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Bacillus subtilis

Antifungal Activity of
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Azo Dye Biodegradation by
Azoreductase from *Bacillus megaterium*

Anti-hyperlipidemic Effects of
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Abstracts



International Conference on Implications of Biotechnology on Biodiversity and its Conservation

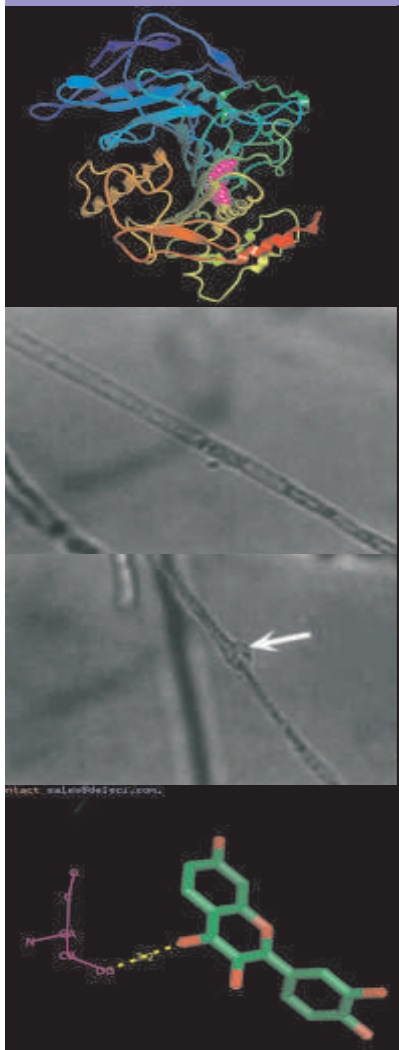
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Leptospira sp. OmPL1 Structural Prediction

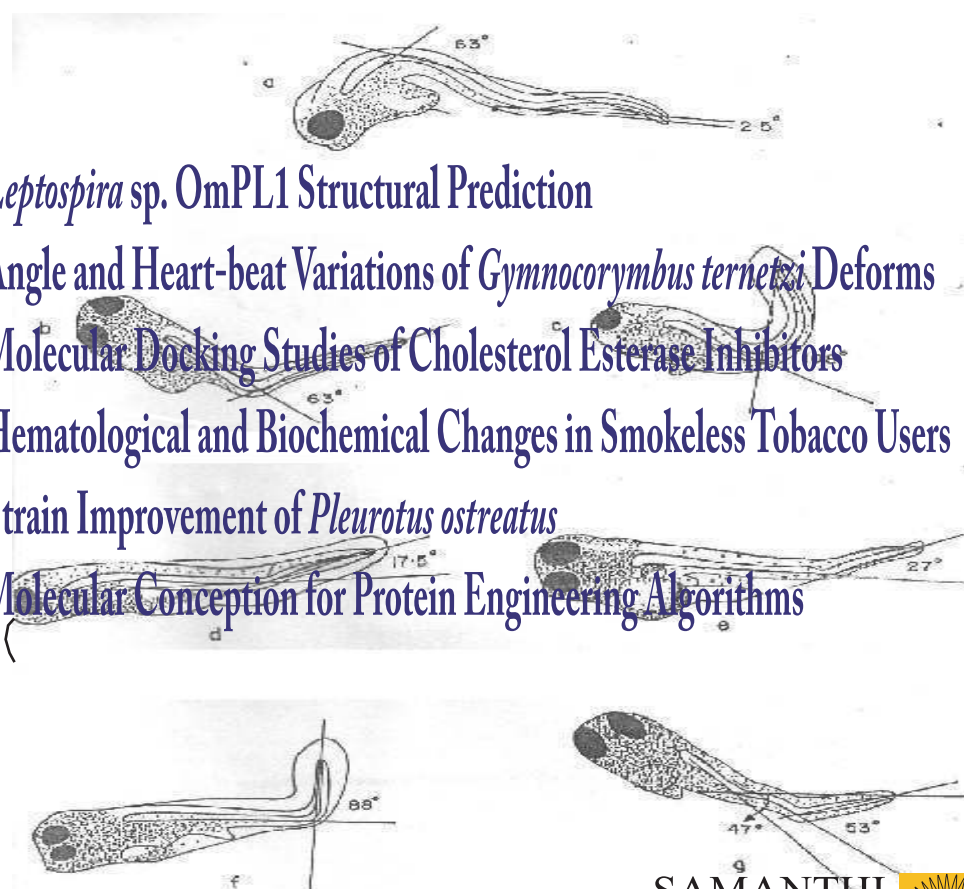
Angle and Heart-beat Variations of *Gymnocorymbus ternetzi* Deforms

