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Isolation and Regeneration of Protoplasts from *Laccaria Fraterna* - an Ectomycorrhizal Fungus

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Abstract

An efficient method for high yields of protoplast isolation and regeneration and viability was achieved in *Laccaria fraterna*, an ectomycorrhizal fungus. In this study, we standardized the optimal conditions such as mycelial age, temperature, pH and osmotic stabilizers for the release and regeneration of protoplasts in *L. fraterna*. Maximum number of protoplasts (5.1×10^8) was isolated from 4 day old mycelia suspended in an osmotically stabilized MMC buffer (pH 5.0) with 0.5 M mannitol and 10 mg/mL Novozyme 234. Maximum yields of protoplasts were released from the mycelium using Novozyme 234 after 3 h. Protoplasts exhibited two kinds of regeneration patterns in liquid media and one in solid medium. Almost all the protoplasts were nucleated and viable as observed by acridine orange and fluorescein diacetate staining. The regeneration frequency was as high as 36% under optimal conditions. When colonies from regenerated protoplasts were inoculated with *Eucalyptus globulus*, few plants showed ectomycorrhizal association. Results of this study indicate that this fungus could be potentially used in transformation, protoplast fusion and other genetic studies.

Keywords: Ectomycorrhizal Fungus, *Laccaria fraterna*, Novozyme 234, Protoplasts, Regeneration Patterns.

1. Introduction

Ectomycorrhizal Fungi (EMF) that live in symbiotic association with roots of most terrestrial plants play an important role in forest ecosystems[1]. The ubiquity of mycorrhizal associations and their widespread occurrence in natural ecosystems is definite evidence that the association confers fitness on the host as well as on the fungus. Although physiological and ecological studies of ectomycorrhizal fungal associations have been extensively studied by several researchers, genetic and molecular aspects have not been adequately explored[2].

In recent years, protoplasts have become a valuable experimental tool in physiological, biochemical and genetic manipulation of fungi[3]. Various factors such as age of mycelium, osmotic stabilizer, temperature, pH, digestion time, and lytic enzyme concentration are critical determinants of success in protoplast isolation and regeneration[4]. Optimization of conditions for the isolation, regeneration and fusion of protoplasts in *Saccharomyces cerevisiae* has opened new avenues for genetic and molecular biology studies in several filamentous fungi[5]. Although protocols for the isolation of protoplasts from basidiomycetous fungi have been standardized[6], isolation and regeneration of the protoplasts from EMF has been initiated only since 1986[7-10].

The ectomycorrhizal fungus *Laccaria fraterna* grows in tropical regions and is widespread throughout the world, where *Eucalyptus* and other ectomycorrhizal associated plants are grown[11]. *L. fraterna* has several characteristics typifying a 'weed-like growth' habit frequently dominates disturbed and farm sites having negligible ectomycorrhizal flora soon after trees are introduced. It produces large number of basidiomes and is a major early colonizing component of the mycorrhizal fungal population. Despite their dominance, mycorrhizal association in

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L. fraterna has not been adequately explored. The objectives of the present study were to: 1) standardize the optimal conditions for the isolation and regeneration of protoplasts from *L. fraterna,* 2) study the growth on various carbon sources to evaluate efficient cellulose degrading substrates 3) study the *in vitro* ability of regenerated protoplasts in establishing ectomycorrhizal association with *Eucalyptus globulus* seedlings.

2. Methods and Materials

2.1 Growth Culture

The ectomycorrhizal fungus, *L. fraterna,* was obtained from the culture collection of the Centre for Advanced Studies in Botany, Chennai, India. Fungal cultures were maintained on Modified Melin-Norkrans (MMN) medium[12] at 28 ± 2°C in the dark. Mycelial discs of 0.9 mm were grown on MMN broth in which glucose was replaced by different carbon sources such as cellulose, cellobiose, Carboxy Methyl Cellulose (CMC), lactose, maltose and fructose. Growth of the fungus was measured after 30 days at 28 ± 2°C in the dark. Cultures grown in glucose served as control.

