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A study on strain improvement of *Fomitopsis feei* by protoplast fusion technology

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Abstract

Protoplast fusion technology was used for the formation of improved strain from a brown rot fungus, *Fomitopsis feei* and a white rot fungus, *Pycnoporous* sps. for the enhanced production of exopolysaccharide. Self-fusion of *Fomitopsis feei* and intergeneric hybridization of *Fomitopsis feei* with *Pycnoporus* sp. were researched. A combination of chitinase and lysing enzymes were used for the release of protoplasts. Fusion was successful with the formation of visible stable morphological regenerated colonies after several subcultures. Mycelial growth and hyphal size of wild species and fusants were significantly different from those of the parental strains. Although, Self and intergeneric protoplast fusants were produced successfully but were not best producers of exopolysaccharide compared to wild fungi. Even though, strain improvement towards highest production of exopolysaccharide is not successful, this study shows the possibility of fusion between these two mushrooms and could be useful in other research areas.

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Introduction

Mushrooms comprise an extremely abundant and diverse world of fungi. Mushrooms are being evaluated for their nutritional value and acceptability as well as for their pharmacological properties. They make up a vast and yet largely untapped source of powerful new pharmaceutical products. Polypore fungi are the major source of biologically active natural products among the species of the diverse fungal phylum Basidomycota. Very little research was done on brown rot fungi for biologically active compounds compared to white rot fungi. Some of these brown rot fungi are inedible but are having potential importance in medicinal use. Hence, research on brown rot fungi encourages people to realize and appreciate the value of biologically active compounds from them.

Fomitopsis feei, is an inedible brown rot fungus belongs to polyporales. Its growth pattern, molecular identification and biomolecule analysis was researched and published (Hima Bindu and Singara Charya, 2017). During our screening for exopolysaccharide production by mushrooms from Warangal district, Telangana State, India, *Fomitopsis feei* showed good result (Unpublished). Composition of culture medium and other environmental factors were optimized during physiological studies for more production of exopolysaccharides (Unpublished).

In order to further improve its exopolysaccharide production protoplast fusion technology was followed. Protoplast fusion technology is extensively using for the developing of inter generic, intra generic, inter specific and intra specific supra hybrids with higher potentiality than their parental strains. There were reports on interspecific hybridization of protoplasts in white rot fungi (Yoo *et al.,* 2002; Yanai *et al.,* 1995) such as, *Ganoderma lucidum*, *Pleurotus ostreatus* and *Grifolia umbellate*. Hence in the present study mycelial culture of *Fomitopsis feei* was used for strain improvement for the production of polysaccharides with protoplasts of a white rot fungus *Pycnoporus sp*. As self-fusion is also one way to improve the production of exopolysaccharide, self-fusion of *Fomitopsis feei* protoplasts was also researched along with intergeneric hybridization. This is the first report of research on its kind on these two fungi.

Materials and methods

Protoplast isolation

Five mycelial discs (10mm) from actively growing vegetative 7-day-old cultures of both the fungi viz., *Fomitopsis feei* and *Pycnoporous* sps. DIS 343d (DQ327660.1) were inoculated individually in Erlenmeyer flasks (250mL) containing sterile production broth (50mL) and incubated at 28±2^{o}C on a rotary shaker (Remi, India) at 150rpm for 4 days. The mycelia were harvested by centrifugation, filtration and washed twice with sterile water. The harvested mycelia (100mg) were aseptically transferred to 1mL of osmotic stabilizer (0.6mL of potassium chloride in 0.1M phosphate buffer, pH 6.0) and 1mL of enzyme mixture (1 mg/mL in 50mM phosphate buffer, pH 6.1 of chitinase enzyme and 15mg/mL of lysing enzyme in 50mM phosphate buffer, pH 6.1). The mycelia in enzyme mixture were incubated on a rotary shaker (120 rpm) (Remi, India) at room temperature (28±2^{o}C) for 3 hrs. (Lalithakumari, 2000). Both the flasks were checked for every one hour for the release of protoplasts. After the incubation, the mycelia remnants of both mushrooms were removed by filtration through layers of sterile filter papers and the suspended protoplasts were precipitated at 2000rpm for 20 minutes by centrifugation. The pellet collected were washed twice with osmotic stabilizer and then suspended in the osmotic stabilizer.

