glycol (molecular weight 6000), they are seen to adhere with each other followed by visible shrinking which is indicative of the high degree of osmotic pressure within the solution. Stages of fusion of two protoplasts are documented after 35 minutes of incubation of the protoplasts with polyethylene glycol under a phase contrast microscope (Figure 3). However, they could not be specifically selected on a solid medium due to lack of distinctive genetic markers. This is the reason why we could only generate quantitative data on the rate of self-fusion of protoplasts in *A. oviformis*.

The impetus for the use of fusion techniques with fungal protoplasts has originated from identical work on animal and plant cells (McManus *et al.*, 1994; Zimmerman *et al.*, 1981). Spontaneous fusion of protoplasts in fungi has been also reported earlier (Peberdy, 1979). In these studies, it has been demonstrated that the use of polyethylene glycol (molecular weight of 4000 to 6000) enhances the fusion efficiency of the protoplasts by thousand fold. However, the concentration of this chemical constituent (polyethylene glycol) is critical since low concentration fails to cause fusion while high concentration makes the environment hypertonic causing the protoplast to shrink resulting in low frequency of fusion.

The fusion of protoplasts is equally applicable to the question of transmission of cytoplasmic genetic determinants such as mitochondria. The use of petite strains of yeast has provided an interesting system to demonstrate mitochondrial transfer. The protoplast of *Kluveromyces* could be successfully fused to restore respiratory competence to petite micro colony cells and to establish a preliminary genetic analysis of respiratory deficient mutants (Peberdy, 1979). In view of these observations it seems possible to restore respiratory or other similar deficiencies that are caused due to cytoplasmic genetic determinants in economically important fungi such as *A. oviformis*, through the process of self fusion of protoplasts. However, suitable auxotrophic markers must be present in the parent strains such that defined media incorporated with appropriate amino acids can be employed during selection of true fusants.

One of the significant advantages in developing protocol for viable protoplast generation and reversal is in establishing a gene delivery system with the help of electro-transformation technology. With techniques for gene-searching and engineering developing at a rapid pace, it would soon be a necessity to have a proper gene delivery system in such economically important fungus in order to generate genetically improved strains. In this context, the findings of this study are significant and have the potential to contribute in the process of development of a gene delivery system in this important species of nematophagous fungus.

D. parvispora mycelia did not respond to Novozyme 234 since no cell wall degradation is

observed even after extended incubation of the mycelial mass with this enzyme under varying concentrations of different osmotic stabilizers. Therefore, extended study with different lytic enzymes such as chitinase or laminarinase (Gautam *et al.*, 1996) applied singly or in combination need to be explored to identify appropriate treatment conditions for this specific fungus.

CONCLUSION

A protocol for generating viable protoplasts from freshly grown mycelia of the nematophagous fungus *A. oviformis* is reported. In the process, a wide range of osmotic stabilizers are tested to conclude that Sodium Chloride at a concentration of 0.6 molar generated the maximum number of viable protoplasts when 24 hours-old fungal mycelia are treated with the lytic enzyme Novozyme 234 for 35 minutes. Self fusion of *A. oviformis* in presence of Polyethylene Glycol (molecular weight=6000) as well as individual protoplast regeneration into mycelia are demonstrated. The significance of these findings are in the context of strain improvement of *A. oviformis* previously identified as nematode-trapping biological control agent is discussed.

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