Molecular Docking Studies of Cholesterol Esterase Inhibitors

Hematological and Biochemical Changes in Smokeless Tobacco Users

Strain Improvement of *Pleurotus ostreatus*

Molecular Conception for Protein Engineering Algorithms



Strain Improvement in Lignocellulolytic Mutants of *Pleurotus ostreatus* by Protoplast Fusion

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Abstract

Protoplast fusion technology, a viable option for strain improvement in higher basidiomycetes was applied for further and stable improvement in the lignolytic activities of a white-rot fungus *Pleurotus ostreatus*. The two mono-caryotic parent strains of *P. ostreatus* – POM₁ (U.V.irradiated mutant) and POM₂ – (X-ray irradiated mutant) were isolated and used for protoplast isolation and fusion. The amounts of protoplasts obtained in POM₁ and POM₂ were 4.59 x 10⁶ and 4.65 x 10⁶ protoplasts/ml respectively. Among the 357 colonies isolated after protoplast fusion only 3 possessed clamp connections and chosen as fusants (POFU₁, POFU₂ and POFU₃). Of the three fusants POFU₂ showed maximum growth rate on malt extract agar plates incubated at room temperature. In malt extract broth after 14 days of incubation the maximum enzyme activity was recorded (116 U/ml) in the culture filtrate of POFU₂. More than two fold increase in laccase activity was recorded in two fusants- POFU₂ and POFU₃ as compared to the parent strains. In laccase isozyme analysis, fusants showed bands common to either of the parental strains or to both. Further non- parental new bands were observed in the laccase isozyme patterns of POFU₂ and this fact indicates that the isolates were fusants between POM₁ and POM₂.

Keywords: Protoplasts; *P. ostreatus*; Lignolytic enzymes; Mutants

Introduction

Protoplast fusion offers great potential for the improvement of industrially important microorganisms. Protoplast mediated genetic transformation provides great potential for mushroom strain improvement with desired characters. It was proved potentially useful method for the improvement of desired traits in higher basidiomycetes (Gold *et al.*, 1983). Further, it is an important approach to both conventional inter-strain hybridization and to generate hybrids in mushrooms (Peberdy 1991; Bok *et al.* 1994). This technology has been particularly useful for the creation of mutations or for other genetic engineering methods with some fungi or higher organisms in which a few or no plasmids have been identified (Muralidhar *et al.*, 2003). Inter specific hybrids constructed by protoplast fusions in several species of edible mushrooms have been reported and the control of their vegetative and generative phenotypes by nuclear genes has been investigated by culturing tests, isozyme characterization and DNA fingerprinting analysis (Tamai *et al.* 1990; Sunagawa and Ura, 1992; Eguchi and Higaki, 1995). These techniques have been widely used for the enhanced activity in the conversion of cellulose to ethanol (Knowles *et al.* 1987), strain improvement for alcohol fermentation (Fukuda and Kimura, 1991), citric acid producing strains of *Aspergillus niger* (Kirimura *et al.*, 1987), concomitant synthesis of cellulase and citric acid (Kirimura *et al.*, 1990).

To evaluate the lignocellulolytic activities of edible mushrooms, various techniques have been applied: mating among monokaryotic hypha (Fukuda *et al.*, 1995), protoplast fusion between inter and intra species

(Fukuda *et al.* 1995; Yan *et al.* 1996) and gene transformation (Godfrey *et al.*, 1994). *Pleurotus ostreatus* is an edible basidiomycetes of increasing biotechnological interest due to its ability to degrade both wood (Marzullo *et al.*, 1995) and chemicals related to lignin degradation products (Bezalel *et al.* 1997). Furthermore, this fungus produces secondary metabolites having pharmaceutical application (Kurashige *et al.* 1997; Bobek *et al.* 1993) and some proteins of potential industrial use (Shin *et al.* 1997; Wessels 1997). This biotechnological interest in *P.ostreatus* has fueled research on different aspects of its molecular biology.

