ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com ***INTERNATIONAL JOURNAL OF ADVANCED RESEARCH***

RESEARCH ARTICLE

DYE DEGRADATION EFFECT OF AN INTERGENERIC HYBRID BETWEEN *GANODERMA LUCIDUM* AND *PLEUROTUS FLORIDA*

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Manuscript Info

Manuscript History:

Received: 12 October 2013
Final Accepted: 22 October 2013
Published Online: November 2013

Key words:
Ganoderma lucidum, Pleurotus florida, protoplast fusion, Reactive dyes degradation.

Abstract

Synthetic dyestuffs present a challenge to conventional physico-chemical and biological treatment methods. The study focus on the decolorization of three different textile dyes (Reactive Red 2, Reactive Black 5 and Reactive Yellow 44) by a hybrid mushroom attained by somatic hybridization between an edible mushroom *Pleurotus florida* and a medicinal mushroom *Ganoderma lucidum* and the effect was compared with that of the parents. Maximum amount of protoplasts were released when 5 days old mycelial culture was agitated with 15 mg of lytic enzyme in an osmotic stabilizer (0.6 M $MgSO_4$.$7H_2O$ in 0.05 M Sodium Maleate buffer pH 5) for 18 hrs. After the NTG treatment and mycelial regeneration, arg^- and phe^- auxotrophic mutants of *Ganoderma lucidum* and *Pleurotus florida* respectively were selected as fusants. A hybrids was obtained when the fusants were incubated in 40% PEG succeeded by screening. The hybrid showed higher rate of decolorization for Reactive Black 5 and Reactive Yellow 44 and rapid decolorization in Reactive Red 2, than the parent strains.

Introduction

Synthetic dyes are extensively used in several industries including textile, paper, printing, cosmetics and pharmaceuticals (Marmion, 1991). The total world colorant production is estimated to be 8, 00,000 tons per year and atleast 10% of the used dye stuff enter the environment through wastes (Palmieri et al., 2005; Levin et al., 2004). Decolourisation of textile dye effluent does not occur when treated aerobically by municipal sewage systems (Willmott et al., 1998).

Colour can be removed from effluent by chemical and physical methods including absorption, coagulation-flocculation, ion exchange oxidation and electrochemical methods (Lin *et al*., 1994). The above ways for clean up are expensive, which limit their application (Moreira et al., 2000). Alternatively, dye decolourisation using microbial enzymes has received great attention in recent years due to its efficient application (Abadulla et al., 2000). A number of biotechnological approaches have been suggested by recent research as of potential interest towards combating pollution by dyes in an ecofriendly manner. They include the use of bacteria and fungi or often in combination with physico- chemical processes (Willmott et al., 1998). Though bacteria could utilize dyestuff under anoxic conditions, the disadvantage of this method is the production of aromatic amines by these organisms. These amines may be toxic and carcinogenic (Meyer, 1981). Aerobic bacteria usually tend to be specific towards a particular dye (Reddy, 1995).

White rot fungi are unique among eukaryotic and prokaryotic microorganisms, because they possess a very powerful extra cellular oxidative enzymatic system (LDS) (Rigas and Drista, 2006). Their extracellular ligninolytic system involve enzymes such as Manganese peroxidases (MnP), Lignin peroxidases (LiP) and Laccases (Lac), which are involved not only in the degradation of lignin, but also in the degradation of various xenobiotic pesticides, PCBs and PAHs (Pointing, 2001; Barr and Aust, 1994; Kubatova et al., 2001; Wesenburg et al., 2003; Jarosz-Wilkolazka et al., 2002; Kirk and Farrell,1987).They being non-specific can attack a wide variety of complex aromatic dyestuffs(Barclay et al.,1990; Nagai et al.,2002; Boer et al., 2004; Kamitsuji et al., 2005)

Since the enzymes are extracellular, the substrate distribution limitation into the cell generally encountered in bacteria, is not observed. Further, the extracellular enzyme system enables white rot fungi to tolerate high pollutant concentration (Kapdan et al., 2000; Rodrignez et al., 2004).

Revankar and Lele (2006) have reported that a new isolate of white rot fungus, *Ganoderma* sp. WR-1 showing very high laccase producing ability. Asgher et al.,(2006) has reported decolorization of some reactive textile dyes by *Ganodema lucidum* and *Pleurotus osteatus*, Yet there is no reports on decolorization using fungal hybrids.