2.2 Protoplast Isolation

L. fraterna mycelia grown in MMN broth for one week were used for protoplast isolation. Mycelia were macerated using a blender for one minute, inoculated into fresh MMN broth and incubated for 4 days at 28 ± 2°C under stationary conditions. One gram fresh weight mycelium of the fungus was harvested, washed once in sterile distilled water, twice with osmotically stabilized MMC solution (0.5 M mannitol, 0.05 M maleic acid, 0.05M $CaCl_2$, pH 5.6) and incubated in an osmotically stabilized MMC buffer with 10 mg/mL Novozyme (Interspex, CA, USA) for 6 h at 30°C under continuous shaking (100 rpm). The effect of five different osmotic stabilizers such as mannitol, sorbitol, sucrose, KCl and $MgSO_4$ at a concentration of 0.5 M were tested. In addition, parameters such as age of mycelium (0–7days), pH of the medium (pH4.0–pH6.0), and incubation temperature (20°C–35°C) were tested. During incubation, samples were collected at regular intervals and protoplasts were separated by filtration through 44 µm mesh to remove hyphal fragments. The filtered solution was centrifuged at 1,500 g for 10 minute, washed once and re-suspended in MMC buffer. Protoplast yield was measured by counting 10 µL of the protoplasts suspension in a Fuchs-Rosenthal haemocytometer.

2.3 Regeneration of Protoplasts

MMN broth supplemented with 0.6 M mannitol was used for regeneration of *L. fraterna* protoplasts. Protoplasts were suspended in 10 mL of MMN broth supplemented with osmotic stabilizer and incubated at 25°C without shaking. After 6–7 days, cells were collected by centrifugation at 1,500 g for 10 minute, re-suspended in 1 mL of osmotically stabilized MMN broth, and spread on the surface of MMN regeneration medium using a Drigalsky loop. Protoplasts were incubated at 25 ± 2°C in dark until colonies developed. Protoplasts plated in the absence of osmotic stabilizer served as control. The number of regenerated colonies was counted using an inverted microscope after 1 week of incubation and results were recorded.

2.4 Characterization of *L. fraterna* Protoplasts

The integrity (viability) of the protoplasts was evaluated by acridine orange and fluorescein diacetate (FDA) staining. One drop of acridine orange (0.1%) was added to the protoplast suspension (1.5 mL), and incubated in dark for 15 minute at room temperature. The suspension was then washed twice with MMC buffer, centrifuged at 100 g for 10 minute, resuspended in same MMC buffer, observed under a light microscope (Carl Leitz photomicroscope) at 200 X[13]. The protoplasts were suspended in FDA (0.01% w/v) for 5 minute at room temperature, and observed under UV microscope. (FDA and acridine orange: excitation 450–490 nm, emission ≥515 nm)[14].

2.5 Establishment of Ectomycorrhizal Association

The in colonies from regenerated protoplasts were observed for their ability to form ectomycorrhizal association with *E. globulus* using the *in vitro* synthesis technique[15]. A test tube measuring 300 × 38 mm in length and diameter was used as an *in vitro* planting chamber. The tube was lined with blotting paper (14 × 22 cm) and 100 cm^3 of vermiculite was added. The tube was sealed with cotton plug and covered with aluminum foil which was removed later and replaced with 100 mL sterile glass beaker. The tubes were sterilized twice for 60 minute with a 48 h interval. Following this, 70 mL of sterile MMN broth was added aseptically to the tube. Seeds of *E. globulus* were surface sterilized with 30% H_2O_2 for 25 minute, washed thrice with sterilized water and germinated on MMN agar

in 100 mL flasks. After a week, a single uncontaminated seedling was placed aseptically in the test tube and two 6 mm diameter agar discs from the margin of the regenerated protoplast colonies were placed close to the stem. The test tubes were then placed in a growth chamber 25 ± 2°C, with 12 h light. Three replicate treatments were maintained with six plants in each group (manipulated and non-manipulated fungus). Mycorrhizal association was evaluated by sectioning, staining and observation of roots after 15, 30, 60 and 90 days of inoculation.

2.6 Statistical Analysis

All the parameters were analyzed using one-way ANOVA and LSD test at (0.05 level) by SPSS 9.0 and means were compared for significance.

