

Strain Improvement of *Pleurotus Species* by Protoplast Fusion

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Abstract

Objective: To improve the strain of *Pleurotus* mushroom by protoplast fusion technique

Methods: The white rot fungus *Pleurotus* species was isolated from fresh fruiting body of Oyster mushroom by spore print technique and was cultured on Potato dextrose agar. The selected strain was identified based on the morphological and cultural characters on Potato dextrose agar. The protoplasts were isolated from both wild and UV mutant strains of *Pleurotus* species. These were examined microscopically and the isolated protoplasts of wild and mutant strains of *Pleurotus* were counted by haemocytometer.

Protoplast fusion between wild and UV mutant *Pleurotus species* was carried out by isolating protoplasts from 4-day-old monokaryotic mycelia cultured on malt extract broth. The mycelia were then agitated at 100rpm for 2h in 1ml osmotic stabilizer (0.6 M MgSO₄·7 H₂O in 0.05 M sodium maleate buffer, pH 5). The freshly prepared protoplasts were then mixed and incubated in 40% PEG (Polyethylene glycol 6000)/0.05 M CaCl₂·2H₂O for 20 minutes at room temperature. All protoplasts were regenerated on Regeneration Medium for 7-12 days. The regenerated colonies were detected and fusants were selected based on their clamp connections on their mycelia.

Results: The fusants were proved to be hybrids of wild and UV mutant strain of *Pleurotus species*. Their mycelia were significantly faster in growth and larger in size than the parental strains. They showed bands common to their parents when laccase was used for isozyme studies.

Conclusion: Fusants possessing clamps was selected and proved to be hybrids of wild and mutant strains of *Pleurotus species* by comparing their mycelial growth, hyphal size and isozyme patterns. Employing the fungal strains, which show synergistic associations in co culture for the development of protoplast fusants with the desirable characters of both the parents, will be a viable option for various industrial and medicinal applications of laccase.

KEY WORDS: *Pleurotus*, protoplast fusion, laccase, strain improvement

Pleurotus mushrooms, commonly known as oyster mushrooms which associated with members of *Apiaceae* family. They grow widely in tropical and subtropical areas, and are easily artificially cultivated. They are healthy foods, low in calories and in fat, rich in protein, chitin, vitamins and minerals. They also contain high amounts of gamma amino butyric acid (GABA) and ornithine. GABA is a non-essential amino acid that functions as a neurotransmitter whereas ornithine is a precursor in the synthesis of arginine. *Pleurotus ostreatus* was able to alleviate the hepatotoxicity induced by chloroform in rats. It was also reported that the extract from *Pleurotus ostreatus* appeared to protect major organs such as the liver, heart and brain of aged rats against oxidative stress.^[1] The oyster mushroom is one of the few known carnivorous mushrooms. Its mycelia can kill and digest nematodes, which are believed to be way in which the mushrooms obtain nitrogen. There are about 40 species of *Pleurotus* mushrooms and they rank second among the important cultivated mushrooms in the world.

Recent studies on various *Pleurotus species* have shown a number of therapeutic activities, such as antitumor, immunomodulatory, antigenotoxic, antioxidant, anti-inflammatory, hypocholesterolaemic, antihypertensive, antiplatelet-aggregating, antihyperglycaemic, antimicrobial and antiviral activities. A water extract of *Pleurotus ostreatus* exhibited the most significant cytotoxicity by inducing apoptosis of human carcinoma cells, when compared to many other mushroom extracts. It has been suggested that the active compounds in the extract were water-soluble proteins or polypeptides^[2]

Mushrooms contain substances that exert direct or indirect antiviral effects as a result of immunostimulatory activity^[3] Inhibitory activity against human immunodeficiency virus (HIV)-1 reverse transcriptase has recently been demonstrated for *Pleurotus sajor-caju* and *Pleurotus pulmonarius* hot water extracts^[4]. Anti-HIV activity was also demonstrated for an ubiquitin-like protein isolated from *Pleurotus ostreatus* fruiting bodies^[5]. Some finding leads to the possible application of white oyster mushroom as a natural antioxidant source^[6].

Strain improvement in the field of microbiology is mainly done to improve the microbes' productivities. The main purpose of strain improvement is to increase productivities, to change unused co-metabolites, to improve the use of carbon and nitrogen sources, to improve morphology of cells in order to separate the cells and its products. Mutagenesis is a process of mutation in strain improvement of microorganism. A treatment to microorganism will cause an improvement in their genotypic and phenotypic performances. Mutation can be random or direct. Mutation may be performed by physical, chemical or combination of physical and chemical method. Ultra violet radiation (UV- light) has been reported as one of the best physical method of strain improvement for better yield performance^[7, 8]. This method has been employed in improving enzyme production in *Aspergillus niger*, *Rhizopus oryzae*^[9] mycelia cell and sporophore production in *Pleurotus florida* and *Pleurotus sajor-caju*^[10].