With this background, the present work was aimed to isolate the protoplast from developed mutants of *P. ostreatus* (POM₁-U.V. irradiated mutant and POM₂-X-ray irradiated mutant) and to carry out the intra-strain protoplast fusion with the objective of investigating the possible enhancement of the extra- cellular lignolytic enzyme (Lignin Peroxidase-LiP and Laccase-Lac) production in the fusant progenies.

Materials and Methods

The *Pleurotus ostreatus* was collected from the surrounding forest area, and then cultured, purified, identified and preserved in Kakatiya University culture collection centre (Ac No. 207). Mutant strains of *Pleurotus ostreatus* were obtained with the objective of enhancing their lignolytic activity. For the development of mutants, actively growing *P. ostreatus* was inoculated in to the central part of the sterile malt agar plates and incubated for 3 days at 37°C. After sufficient growth, the plates were exposed to U.V. rays and X-rays. In the UV irradiation, the

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plates were exposed for 10, 20, 30, 40, 50 and 60 min. at an intensity of $83\mu\text{ Wcm}^{-2}$. During X-ray irradiation, the plates were exposed for 0.4, 0.8, 1.2, 1.6, 2.0 and 2.4 seconds at 40 mA and 70 KV. Unexposed plates were considered as control. After the exposure all the plates including control were further incubated at 37°C for 4 days and approximately 2mm^2 of the fungal mycelium from the spreading edge of each plate was sub-cultured using a separate sterile malt agar slant. All the experiments are conducted in triplicate. As the mutant strains of *P.ostreatus*, POM₁ and POM₂, were proved to be potential in effective biodelignification, both these mutants were used for further and stable strain improvement programme by protoplast fusion technique (Vijaya *et al.* 2006).

Two monokaryotic strains of *P.ostreatus* POM₁ and POM₂ (obtained by mutations) were maintained on malt agar plates at 27°C . (Malt extract - 3 %, NH₄Cl - 0.01 %, agar - 2 %, pH-5.7). Mycelia to be used for the preparation of protoplasts were grown on malt extract agar (MEA) plates. The 5-day cultured mycelial mats were punched with a borer (0.5 cm) and 5 such plugs of mycelia were transferred to Petri dishes containing MEA covered with uncoated cellophane and incubated at 28°C in the dark. The mycelia were removed from the cellophane after 5 days of incubation. This is transferred in to the malt extract broth in a 250 ml Erlenmeyer flask. To this 50 glass beads (0.5 cm diameter) were added and placed on a shaker with the agitation speed of 200 rpm at 23°C for 5 days. The resultant protoplasts were separated from the mycelial fragments by transferring 0.3 g of the mycelia on to 3 ml of the sterilized lytic enzyme solution (2 % cellulose and 1 % Novozyme 234) in test tube and sterilized at 100 rpm at 30°C for 2 hrs (Hashiba, 1992). The residual mycelium was removed by filtration through a Millipore filter and the suspended protoplasts were precipitated at $1000 \times \text{g}$ for 5 min.. The resultant protoplasts were then washed three times with a fusion medium (0.1 μM CaCl₂, 0.5 μM MgCl₂ and 0.5 M mannitol). Protoplasts obtained from POM₁ and POM₂ were counted by using a haemocytometer, after suspending in 0.6 M sucrose.