3. Results

All the different carbon sources tested in this study supported fungal growth; however, the fungus grew profusely (≤50 mm) in MMN medium amended with cellobiose (Table 1) in addition to glucose. Lactose, fructose and maltose supported a moderate growth (≤25 mm), while CMC, cellulose and starch (≤15 mm) supported poor growth. Cellobiose as the carbon source induces specific cell wall degrading enzyme activities such as cellobiohydralase, cellobiose oxidase and β-D-glucosidase. These enzymes were higher in fungal mycelia grown on cellobiose. These specific enzymatic activities are a prerequisite for the wood–degrading fungi[16] and have been well documented in some litter–decomposing ectomycorrhizal fungi[17].

Although mycorrhizal fungi have numerous applications, very few studies on protoplast isolation and characterization have been reported[18]. One of the objectives of the present study was to determine the influence of various concentrations of Novozyme 234, age of the culture, temperature, pH, osmotic stabilizers, and rotation/minute of shaker on yield of *L. fraterna* protoplasts. Results of our study showed that higher concentration of Novozyme (10 mg/mL) released 5.1×10^8 protoplasts/g fresh weight mycelium of *L. fraterna* in a short digestion time (45 minute to 3 h). Protoplasts were rounded, intact and arranged in a linear fashion inside the hyphal fragments within 30–40 minute of incubation. After 45 minute to 3 h of incubation, the mycelial walls were digested and circular separated protoplasts were observed (Figure 1 a, b, c). Maximum release of protoplasts occurred at 3 h of incubation and a decline in yield was observed thereafter.

Table 1. Effect of carbon sources on growth of *L. fraterna*

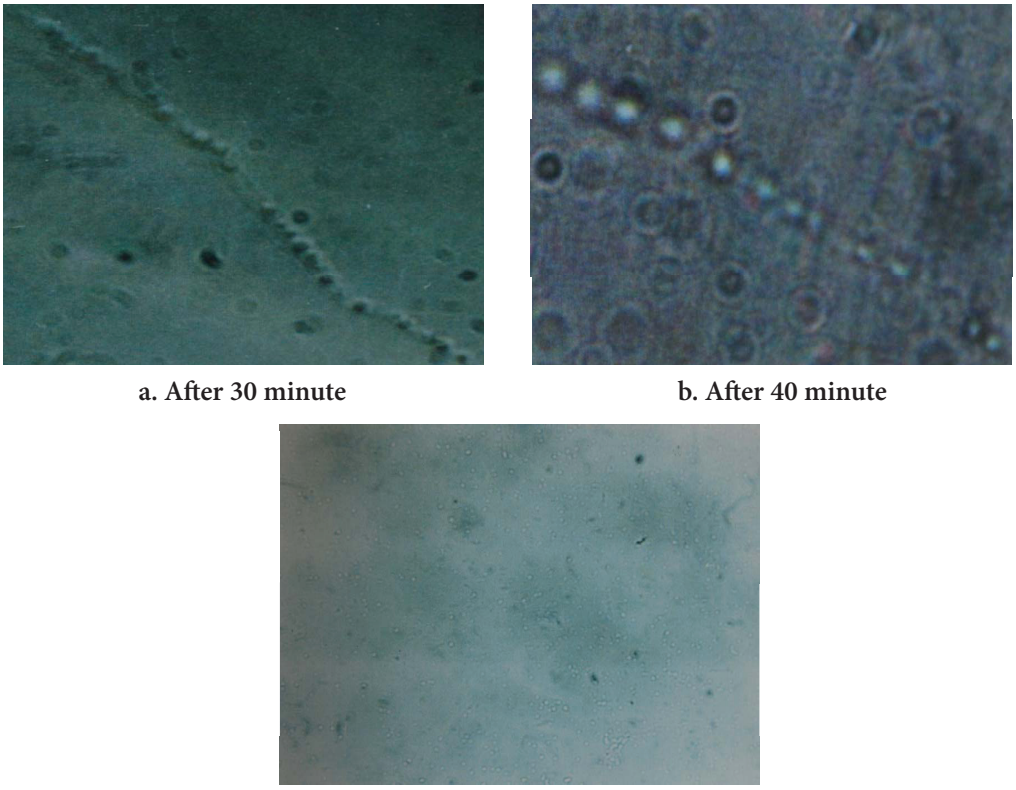
Carbon Sources	Growth
Cellulose	+
Cellobiose	+++
CMC	+
Lactose	++
Fructose	++
Maltose	++
Starch	+