Protoplast fusion an important tool in strain improvement for bringing genetic recombination and developing hybrid strains in filamentous fungi. Protoplast fusion has been used to combine genes from different organisms to create strains with desired properties. These are the powerful techniques for engineering of microbial strains for desirable industrial properties. Protoplast fusion would continue to be an existing area of research in modern biotechnology^[11]. Strain improvement of this *Pleurotus* species was done by protoplast fusion technique. Protoplast fusion has been used as a method to create mushroom hybrids especially when conventional method cannot be achieved. As conventional hybridization, protoplast fusion can be performed intraspecifically^[12, 13], interspecifically^[14, 15], intergenerically^[16, 17] and even interheterogenerically^[18, 19].

Hence, protoplast fusion is of current interest because of its applications in pure and applied genetics. Protoplast fusion technology is applied for developing interspecific, intraspecific and intra-generic suprahybrids with higher potentiality than their parental strains. Protoplast fusion has proved a feasible method for inter-specific and inter-generic hybridization for strain improvement among edible mushrooms^[20]. Protoplasts have been studied and prepared in large number of edible mushrooms^[21] but their protoplast yields have been found poor as compared to non-basidiomycetous fungi^[22]. Induced protoplast fusion can overcome vegetative incompatibility and produce hybrids with the combined properties of both parents^[23]. Protoplast fusion technology, a viable option for strain improvement in higher basidiomycetes^[24, 25].

1. Materials and Methods

1.1. Sample Collection

The fruiting bodies of Oyster mushroom (*Pleurotus species*) were harvested from Dr.MGR Janaki College of Arts And Science for Women, Chennai.

1.2. Cultivation of fruiting bodies of *Pleurotus species*

The mycelia of *Pleurotus* species on MEA slant were sub cultured onto boiled sorghum seeds. Then incubated at room temperature until they grew over the seeds. The seeds were sprayed on the ready prepared mushroom bed and incubated in a moist area until fruiting bodies occurred. The shape and color of the fruiting bodies including the color of their spore prints were determined and photographed.

1.3. Spore print preparation and single spore isolation

A small piece (about 2.5x2.5 cm) was cut from the fresh mushroom cap. Then it gills were stucked on a sterile lid of a petridish with the help of paraffin or sticky tape. Then it was placed on top of a sterilized beaker lined with 1x1 cm of sterilized filter paper. These spores were then allowed to discharge onto the pieces of paper for 3-10 hours before the lid was replaced with a new sterilized one. Then the beaker with spore prints on pieces of paper had been kept in a refrigerator until single basidiospore isolations were performed.

1.4. Selection of UV mutants by performing UV mutagenesis

The pure culture of *Pleurotus* species on Potato dextrose agar plates were exposed to UV- light for 90mins. The mutants were sub cultured on the Potato dextrose agar with 5% yeast extract agar (YEA), incubated at 25°C for 7days.

1.5. Strain Improvement of *Pleurotus species* by protoplast fusion technique: Protoplast isolation from wild and mutant strains of *Pleurotus*

Four days old monokaryotic mycelia of both wild and mutant strains of *Pleurotus* were transferred onto 50ml of MEB (Malt extract broth) plus 20 glass beads (0.50cm in diameter) in a 250ml Erlenmeyer flask. It was then placed on a shaker with the agitation speed of 200 rpm, at 25°C for 4 days.

Protoplasts were separated from the mycelia using a method modified from Hashiba (1992). This method was done by adding 0.3 g of the mycelia onto 3ml of the sterilized lytic enzyme solution in a test tube. Then it was kept in a shaker at 100 rpm at room temperature for 2 hours. Then the mycelial remnants were removed by filtration through a Millipore membrane filter. The suspended protoplasts were then washed twice with the osmotic stabilizer. These were then finally suspended in 5ml of the osmotic stabilizer. Protoplasts obtained in wild and mutant strains of *Pleurotus* were counted using a haemocytometer.

1.6. Fusion of protoplasts from wild and mutant strains and regeneration of protoplasts

One milliliter of each of the freshly prepared protoplasts of both wild and mutant strains of *Pleurotus* was mixed in a test tube. This was then centrifuged at 3500 rpm for 10 minutes. The supernatant was rinsed off. To this 1 ml of sterilized PEG (40 g PEG in 100ml 0.05 M CaCl₂. 2H₂O) was added and incubated at room temperature for 20 minutes by shaking the tubes every 5 minutes. Another

9ml of the osmotic stabilizer was then added to the tube and centrifuged at 3500 rpm for 10 minutes. Then the supernatant was rinsed off and the "mixed protoplasts" were washed twice with the osmotic stabilizer. Then the protoplasts solution was then diluted to 1×10^4 protoplasts/ml and 0.1ml of the suspension was used for protoplast regeneration by culture it on a plate of Regeneration Medium. The plate was incubated at 25°C until colonies occurred. Then each colony was isolated day by day onto a MEA slant.

