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Full Length Research Paper

Strain improvement in dye decolourising mutants of *Mucor mucedo* by protoplast fusion

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The amounts of protoplasts obtained in the developed mutants of *M. mucedo* MMM_1 (U.V. irradiated mutant) and MMM_2 (ethyl methyl sulfonate treated mutant) which are very effective decolourisers were 5.23×10^6 and 5.65×10^6 protoplasts/ml respectively. Among the 385 colonies isolated after protoplast fusion only 3 possessed clamp connections and chosen as fusants ($MMFu_1$, $MMFu_2$ and $MMFu_3$). Of the 3 fusants, $MMFu_3$ showed maximum growth rate on potato dextrose agar plates incubated at room temperature. The fusant $MMFu_3$ showed very good increase in the production of three enzymes protease (1.90 U/ml), peroxidase (1100 U/ml) and laccase (200 U/ml) when compared to the two parent strains proving that the higher enzymatic secretions are responsible for the decolourisation activity. In protease isozyme analysis, fusants showed bands common to either of the parental strains or to both. Further non parental new bands were observed in the protease isozyme patterns of $MMFu_3$. This fact indicated that the isolates were fusants between MMM_1 and MMM_2. The fusant $MMFu_3$ showed the maximum decolourisation of crystal violet up to 95% and malachite green up to 84% after 10 days of incubation. The results clearly indicated that the protoplast fusants showed improvement in the decolourisation efficiency in both the cases of crystal violet and malachite green.

Key words: Protoplasts, *Mucor mucedo*, fusants, protease, decolourisation activity.

INTRODUCTION

Fungal biomass has huge capability of treating effluents discharged from various industries. Fungal systems appear to be most appropriate in the treatment of coloured and metallic effluents (Ezeronye and Okerentugba, 1999). Thus, more technically advanced research efforts like protoplast fusion are required for exploiting fungal species and improvement of practical application to propagate the use of fungi for bioremediation of industrial effluents. Protoplast fusion proved potentially useful method for the improvement of desired traits in higher fungi since Gold et al. (1983).

Intraspecific hybridization between *Mucor pusillus* strains, as well as interspecific hybridization between *Mucor pusillus* and *Mucor miehei* was brought about by protoplast fusion. Later, intraspecific prototrophic hybrids of *M. pusillus* developed by protoplast fusion were showed to exhibit distinctly higher productivity of milk clotting protease than the parental strains (Hamamoto et al., 1986). Electrical parameters were optimized for induction of fusion of protoplasts formed from two auxotrophic *Mucor circinelloides* strains (Somogyvari et al., 1996). Spheroplasts from the auxotrophic strains of *Mucor racemosus* were mixed and fused in the presence of PEG and $CaCl_2$ (Lasker and Borgia, 1980). Strains of *Mucor* are currently of growing interest because of their ability to produce industrially important enzymes, e.g., protease (Outtrup and Boyce, 1990) and their excellent applicability in microbial bioconversions. This biotechnological interest in *M. mucedo* 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 *Mucor mucedo* (MMM_1 and MMM_2) and carry out the intrastrain protoplast fusion with the objective of investigating the possible enhancement of the decolourisation activity in the fusant progenies.

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MATERIALS AND METHODS

M. mucedo used in this study was isolated from dye amended soils around textile dye industries on Tenali road at Mangalagiri, 10 km away from Vijayawada, Andhra Pradesh. The developed mutants of *M. mucedo* {MMM_1-U.V. irradiated mutant and MMM_2-EMS (ethyl methyl sulfonate) treated mutant} proved to be very effective decolourisers, both these were used for further and stable strain improvement by protoplast fusion technique (Vijaya et al., 2005).

Protoplast isolation and fusion

Two strains of *M. mucedo* MMM_1 and MMM_2 (obtained by mutations) were maintained on potato dextrose agar plates at 37°C and transferred into the potato dextrose 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 30°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% chitinase and 1% Novozyme 234) in test tube and shaken at 100 rpm at 30°C for 2 h (Hashiba, 1992). The resultant protoplasts were then washed 3 times with a fusion medium (0.1 µM $CaCl_2$, 0.5 µM $MgCl_2$ and 0.5 M mannitol). Protoplasts obtained in MMM_1 and MMM_2 were counted by using a haemocytometer, after suspending in 0.6 M sucrose. Protoplasts from each strain were mixed at a ratio of 1:1. One ml of each of the freshly prepared protoplasts of MMM_1 and MMM_2 was mixed in a test tube and centrifuged at 1000 x g for 10 min.