Protoplast regeneration was performed in malt agar medium, osmotically supported by 0.6 M sucrose. Protoplasts were diluted in 0.8 % (wt / vol) low-melting point agarose supplemented with 0.6M sucrose kept at 35°C to final concentration of 1×10^5 protoplasts/ml. Regenerated mycelia from protoplasts were examined by microscopy ($\times 400$) to select the ones without clamp connection, which are the monokaryotic cultures for protoplast fusion. Protoplasts from each strain were mixed at a ratio of 1:1. One ml of each of the freshly prepared protoplasts of POM₁ and POM₂ was mixed in a test tube and centrifuged at $1000 \times \text{g}$ for 10 min. The supernatant was rinsed off and 1 ml of sterilized PEG (40 % in 0.05 M CaCl₂, 2H₂O) was added to the protoplasts in a test tube and incubated at room temperature for 20 min. by shaking the tube every 5 min. Another 9 ml of the osmotic stabilizer was then added to the tube before centrifugation at $1000 \times \text{g}$ for 10 min. The supernatant was rinsed off and the mixed protoplasts were washed twice with the osmotic stabilizer. The solution was then diluted to 1×10^4 protoplasts/ml and 0.1 ml of suspension was plated on a regeneration medium (20 g malt extract, 20 g glucose, 1 g peptone and 30 g agar in 1000 ml of 0.6 M sucrose) followed by overlaying with the same kind of medium, but with a concentration of agar of only 5 g. The plate was then incubated at 25°C until colonies are developed. Each colony was isolated day by day and transferred on to a malt extract agar slant.

The colonies were screened by examining microscopically for clamp connections on their hyphae. The colonies with clamp connections on mycelia were selected as 'fusants' and were individually sub- cultured on

MEA slants. About three fusants (POFu₁ to POFu₃) were selected based on their clamp connections. Original parents POM₁ and POM₂ were also selected from the mixture and plated on malt extract agar medium. The characteristic studies such as size of the zone, mycelial growth, hyphal size, isozyme patterns and enzyme estimations were carried out. The fusant, non-fusant and parent strains were examined for the production of cellulolytic and lignolytic enzymes by growing in malt extract broth and incubation for 7, 14 and 21 days at 37°C . Culture filtrates were obtained by filtration through Whatman No.1 filter paper and filtrates were centrifuged at $10,000 \times \text{g}$ at 4°C . These dialysed culture filtrates were used as enzyme sources.

The recovered fusants, non-fusants and parents were subjected to isozyme analysis for characterization. Poly acrylamide gel was used for the electrophoresis and a method modified from that of Pasteur *et al.* (1998) was followed. The mycelia of each strain were cultured on malt extract broth, pH 7.0 at 25°C for 14 days. 200 mg of each mycelium was harvested by filtering with two layers of white muslin cloth and then washed twice with sterilized distilled water followed by homogenization in an extraction buffer (0.94 % sodium phosphate monobasic, 1.61% sodium phosphate dibasic, 0.015 % dithiothreitol and 19.81 % sodium lauryl sulfate). The homogenate was centrifuged at $12000 \times \text{g}$ at 4°C for 30 min. and the supernatant was collected and used as a sample for electrophoresis. 10 ml of this supernatant was mixed with 5 ml of the sample buffer (pH 6.8, Tris-HCl- 0.6 M, glycerol - 10 % and bromophenol blue - 0.025 %) 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. Laccase bands were stained with 5 mM p-phenylene diamine and photographed.

Results and Discussion

P. ostreatus was selected for the present study among many white-rot fungi based on its positive qualities like production of lignolytic enzymes, non-toxicity, nutritive value, easy growth, medicinal properties etc. With a view to enhance its selective delignifying capacity mutations were induced and two positive mutants (POM₁, POM₂) were developed (Vijaya *et al.* 2005). To further enhance the lignolytic enzyme production abilities of POM₁, POM₂ and its use in fodder technology, protoplast fusion technology was applied.

The amounts of protoplasts obtained from POM₁ and POM₂ were 4.59×10^6 and 4.65×10^6 protoplasts/ml respectively. The number of colonies isolated after protoplast fusion of POM₁ and POM₂ were 357. Among these only three possessed clamps and thus were chosen as fusants and named as POFu₁, POFu₂ and POFu₃ (Figures 1 and 2).