+ – Poor growth (≤15 mm radial growth)
++ – Moderate growth (≤25 mm radial growth)
+++ – Rich growth (≤50 mm radial growth)

Age of the fungal mycelium is known to play an important role in the release of protoplasts. In a study by Peberdy[19], protoplast yield was shown to be depended on the relative proportion of young and old hyphae. While young hyphae were easily digested by enzymes, older hyphae were not, and this affected the yield. Similar results were observed in the present study. Four day old cultures of *L. fraterna* released 5.1×10^8 protoplasts/g fresh weight mycelium, while the protoplast production was lower when hyphae were older (Figure 2).

The temperature most suitable for cell wall digestion was 30°C resulting in the production of 5.1×10^8 protoplasts/g fresh weight mycelium of *L. fraterna*. At 20 and 25°C, there was a steady increase in the protoplast yield. Maximum increase was observed at 30°C, while at 35°C fewer protoplasts were released with a decline in yield (Figure 3). The release of protoplasts was also profoundly influenced by pH of the medium. At optimum pH (5.0) as many as 5.1×10^8 protoplasts/g fresh weight mycelium was released. However, at a pH above and below 5.0, cell lysis and decrease in yield was noted. During an incubation period for 24 h the pH of the mycelial suspension showed a transient change from pH 5.0 to 5.5 then to 4.7. A centrifugal speed of 100 rpm favored more yield of protoplasts than at 75, 125 and 150 rpm and stationary state (data not shown). But Stülten et al.[18] observed that 80 rpm released maximum number of protoplasts from *C. geophilum*.

Osmotic stabilizers such as inorganic salts, sugars and sugar alcohols were used to stabilize and release maximum number of protoplasts from the mycelium. Maximum number of protoplasts was released in mannitol, followed by other osmotic stabilizers such as sucrose,



a. After 30 minute

b. After 40 minute

c. After 1 h

Figure 1. Effect of Novozyme 234 on mycelial fragments of *L. fraterna* (1200 X). 10 mg/mL Novozyme 234 released 5.1×10^8 protoplasts/g fresh weight of 4 day old at 30°C in MMC buffer pH 5.6 within a short digestion time (45 minutes to 3 h).

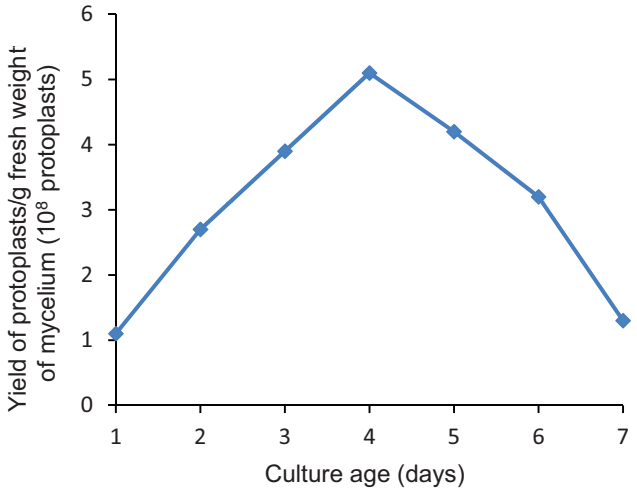


Figure 2. Influence of culture age on release of protoplasts from *L. fraterna*. Four day-old cultures of *L. fraterna* released 5.1×10^8 protoplasts/g fresh weight mycelium, while the protoplast release was lower in the older hyphae (5–7 days).

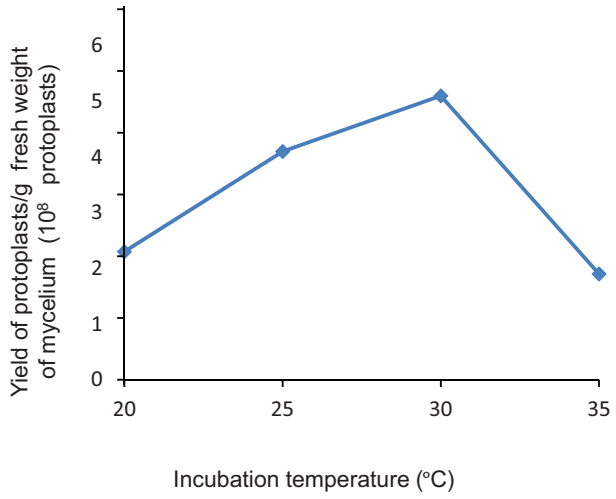


Figure 3. Effect of temperature on the release of protoplasts from *L. fraterna*. There was a steady increase in the protoplast yield starting at 20°C. Maximum numbers of protoplasts released at 30°C, while fewer protoplasts were released at 35°C.