The supernatant was rinsed off and 1 ml of sterilized PEG (45% in 0.05 M $CaCl_2$, $2H_2O$) 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 x 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 x 10^4 protoplasts/ml and 0.1 ml of suspension was plated on a regeneration medium (200 g potato, 20 g dextrose and 20 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 37°C until colonies developed. Each colony was isolated day by day on to a potato dextrose agar slant.

Selection of fusants and proof of hybridization

The colonies were screened by examining microscopically for clamp connections on their hyphae. The colonies with clamp connections on mycelia were selected as 'fusants'. About 3 fusants ($MMFu_1$, $MMFu_2$ and $MMFu_3$) were selected based on their clamp connections and plated on potato dextrose agar medium. The characteristic studies were mycelial growth, hyphal size, enzyme estimations, isozyme patterns and decolourisation activities.

In vitro production of protease, peroxidase and laccase

The fusant and parent strains were examined for the production of decolourising enzymes by growing in potato dextrose broth and incubation for 10 days at 37°C. Culture filtrates were obtained by filtration through Whatman No.1 filter paper and filtrates were centrifuged at 10,000 x g at 4°C. These dialysed culture filtrates were used as enzyme sources.

Estimation of protease, peroxidase and laccase (phenol oxidase)

Protease enzyme was estimated as per the procedure suggested by Nanniperi et al. (1980). The peroxidase enzyme from the fungal cultures was estimated as per the method suggested by Tein and Kirk (1984). The laccase enzyme from the fungal cultures was estimated as per the method adopted by Lobos et al. (1994).

Protease isozyme pattern determination

The recovered fusants and parents were subjected to isozyme analysis for characterization. Poly acrylamide gel electrophoresis was performed following the method of Pasteur et al. (1998).

Decolourisation assay

Decolourising activity was expressed in terms of percent decolourisation as described by Yatome et al. (1993). The decrease in absorbance was monitored at A_{590} and A_{616} nm for crystal violet and malachite green respectively. Decolourisation was calculated according to the following formula:

$$D\% = 100 \times \frac{(A_{ini} - A_{fin})}{A_{ini}}$$

Where D = decolourisation, A_{ini} =initial absorbance, and A_{fin} = final absorbance of dye, after incubation time.

RESULTS

M. mucedo was selected for the present study among many soil fungi based on dye agar plate method where it showed very good decolourisation capability. With a view to enhance its selective decolourisation capacity mutations were induced and two positive mutants (MMM_1, MMM_2) were developed. To further enhance the decolourisation abilities of MMM_1, MMM_2 and their use in biotreatment technology, protoplast fusion technology was applied.

The amount of protoplasts isolated and selection of fusants

The amounts of protoplasts obtained in MMM_1 and MMM_2 were 5.23 x 10^6 and 5.65 x 10^6 protoplasts/ml respectively. The number of colonies isolated after protoplast fusion of MMM_1 and MMM_2 were 385. Among these only 3 of them possessed clamps and thus were chosen as fusants and named as $MMFu_1$, $MMFu_2$ and $MMFu_3$ (Figure 1).

Mycelial growth and hyphal size

Mycelial growth and hyphal size of parents and fusants were measured by culturing on PDA at 37°C for 7 - 9 days and the data is presented in Table 1. The results clearly indicated that the fusants which are dikaryotic grow faster and have larger hyphae than the monokaryotic