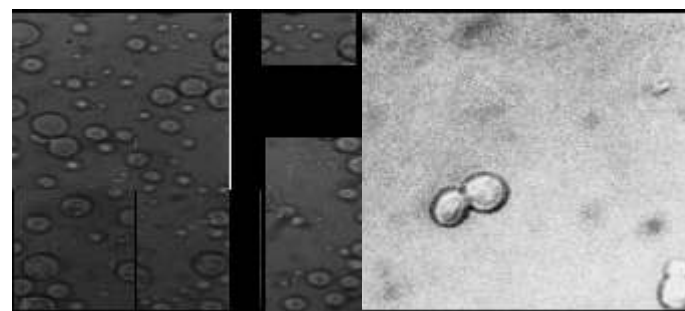


Figure 1. Isolation of protoplasts in *P. ostreatus*. (Released protoplasts observed after 3 hrs of incubation ($\times 400$) (Left). Fusion of protoplasts of *P. ostreatus* after treatment with polyethylene glycol ($\times 400$) (Right).

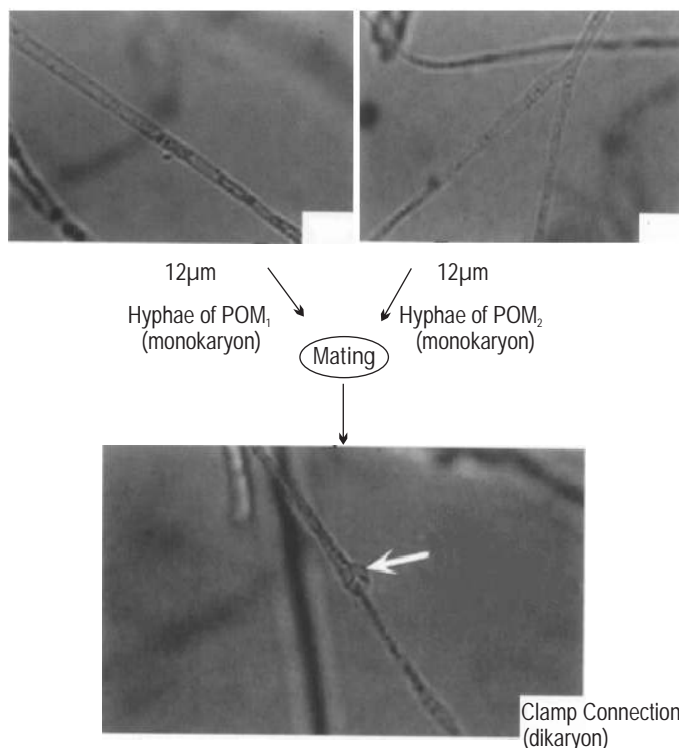


Figure 2. Cross of POM₁ and POM₂

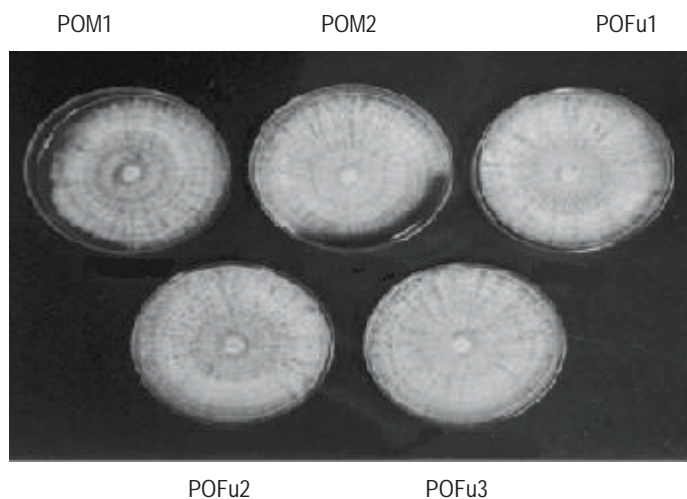


Figure 3. Colony morphology of POM₁, POM₂, POFu₁, POFu₂ and POFu₃ on malt agar plates.