sorbitol, $MgSO_4$ and KCl (Figure 4). The molarity of the osmotic stabilizer was also critical in influencing the yield of protoplasts. Highest yield of protoplast was obtained when mannitol was used at 0.5 M. At this molarity, protoplasts were stable and did not lyse at room temperature for at least 48 h.

In general, frequencies of protoplast regeneration in ectomycorrhizal fungi have been reported to be comparatively low. One of the reasons for this low regeneration rate is attributed to the absence of nuclei in the protoplast[19]. In the present study, almost all protoplasts were nucleated as revealed by acridine orange staining (Figure 5a), and exhibited plasma membrane integrity close to 100% with FDA (Figure 5b). Some protoplasts had more than one nucleus as revealed by acridine orange staining and the regeneration frequency was 36% depending on the culture conditions. More than 60% of protoplasts were nucleated and plasma membrane integrity was close to 100%.

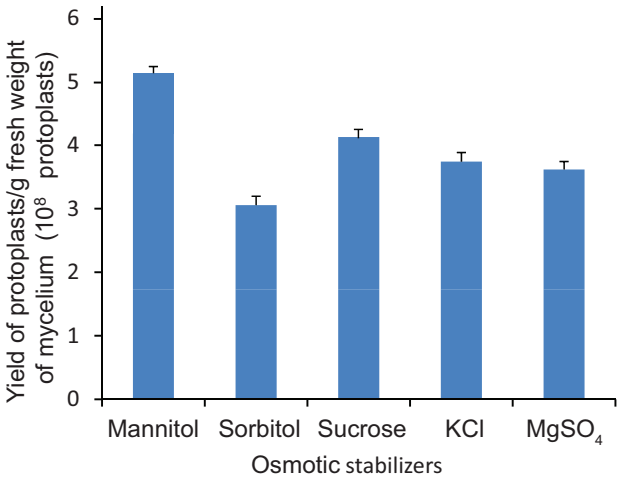
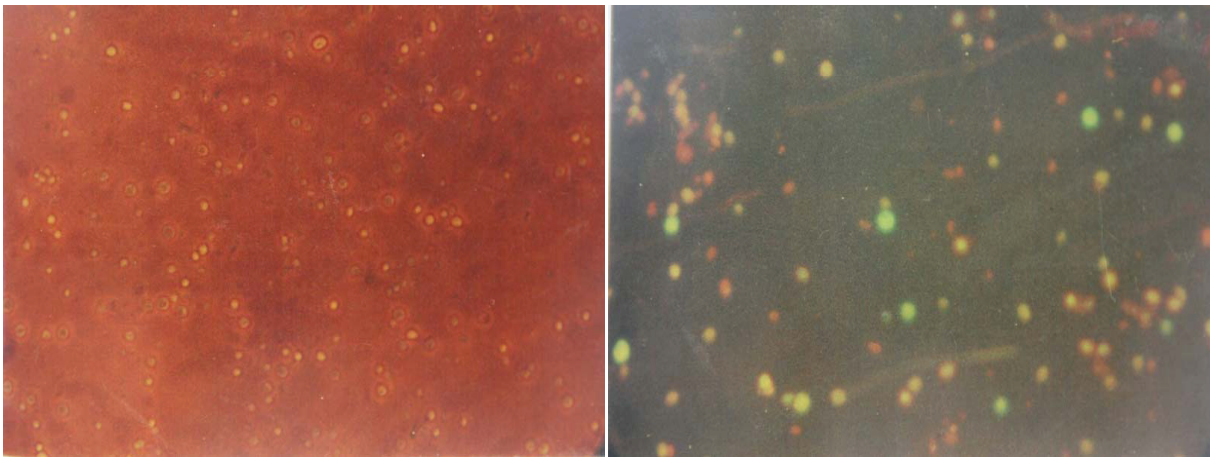


Figure 4. Effect of osmotic stabilizers on protoplasts release from *L. fraterna*. Maximum number of protoplasts was released in Mannitol, followed by other osmotic stabilizers such as Sucrose, Sorbitol, $MgSO_4$ and KCl. Results shown represent the means of three replicates. Standard deviation was ±0.09 to 0.14.