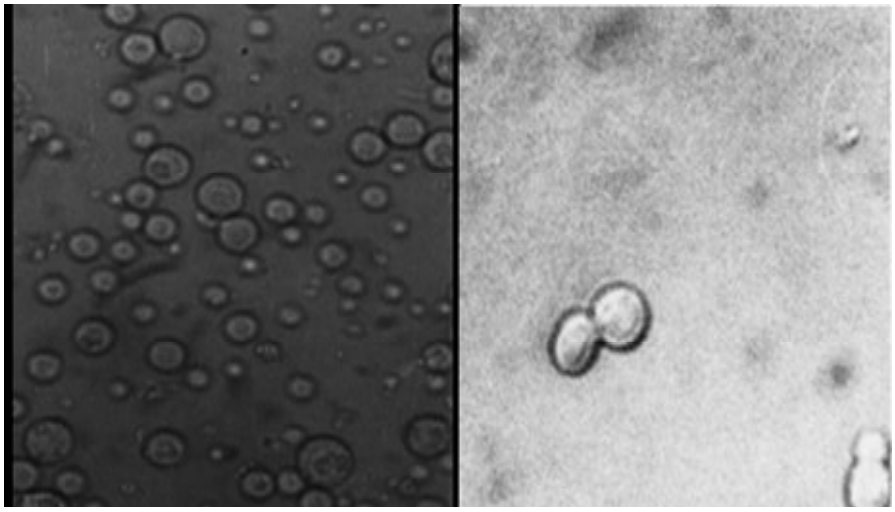

Figure 1. Isolation of protoplasts in *M. mucedo*. Left: Released protoplasts observed after 3 h of incubation (×400). Right: Fusion of protoplasts of *M. mucedo* after treatment with polyethylene glycol (×400).

Table 1. Mycelial growth and hyphal size of fusants and their parental strains after 5 and 10 days of incubation on PDA at 37°C.

Strain	Diameter of colony (cm)		Degree of aerial mycelium growth
	5 days	10 days	
MMM_1	5.2 ± 0.14	8.8 ± 0.12	+ +
MMM_2	5.3 ± 0.07	8.6 ± 0.05	+ +
$MMFu_1$	5.0 ± 0.12	9.2 ± 0.10	+ + +
$MMFu_2$	4.8 ± 0.10	9.8 ± 0.11	+ + + +
$MMFu_3$	5.0 ± 0.07	9.4 ± 0.06	+ + +

+ + = Moderate growth, + + + = good growth, and + + + + = excellent growth.

Table 2. Enzyme production by the parents and fusants of *Mucor mucedo* after 10 days of growth.

Fungal strain	Protease (U/ml)	Peroxidase (U/ml)	Laccase (U/ml)
MMM_1	1.46	900	100
MMM_2	1.48	1000	100
$MMFu_1$	1.40	900	200
$MMFu_2$	1.42	1000	100
$MMFu_3$	1.90	1100	200

parental strains. These results were strongly supported by the studies of Abe et al. (1982) and Prabhavathi et al. (2006).

In vitro production of protease, peroxidase and laccase by fusants and parents

To investigate the effect of protoplast fusion on the production of decolourising enzymes, the fusants and parent strains were inoculated onto PDA and incubated at 37°C for 10 days and the obtained data is presented in Table 2. The results indicated that the enzyme activity of the fusant $MMFu_3$ was increased remarkably (>50%) when compared to parent strains. The fusant $MMFu_3$ showed very good increase in the production of three enzymes protease, peroxidase and laccase when compared to the two parent strains proving that the higher enzymatic