1.7. Selection of fusants

The colonies were then screened by examining microscopically for clamp connections on their hyphae. Those colonies with clamps on mycelia were selected as "fusants" and individually sub cultured on MEA slants.

1.8. Proofs of hybrids

Then the fusants were further studied for evidence of hybridization by checking the following characteristics in comparison with their parental strains. The characteristic studies were mycelial growth, hyphal size and isozyme patterns.

1.8.1. Determination of mycelia growth and hyphal size

The mycelia from each fusants and parental strains were sub cultured on MEA plates. Then it was incubated at room temperature for 4 days. The hyphal width and hyphal size were measured microscopically. Then the results were analyzed statistically. (2 Way ANOVA)

1.8.2. Determination of isozyme patterns by electrophoretic analysis

The mycelia of each strain were cultured on malt extract broth, pH 7 at 25°C for 20 days. Then they were filtered with two layers of muslin cloth. These were then washed twice with sterilized distilled water. The mycelia were then picked up into a micro centrifuge tube in which extraction buffer for enzyme extraction was added. The tube was then centrifuged at 3500 rpm at 4°C for 30 minutes. Then the supernatant was kept at -20°C. Electrophoresis analysis was performed. This was performed by mixing 15µl of the supernatant with 5µl of the sample buffer (pH 6.8, 0.6M Tris-HCL, 10% glycerol and 0.025% bromophenol blue) before loading the liquid of each strain into each slit on the acrylamide gel in the electrophoresis set. The gel was then taken up and stained with substrate solution of laccase enzyme.

2. Results

2.1. Isolation and identification of *Pleurotus* species

The white rot fungus *Pleurotus* species was isolated from fresh fruiting body of Oyster mushroom by spore print technique which was shown in Plate 1 and Plate 2 respectively and was cultured on Potato dextrose agar. The selected strain was identified based on the morphological characters, which was tabulated on Table 1 and Cultural characters on Potato Dextrose Agar, which was shown on Plate 3.

Table 1: Identification of *Pleurotus* species

S.NO.	CHARACTERSTICS	RESULTS
1.	Pileus	Oyster cell shaped pileus.
2.	Stripe	5-14cm diameter, grayish white to brownish white stripe.
3.	Cap	Continuous with cap at one side.
4.	Lobes	Well developed.
5.	Margin	Incarved.



Plate1: Fresh fruiting Bodies of *Pleurotus* species



Plate 2: Spore print of Oyster Mushroom



Plate 3: Growth of *Pleurotus* species on Potato Dextrose agar

2.2. Protoplast isolation from wild and mutant strains of *Pleurotus* species:

The protoplasts were isolated from both wild and mutant strains of *Pleurotus* species. These were examined microscopically and the isolated protoplasts of wild and mutant strains of *Pleurotus* were counted by haemocytometer. This was shown on Plates 4 and 5 (Wild strain) and 6 and 7 (Mutant strain) respectively. The results were tabulated in Table 2.

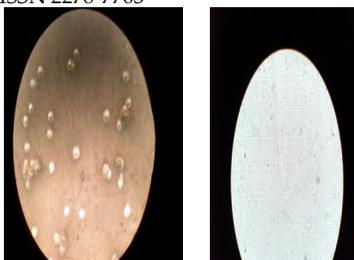


Plate 4: Microscopic Appearance of
Plate 5: Haemocytometer counting
Protolast- Wild strain

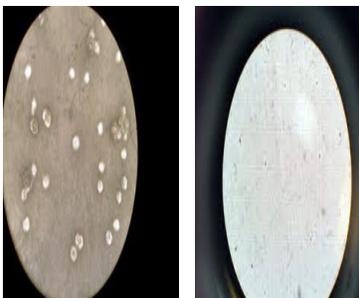


Plate 6: Microscopic Appearance of
Plate 7: Haemocytometer Counting
Protoplast - Mutant strain

Table 2: Enumeration of Protoplasts by Haemocytometer

S. NO.	STRAINS	NO. OF PROTOPLAST
1	Wild	3.26×10^6
2	Mutant	4.56×10^6

2.3. Fusion of protoplast from wild and mutant strains and regeneration of protoplasts:

The isolated protoplasts of both wild and mutant strains of *Pleurotus* were fused by PEG and this was examined microscopically and shown in Plate 8. The fused protoplasts were grown in Regeneration medium for 4 days and after incubation small, round, mycelial growth were observed, which was shown in Plate 9.