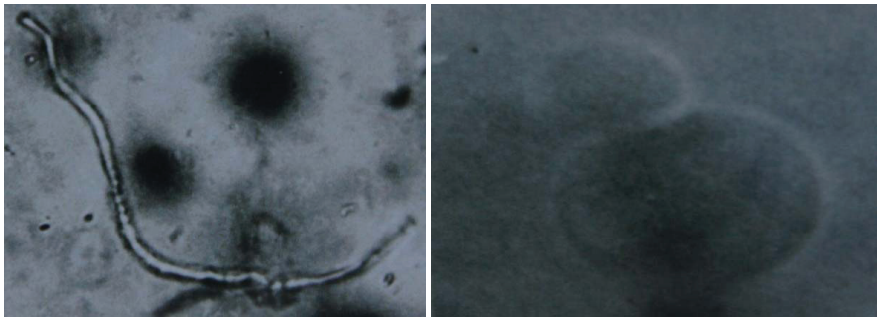
When protoplasts were inoculated into osmotically stabilized media, a proportion of the population reverts to normal cell morphology of the organism called as regeneration[3]. Ectomycorrhizal fungi are known to exhibit four different kinds of regeneration patterns either in liquid medium or in soft agar medium[18]. In the present study, *L. fraterna* produced germ tube like structure (Figure 6a) and yeast like growth (Figure 6b) in liquid media. On soft agar plates pertrophy similar to what Stülten et al.[18] observed on *C. geophilum* was noticed (Figure 6c).

In *in vitro* assays, *E. globulus* seedlings inoculated with colonies from regenerated protoplasts (Figure 7) formed mycorrhizal association and analyzed after 60 days. Only few plants (4 out of 18 plants) showed mycorrhizal association. In these few associations, limited root branching and pinnate mycorrhizas which were orange white in colour were observed. When young, a thin mantle with simple prosenchymatous tissues and a shallow row of hartig net

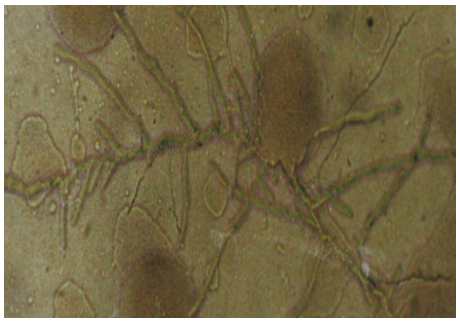


a. Stained with acridine orange **b. Stained with FDA**

Figure 5. Staining of protoplasts from *L. fraterna* (1200 X). Integrity of *L. fraterna* protoplast as evidenced by following staining procedures. a. Acridine orange staining. Almost all protoplasts were nucleated. **b. Staining with FDA.** All the protoplasts exhibited plasma membrane integrity close to 100%.



a. Showing germ tube like structure **b. Showing yeast like structure**



c. Protoplasts showing pertrophy

Figure 6. **Regeneration of protoplasts from *L. fraterna* on MMC medium (1200X). a. Regenerated protoplasts with germ tube like structure.** *L. fraterna* produced germ tube like structure in MMC liquid medium. **b. Regenerated protoplasts showing yeast like structure.** Regenerated *L. fraterna* protoplasts showing yeast like structure in an osmotically stabilized MMC liquid medium. **c. Regenerated protoplasts showing pertrophy.** On soft agar MMN plates, regenerated *L. fraterna* protoplasts showing pertrophy structure.