Strain	Diameter of colony (cm)		Degree of aerial mycelium growth	Hyphal width µm	
	5 days	10 days		5 days	10 days
POM ₁	4.2 ± 0.14	8.8 ± 0.12	++	1.52	2.42
POM ₂	4.3 ± 0.07	8.6 ± 0.05	++	1.20	2.40
POFu ₁	4.0 ± 0.12	9.2 ± 0.10	+++	1.10	2.92
POFu ₂	3.8 ± 0.10	9.8 ± 0.11	++++	1.01	3.10
POFu ₃	4.0 ± 0.07	9.4 ± 0.06	+++	1.20	3.00

Table 1. Mycelial growth and hyphal size of fusants and their parental strains after 5 and 10 days of incubation on MEA at 27°C.

Mycelial growth and hyphal size of parents and fusants (Figure.3) were measured by culturing on MEA at 27°C for 7-9 days and the data was presented in Table.1.

The diameter of each colony was determined for at least 10 replications to represent mycelial growth. Hyphal width was measured microscopically as the hyphal size using a calibrated eye piece micro meter for 100 replications. The results were analyzed statistically. The results clearly indicated that the fusants which are dikaryotic grow faster and have larger hyphae than the monokaryotic parental strains. These results were strongly supported by the studies of Toyomatsu and Mori (1987), Abe *et al.* (1982) and Prabhavathi *et al.* (2006). Kitamoto *et al.* (1988) reported that when protoplast isolations were performed in various filamentous fungi, Basidiomycotina including *P. ostreatus* gave the highest production of protoplasts. However, production of protoplast varies with the factors like species, age, lytic enzymes, osmotic stabilizers, incubation time and temperatures (Yamada *et al.* 1983; Peberdy, 1989; Peberdy and Fox, 1993).

Strain	Cellulolytic enzymes		Lignolytic enzymes	
	C _x (REA)	C ₁ (mg/mL)	LiP (U/mL)	Lac (U/mL)
POM ₁	56	18.7	52	56
POM ₂	66	23.2	52	58
POFu ₁	100	22.4	90	100
POFu ₂	100	21.4	101	116
POFu ₃	110	22.0	95	101

Table 2. Production of cellulolytic (C_x and C₁) and lignolytic enzymes [LiP : Lignin Peroxidase; and Lac : Laccase] in malt extract broth by fusants and parents after 14 days of incubations.

To investigate the effect of protoplast fusion on the production of lignolytic enzymes, the fusants and parent strains were inoculated in malt extract broth (MEB) and incubated at 27°C for 14 days and the data obtained was presented in Table. 2.

The results indicate that the lignolytic enzyme activity of all the fusants was increased remarkably (>50%) compared to the parent strains. The maximum enzyme activity was recorded (116 unit/ml/min) in the culture filtrate of POFu₂. About two fold increase in laccase activity was recorded in the fusants (POFu₁, POFu₂ and POFu₃) as compared to the parents. Therefore, fungal protoplasts are an important tool in physiological and genetic research and genetic manipulation can successfully be achieved through the fusion of protoplast in filamentous fungi that lack the capacity for sexual reproduction (Peberdy 1989; Hamari *et al.* 1977).

Isolation, fusion and regeneration of protoplasts have been achieved in the genus *P. ostreatus* (Dhitaphichit and Pornsuriya 2005; Fukuda *et al.*, 2007; Lee *et al.* 2000). However limited attempts were made to improve the strain of *P. ostreatus* to enhance the lignolytic enzyme production. Hence, there is ample scope for strain improvement in *P. ostreatus* utilizing this protoplast fusion technique for enhancing the enzyme productions.

To rule out the possibility that the fusants, products of the fusants arose as a result of contamination and in order to determine whether the genomes of both POM₁ and POM₂ were introduced in to the fusant cells, the