Protoplast fusion

Protoplasts were fused by following the method of Stasz *et al.* (1988) in the presence of polyethylene glycol (PEG, mol. wt. 6000, 30%) and $CaCl_2$ (0.05M). To the purified protoplasts of the two test organisms in the osmotic stabilizer,

an equal volume of PEG mixture was added and the mixture was incubated at 28±2°C for 10min. for fusion to take place.

The fusion process was examined under the microscope by taking 10µL aliquot of fusion mixture. Protoplasts from the same strain of *Fomitopsis feei* were also fused for the observation of self-fusion.

Regeneration

0.1mL of fused protoplast were inoculated on Minimal Regeneration Medium (MRM) and incubated at 3-4 days at 28±2°C. Each colony was isolated day by day and sub cultured onto a Malt extract agar slants.

Results and discussion

The process of protoplast formation from mycelium is a means for the production of mononucleotide cells. Protoplast fusion is important in biochemical and genetic engineering studies, but many details regarding its mechanism are not known. Membrane fluidity may affect the rate of protoplast fusion.

Protoplast fusion is normally carried out by selecting parent strains then by preparing, fusing and regenerating protoplasts, by identifying the fusants. Intergeneric and self-fusions were showed in Fig. 1 and Fig. 2 respectively.

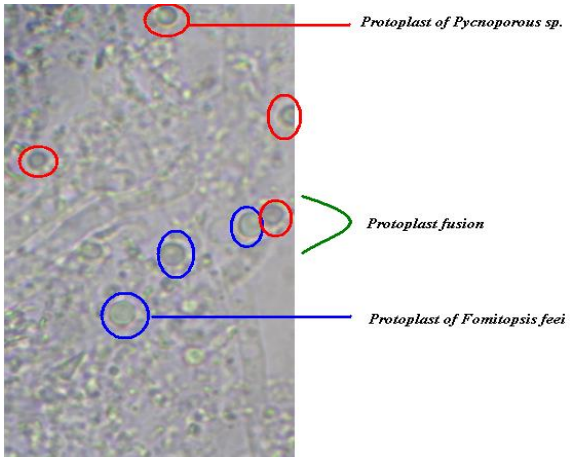

Fig. 1. Intergeneric fusion of protoplasts of *F. feei* and *Pycnoporous* sp.

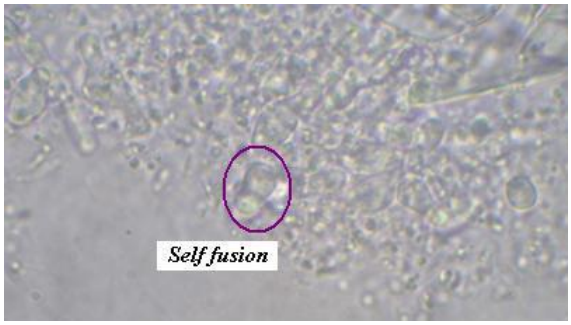

Fig. 2. Self-fusion of protoplasts of *Fomitopsis feei.*

The number of colonies grown on MRM after protoplast fusion of *Fomitopsis feei* and *Pycnoporus* sp. and self-fusion of *Fomitopsis feei* were 14 and 9 colonies respectively (Fig. 3 and Fig. 4). Regenerated colonies became visible morphologically after 3 to 4 days of incubation at 28°C. Similar observations have been made that *Pleurotus florida* and *Pleurotus sajor-caju* required 3 and 4 days respectively and 30°C for regeneration (Zhao and Chang, 1993). The germinating protoplasts developed into filamentous mycelia and exhibited small variation in mycelial morphology from the parent (e.g., in the color of the hyphae, growth speed, and growth of aerial hyphae). There are reports on the fusants exhibiting novel nutrient and biochemical characteristics even though they resembled any one or both the parents morphologically (Zhao and Chang, 1996). This study provided a solution to produce stable fertile mushroom hybrid, which is derived from more than one species.