Plate 8: Microscopic Appearance of fused Protoplast from Wild and Mutant strains of *Pleurotus*



Plate 9: Small, Round, Mycelial growth of fused Protoplast on Regeneration Medium

2.4. Selection of fusant

The colonies from Regeneration medium were screened microscopically and clamp connections between hyphae were observed. The clamps are shown in Plate 10.



Plate 10: Microscopic Appearance of Clamp Connections of Fusants

2.5. Proof of hybrids

The fusants were sub cultured on MEA for 4 days and colonies were observed. The characteristics of mycelial growth, hyphal size and laccase isozyme patterns were determined and compared with parental strains.

2.6. Determination of mycelia growth and hyphal size

The hyphae width and hyphal size of fusants were measured microscopically and the results were analyzed statistically with their parental strains. The results were shown on Table 3 and Plates 11- 16 respectively. Mycelial growth and hyphal size of fusants were significantly different from those of the parental strains which are relevant to the theories that fusants which are dikaryotic grow faster and have larger hyphae than the monokaryotic parental strains.

Table 3: Characteristics features of Fusant and Parental strain (Wild/ Mutant) of *Pleurotus species*

S. No	STRAIN	DIAMETER OF COLONY (cm)	HYPHAL WIDTH (µm)
1.	Parental wild strain	4.65	2.76
2.	Parental mutant strain	3.24	2.43
3.	Fusant	5.32	3.12

The means were significantly different ($\alpha = 0.05$)

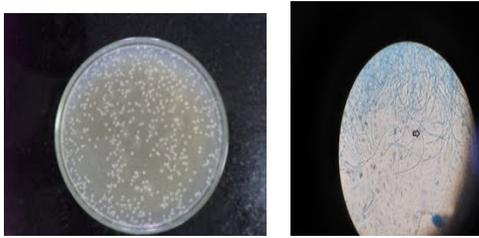


Plate 11 & 12: Mycelial growth and hyphae of Parental Wild strain

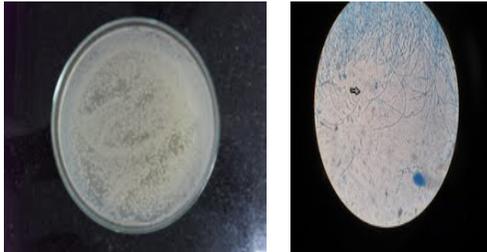


Plate 13 & 14: Mycelial growth and hyphae of Parental Mutant strain

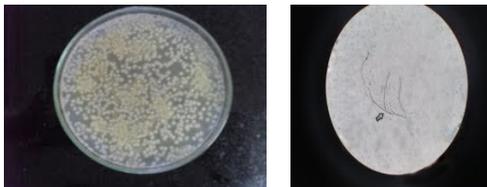


Plate 15 & 16: Mycelial growth and hyphae of Fusant

2.7. Determination of isozyme patterns by electrophoretic analysis

The mycelia of each strain were cultured on Malt extract broth. The samples were then loaded in acrylamide gel and electrophoresis was performed. As a result, different bands were observed. Enzyme electrophoretic pattern was showed in the Plate 17.



Plate 17: Laccase isozyme pattern for *Pleurotus species*

- L1 – Laccase Marker (96 Kb)
- L2- *Pleurotus* wild Strain
- L3- *Pleurotus* Fusant
- L4- *Pleurotus* Mutant strain

10. Discussion

Oyster (*Pleurotus* species) mushrooms are commonly cultivated in Tamilnadu, India, as their cultivation technique is simple and as they can be grown under a wide range of temperatures (25~35°C). Strain improvement in mushrooms is carried out by induced mutations through UV irradiation or chemical mutagens. Protoplast fusion is yet to be developed as a successful strain improvement technique in mushrooms^[26,27]. Development of improved strains for commercial cultivation of edible fungi has been undertaken seriously by the bottom mushroom industry; the production of somatic hybrid sporophores through PEG-mediated protoplast fusion between *Calocybe indica* var. APK2 and *Pleurotus florida*. Significant increase in bio-efficiency and γ -linoleic acid content in these hybrid lines indicated quantitative as well as qualitative improvement of the newly developed somatic hybrids^[28,29]. In the present study, the amounts of protoplast obtained in *Pleurotus* species (Wild) and *Pleurotus* species (Mutant) were 3.26×10^6 and 4.56×10^6 protoplasts/ml, respectively. The results obtained agree to some extent with those published by Pannee Dhitaphichit and Chaninan Pornsuriya (2005)^[30]. Production of protoplast, however, varies with the factors used in the isolation process e.g. species and age of fungal mycelia type and condition of the lytic enzyme and of the osmotic stabilizer^[31].

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