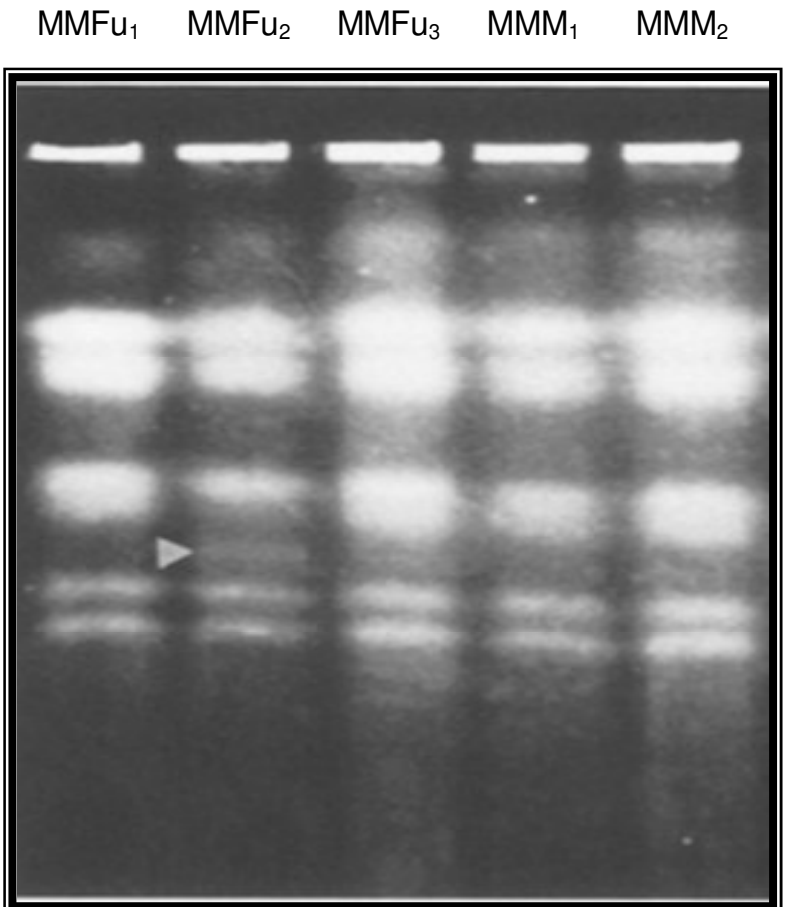

Figure 2. Pulse-field gel electrophoresis pattern of *Mucor mucedo* (fusants $MMFu_1$, $MMFu_2$, $MMFu_3$ and parents MMM_1 and MMM_2). Arrowhead indicates the band of MMM_2 that appeared in $MMFu_2$.

secretions are responsible for the decolourisation activity.

Protease isozyme analysis of *fusants* and parents

To rule out the possibility that the fusants and products of the fusants arose as a result of contamination and in order to determine whether or not the genomes of both MMM_1 and MMM_2 were introduced in to the fusant cells, the protease isozyme patterns of mycelial extract were compared with those of the parental strains. The results were shown in Figure 2. Differences in the isozyme patterns were observed between the parental strains. The isozyme patterns of the fusant showed bands common to either of the parental strains or to both.

Protease isozyme analysis isolates showed bands that also appear in both MMM_1 and MMM_2. The isolates showed one band that appear in MMM_1 and one or few bands that appear in MMM_2. This fact indicates that the isolates were fusants between MMM_1 and MMM_2. There were also isolates that showed bands of only one of the fusion parents.

Decolourisation activity of the three protoplast fusants with crystal violet and malachite green

The decolourisation efficiency of the protoplast fusants $MMFu_1$, $MMFu_2$ and $MMFu_3$ during the decolourisation of two triphenyl methane group dyes (crystal violet and malachite green) was studied. Results proved that the fusant $MMFu_3$ showed the maximum decolourisation efficiency in both the cases of crystal violet and malachite green proving that the decolourisation activity was increased by intraspecific protoplast fusion of two mutant strains of *M. mucedo.* The fusant $MMFu_3$ showed the maximum decolourisation of 95% in crystal violet and 84% in malachite green after 10 days of incubation (Tables 3 and 4).

DISCUSSION

A lot of work was done on strain improvement of different *Mucor* species by protoplast fusion for increasing the production of industrially important enzymes. The present

Table 3. Decolourisation of crystal violet by mutant and fusant strains of *M. mucedo*.

Strain *M. mucedo*	Decolourisation (%)
MMM_1	88
MMM_2	90
$MMFu_1$	75
$MMFu_2$	80
$MMFu_3$	95

Table 4. Decolourisation of malachite green by mutant and fusant strains of *M. mucedo*.

Strain *M. mucedo*	Decolourisation (%)
MMM_1	74
MMM_2	71
$MMFu_1$	63
$MMFu_2$	71
$MMFu_3$	84

working strain *M. mucedo* was isolated from dye amended soils naturally proven to decolourise triphenyl methane dyes. The decolourisation activity of *M. mucedo* was enhanced by protoplast fusion which is a new aspect with *Mucor* strain. It was clear from results that the decolourisation activity was increased by intraspecific protoplast fusion of two mutant strains of *M. mucedo.*

Based on the above investigations, proper conclusions were drawn on the development of biotreatment technology for the bioremediation of dye-contaminated soils and also to reclaim the wastewater. Dyes released by the textile industries pose a threat to the environmental safety. Due to the environmentally friendly techniques bioremediation utilizes, it has been characterized as a soft technology. Its cost-effectiveness and the little disturbance in the environment render this technology a very attractive alternative.

In the present investigation, it was found that the soil fungi adopted to live in the dye contaminated sites were capable of decolourising both the triphenyl methane dyes. Various enzymes involved in the degradation of the dyes have been compiled. Strain improvement by protoplast fusion for production of more efficient biological agents and the application of purified enzymes for decolourisation, which constitute some of the recent advances in this field, have also been reviewed. The studies discussed in this paper indicate fungal decolourisation has a great potential to be developed further as a wastewater treatment technology for small textile or dyeing units. The identification and research of new fungal strains with the aid of molecular techniques will further improve practical application of fungi and it is anticipated that fungal remediation will soon be a reliable and competitive remediation technology.

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