Fig. 3. Regeneration of intergeneric fusants.

Fig. 4. Regeneration of self fusants of *F. feei.*

The results of many researchers had proved that using the enzyme combination was better than using single enzyme on protoplasts preparation of fungus (Gallmetzer *et al.,* 1999; Sun *et al.,* 2001; Xu *et al.,* 2006). The mixture of mycolytic enzymes, i.e. commercial cellulase, crude chitinase and pectinase, KCl (0.6M) as osmotic stabilizer, pH 6 of the phosphate buffer and an incubation time of 3 hours resulted in the maximum release of protoplasts from 3-day-old mycelia of *P. florida* ($5.3\sim5.75\times10^7$ protoplasts/g) and *P. pulmonarius* ($5.6\text{-}6\times10^7$ protoplasts/g) (Eyini *et al.,* 2006). A minimum incubation time of 2-3 hr. was required by *Volvariella volvaceae* (Mukherjee and Sengupta, 1988) but *Lentinus lepideus* exceptionally needed 6 hrs of incubation for maximum release of protoplasts (Kim *et al.,* 2000). PEG induced fusion experiments with *Pleurotus eous* and *Pleurotus flabellatus* produced fusant No.4, which could grow on regeneration minimal medium and minimal medium (Parani and Eyini, 2010).

Hence, in the present study combination of chitinase and lysing enzymes were used for the release of protoplasts. As fused protoplasts were incubated on MRM (Basal Medium), more rapidly growing sectors arose. Mycelial growth and hyphal size of wild species and fusants were significantly different from those of the parental strains (Fig. 5 and Fig. 6) which are relevant to the theories that fusants which are dikaryontic (n+n) grow faster (Toyomatsu *et al.,* 1986) and have larger hyphae (Abe *et al.,* 1982) than the monokaryotic parental strains.

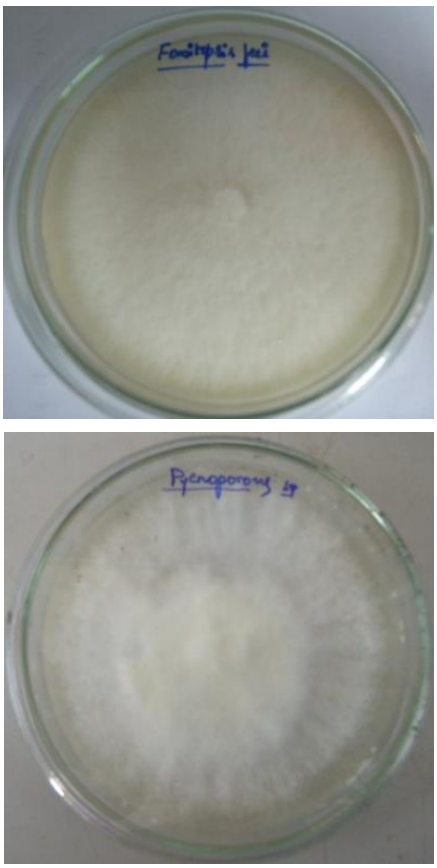

Fig. 5. Growth pattern of wild fungi on MEA agar plates.

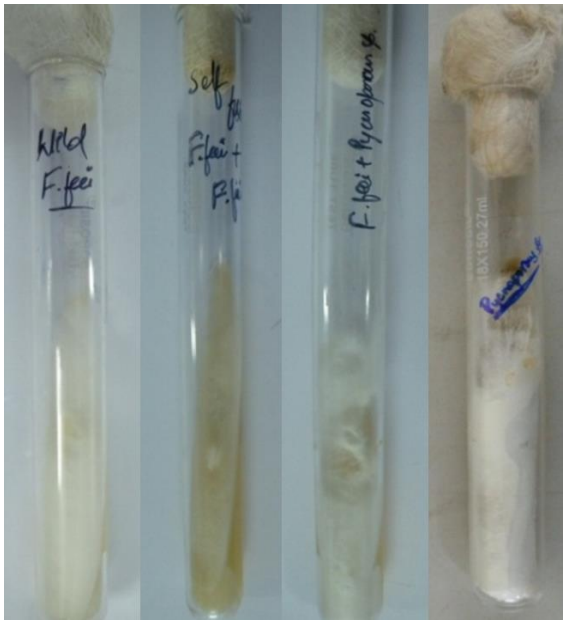

Fig. 6. Morphological growth pattern of wild fungi and fusants in MEA agar slants.