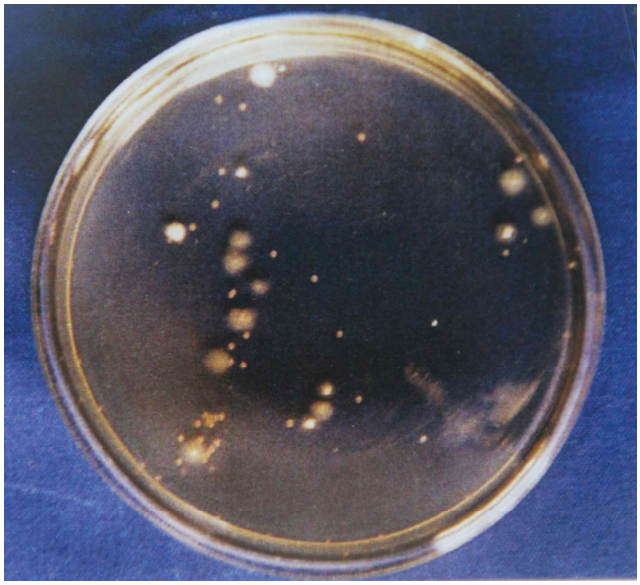


Figure 7. **Colonies from regenerated protoplasts of *L. fraterna*.** Regenerated colonies of *L. fraterna*'s protoplast was counted using an inverted microscope after 1 week of incubation on MMC medium.

were observed. These pinnate mycorrhizas turned brown when old. In general mostly the mycorrhizas showed pinnate branching (Data not shown).

4. Discussion

L. fraterna is an ectomycorrhizal fungus that was introduced from Australia to other countries[20]. It usually dominates in the native hosts, disturbed and farm sites, where the soil is poor in mineral nutrient but rich in organic matter in the form of dead plants[21]. Cellulose is the main polymeric component of these plants and cellobiose is an intermediary product in the degradation of cellulose. In order to digest cellulose, litter–decomposing EMF produce enzymes such as cellobiohydralase, cellobiose oxidase and β-D-glucosidase. Results of the present study showed the ability of *L. fraterna* also produced these enzymes thus dominates the litter. In MMN medium amended with cellobiose *L. fraterna* grew vigorously (≤50 mm). However, other carbon sources supported moderate growth (≤25 mm) and poor growth (≤15 mm).

Cellobiose is known to be a good inducer of these enzymes[16].

Protoplast isolation and characterisization is a powerful tool to study the molecular mechanism in fungi[22]. Although several isolation methods have been standardized in other fungal strains[19], very few studies on characterization of protoplasts in ectomycorrhizal fungi have been conducted[18].

Of the various enzymes, Novozyme 234 is the powerful lytic enzyme used by different researchers throughout the world to isolate protoplasts in fungi. Novozyme 234 released maximum number of protoplasts from the genus *Laccaria,* an ectomycorrhizal basidiomycete[7]. Results of our study showed that higher concentration of Novozyme at 10 mg/mL released 5.1×10^8 protoplasts/g fresh weight mycelium from *L. fraterna* in a short incubation time of 45 minute to a maximum at 3 h. During the initial incubation time of 30–40 minute in lytic enzyme, protoplasts remained intact inside the hyphae. Separated protoplasts were released only after the mycelial cell wall lysed completely after 45 minute of incubation to a maximum at 3 h. Similar findings have been reported by Kropp and Fortin[7] and Barrett et al.[10] where 5 and 10 mg/mL of Novozymes were used to release protoplasts from *L. bicolor* and *L. laccata* respectively. It is known that higher concentration of enzymes release large number of protoplasts in short incubation time[22]. The results of our study corroborates with previous report. An increase in the yield of protoplast was observed up to 3 h after which there was a sharp decline. The reason for this decline could be attributed to the proteolytic activity of Novozyme, which could have destroyed the protoplast membrane[23]. However, the release of protoplasts from cellulolytic fungi is reported to be more rapid than chitinous fungi[24].

Mycelial age is one of the important factors which influence maximum release of the protoplasts[19]. In this study, maximum yield was achieved when inoculum was homogenized, resulting in numerous young hyphae that were susceptible to quick digestion. The yield of protoplast release was proportional to the age of the mycelium. The youngest hyphae (4 days old) released 5.1×10^8 protoplasts/g fresh weight mycelium. Results of this study are in accordance with previous studies by Kropp and Fortin[7]; Barrett et al.[10] who observed similar results in *L. bicolor* and in strains of *L. laccata*. As suggested by Peberdy[19], changes in cell wall composition and thickness as the result of mycelial age could have resulted in the low protoplast yield in older mycelia.