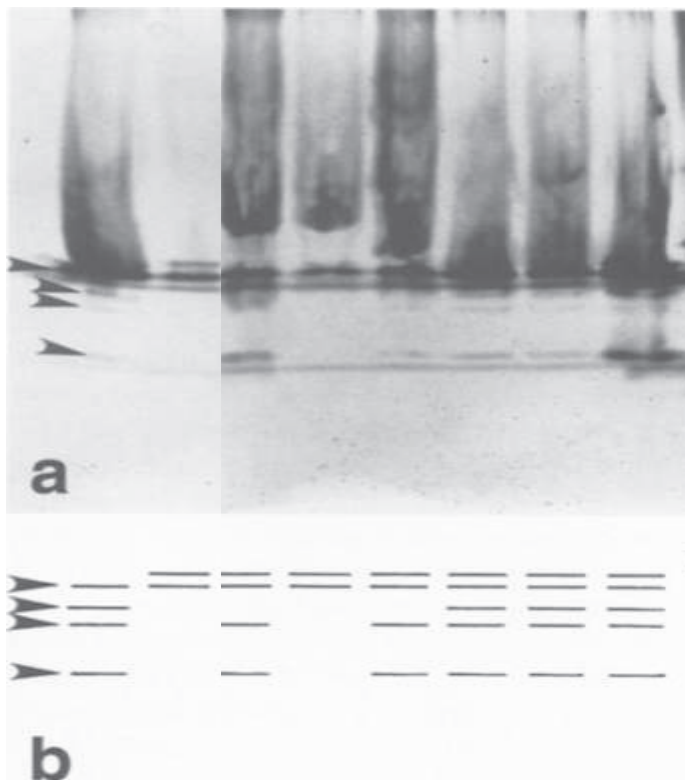


Figure 4. Laccase isozymes of *Pleurotus ostreatus* (fusants POFu1, POFu2, POFu3 and parents POM1 and POM2) (from left side). Arrow-heads indicate bands that also appeared in fusants.

laccase isozyme patterns of mycelial extracts were compared with those of the parental strains. The results were shown in Figure 4.

Differences in the isozyme patterns were observed between the fusants and parental strains. The isozyme patterns of the fusant showed bands common to either of the parental strains or to both. Further more, non-parental, new bands were observed in the laccase isozyme patterns of the fusant as reported in the case of *Penicillium species* (Anne and Peberdy, 1981). These results indicated that the parental genomes may be retained in the fusant cells and also support the occurrence of interaction between the two genomes. Laccase isozyme analysis isolates showed bands that also appear in both POM₁ and POM₂. The isolates showed one band that appear in POM₁ and one or few bands that appear in POM₂ (Figure 4). This fact indicates that the isolates were fusants between POM₁ and POM₂. There were also isolates that showed bands of only one of the fusion parents.

The white rot fungus *P.ostreatus* is an edible basidiomycete with increasing agricultural and biotechnological importance. Genetic manipulations and breeding of this organism are restricted because of the lack of the knowledge about its genomic structure. In general monokaryons are useful for genetic analysis as they are haploid and it is possible to culture in an artificial medium in the laboratory (Miyazaki *et al.*, 2000). Inter specific hybrids constructed by protoplast fusion in several species of edible mushrooms have been reported (Sunagawa *et al.* 1995; Fukuda *et al.* 2007). However, little attention has been made on intra specific fusion. For mushrooms back-crossing is not needed to detect the characters expressed by monokaryons. It is also reported that

monokaryons could be obtained by the preparation and regeneration of protoplasts from dikaryons of edible mushrooms (Ohmasa *et al.* 1987; Peberdy and Fox, 1993).

Previously Das *et al.* (1988) demonstrated the intra strain crossing by protoplast fusion for genetic recombination in *A. niger*. Further, Ogawa *et al.* (1989) achieved intra-specific hybridization in *T. reesei*. Further Prabhavathy *et al.* (2006) used *T. reesei* for intra strain protoplast fusion with the aim of enhancing the extra-cellular CMCase (Carboxy Methyl Cellulase) production. Similar to our studies screening, mutagenesis and protoplast fusion in *A. niger* for the enhancement of extra cellular glucose oxidase (GOD) production was carried by Khattab and Bazaraa (2005). They also noted that a 393.8% improvement in GOD synthesis after applying mutagenesis followed by protoplast fusion in *A. niger*. Pasha *et al.* (2007); Zhao *et al.* (2008) also followed protoplast fusion technology for better utilization of lignocellulosic substrates by fungi. In general it could be noticed that mutagenesis (UV) proved to be an effective technique to enhance enzyme productions. In addition, intra-strain protoplast fusion between higher laccase producing mutants proved effective in achieving superior laccase producing fusants.

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