To ascertain whether fusant Ff1 is phenotypically stable, sub cultured it for several generations. The fusant Ff1 was growing vigorously and the mycelial morphology was also same in every generation on the MEA medium. Growth rate of the fusant was measured by weighing the dried matter of the cells harvested from the cultivation in production medium.

The exopolysaccharide production was also measured. There was no apparent difference in exopolysaccharide production (Fig. 7) and growth rates (Fig. 8) between fusant Ff1 and self fusant of *Fomitopsis feei*. The exopolysacchride production of fusant Ff1 reached its maximum after 7th day of fermentation period but the fungal biomass kept increasing even after 14 days.

This indicated that fusant Ff1 has got the exopolysaccharide producing ability from its parental strain. But these fusants were not efficient in exopolysaccharide production when compared to parental strains. Protoplast fusion experiment between white and brown oyster mushrooms was conducted to obtain an oyster mushroom strain showing high productivity and long storage life but FS1 did not show better storage life compared to its parents (Ira and Agus, 2010).

High enzyme concentrations (Lysing enzyme, 30mg/mL) resulted in the release of a larger number of protoplasts in a shorter digestion period in the previous report (Ishikawa *et al.,* 2010). These findings were in agreement with those reported by Lalithakumari (2000) about the effects of lytic enzyme concentration on the number of protoplasts produced. The combination of enzymes required for complete release of protoplasts was examined.

In this study, only debris of cells was observed in the control sample which is without enzyme. Similarly, only lysing enzyme was not able to isolate the protoplast as no protoplast was observed. In addition, efficacy for protoplast isolation was also very low, when only chitinase is used, as compared to when both chitinase and lysing enzymes were used. The yield of protoplast was much higher when both chitinase and lysing enzymes were used, than the yield obtained from the solution containing only individual enzymes. In this study all the isolated protoplasts were spherical in shape and were clearly observed.

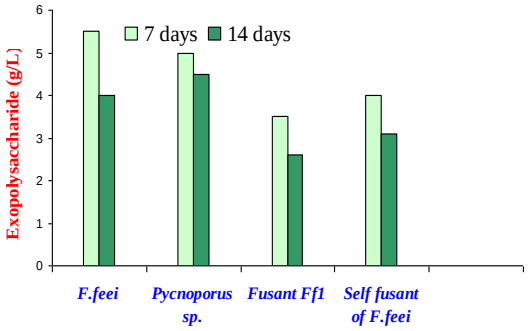

Fig. 7. Comparision of exopolysaccharide production between wild fungi and fusants.

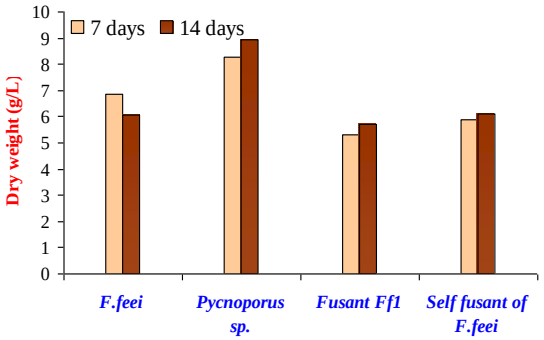

Fig. 8. Comparison of biomass production between wild fungi and fusants.

Conclusion

Strain improvement studies for enhanced exopolysaccharide production showed that mutants were not effective producers compared to wild fungus. Self and intergeneric protoplast fusants were produced successfully but were not good producers of exopolysaccharides than wild fungus. Even though the fusants are not potential for exopolysaccharide production but intergeneric hybrids formation was successful so that other different activities from these hybrids can be researched.

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