Of the various osmotic stabilizers tested in this study, 0.5 M mannitol proved to be most effective in influencing the protoplast yield. Previous studies have shown that osmotic stabilizers such as inorganic salts, sugars and sugar alcohols have a significant influence on protoplast release. In a study by Barrett et al.[10], 0.5 M mannitol was most effective in releasing maximum number of protoplasts from *L. bicolor*, various strains of *L. laccata, C. geophilum 155, H. cylindrosporum, H. circinans, P. tinctorius, S. luteus VT1616* and *T. terrestris*. In *Amanita muscaria*, 0.5 M sorbitol and 0.35 M KCl were found to be equally effective[25]. While in *H. cylindrosporum* no protoplasts were released in the presence of 0.7 M NaCl or KCl[8]. In *C. geophilum SIV*, 0.7 M KCl and sorbitol produced intact protoplasts, while 0.7 M $MgSO_4$ released large protoplasts[18].

The optimum pH for isolation of protoplasts from *L. fraterna* was 5.0. However at pH 4.0, cell lysis was observed due to the after precipitation of proteins. During a 24 h incubation period, the pH of the mycelial suspension showed a transient change from 5.5 to 5.0 to 4.7. Similar observation has also been reported in *C. geophilum*[18]. In addition to pH, temperature also played an important role in influencing protoplast yield from *L. fraterna*. While maximum protoplasts yield for *L. fraterna* was observed at 30°C up to 3 h, at 20°C and 25°C, fewer protoplasts 3.7 and 2.3×10^8 were observed respectively. A considerable reduction from 5.1×10^8 protoplasts/g fresh weight mycelium to 2.7×10^5 was observed when the temperature increased from 30°C to 35°C. The optimum temperature for maximum release of protoplasts from basidiomyceteous fungi has been reported to range between 30°C–35°C[26].

Media used for protoplast regeneration are known to have a significant impact on basidiomycetous and ascomycetous ectomycorrhizal fungi. Like other ectomycorrhizal basidiomycetous fungi, *L. fraterna* regenerated in MMC buffer. Genetic manipulation studies on microbial protoplasts can be challenging if the organism cannot be readily regenerated. In the present study regeneration of *L. fraterna* protoplast was comparatively high (36%) compared to other basidiomycetous ectomycorrhizal fungi where regeneration frequencies were reportedly low. The regeneration rate was less than 1% for *Hebeloma sp*.,[8], *L. bicolor*[7], *L. laccata* and its strains[9, 10] and 2.3% for *C. geophilum SIV* and *1448*[18], while no regeneration was observed in *C. geophilum 155, P. tinctorius 285* and *S. luteus VT1616*[10]. One of the reasons for this low regeneration rate was attributed to the absence of nuclei

in protoplast which cannot regenerate to form vegetative colonies[19]. In the present study, more than 60% of protoplasts were nucleated as revealed by acridine orange staining. Cells exhibited plasma membrane integrity close to 100% with FDA staining resulted in 'weed-like growth'.

Around 23% of *E. globulus* seedlings inoculated with regenerated *L. fraterna* protoplasts showed mycorrhizal association. This is not surprising and similar results have been reported previously by other researchers. Diìas et al.[27] inoculated the colonies from regenerated protoplasts of *S. granulatus* in several plants of *Pinus caribaea var. hondurensis* only few plants were infected. Similar results were also observed by Marx's[28] among *P. tinctorius* isolates. The reason for this low efficiency could be attributed to the repeated subculture in synthetic medium. When compared to other ectomycorrhizal fungi, *L. fraterna* yielded a regeneration rate of nearly 36% under established optimal conditions.

5. Conclusion

The isolation and regeneration of protoplasts from *L. fraterna* is described here for the first time. The regeneration rate of this fungus is a little higher than the range reported for other ectomycorrhizal fungi[7-10,17]. The regeneration rate is suitable for transformation or protoplast fusion experiments.

6. Acknowledgement

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7. Conflicts of Interest

The authors declare no conflicts of interest in this work.

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