Mushrooms are an abundant source of a wide range of useful natural products with biological activities. Protoplasts are now regarded as a useful tool for genetic manipulations (Peberdy 1980 and Ferenczy 1981), which involve fusion leading to somatic hybridization and gene transformation. As conventional hybridization, protoplast fusion can be performed intra-specifically (Kiguchi and Yanagi,1985; Toyomatsu and Mori,1987), inter-specifically (Takehara et al.,1993; Matsumoto et al.,1997), inter-generically (Eguchi et al., 1993; Zhao and Chang, 1996) and even inter-heterogenerically (Eguchi and Higaki, 1995; Toyomatsu and Mori,1987). *Ganoderma* is a polypore mushroom that is soft, corky and flat. The mushroom is too tough to be edible (Wasser, 2005). It has been an economically imported species, particularly in the Far East countries (china, Japan, Korea, etc.,) for over 4000 years. It is widely grown on a commercial scale and is commonly purchased for its medicinal spiritual properties. *Pleurotus florida*, commonly known as oyster fungus, grows wildly in tropical and subtropical rainforests, and can be artificially cultivated. This mushroom is considered as a delicacy and treasured for its flavor and taste. They are rich in nutrition and are claimed to have medicinal value (Zadrazil, 1980). A step has been taken to construct a somatic hybrid which may show recombined characteristics of both *Ganoderma* and *Pleurotus* . A few reports are available on the development of regenerating mushroom protoplasts, but usage of macerozyme, and selection of auxotrophic mutants Arg^- and Phe^- has not been reported. The present study focuses on producing a somatic hybrid between *Ganodema lucidum* and *Pleurotus florida* through protoplast fusion technology and finding its effect on decolorizing three reactive dyes and comparing it with the effect of parents.

Material and Methods

2.1. Reactive dyestuffs:

The three reactive textile dyestuffs were Reactive Red 2 (Red M_5 B), Reactive, Reactive Black 5 (Black B) and Reactive Yellow 44 (Yellow MR), provided by the distribution office at Pallipalayam, Erode, India.

2.2. Strain and culture:

A mycelial culture of *Pleurotus florida* was developed from a local commercial strain and pure culture of *Ganoderama lucidum* (MTCC) were used. Both the strains were cultured in Potato Dextrose broth and Malt Dextrose broth. Mycelia that had grown for 5 days were used for protoplast formation. The cultures were subcultured periodically.

2.3. Culture media:

The basal media contained (g/l of distilled water), Starch 20, Yeast extract 2.5; KH_2PO_4 1.0; NaH_2PO_4 0.05; $MgSO_4$. $7H_2O$ 0.5; $CaCl_2$ 0.01; $FeSO_4$. $7H_2O$ 0.01; $MnSO_4$. $4H_2O$ 0.001; $CuSO_4$. $5H_2O$ 0.002. The pH of the medium was adjusted to 5.5 (Revanker and Lele, 2006).

2.4. Isolation of Protoplast:

Four day-old monokaryotic mycelia of *Ganoderma lucidum* and *Pleurotus florida* was transferred onto 100 ml of Malt extract broth plus 20 glass beads (0.50 cm in diameter) with the agitation speed of 200 rpm, at 25ºC for 4 days. Protoplasts were separated from the mycelia using a method modified from Hashiba (1992) by transferring 0.3 g of the mycelia onto 3 ml of the sterilized lytic enzyme solution having macerozyme in a test tube and shaking it at 100 rpm at room temperature for 16 hrs.

2.5. Effect of lytic enzyme:

Macerozyme was tested for their effect on protoplast release in *Ganoderma lucidum* and *Pleurotus florida*. Macerozyme was used at various concentrations of 8mg, 10mg, and 15mg to digest mycelium. It was prepared in 0.6mol/l sodium Maleate buffer (pH5.8) along with $MgSo_4$.

2.6. Effect of incubation time:

Effect of incubation time in release of protoplast in *Ganoderma lucidum* and *Pleurotus florida* when incubated with macerozyme was analyzed for various periods of 2hr, 10hr, 16hr, and 18hr.

2.7. Effect of Inoculum size:

Freeze dried mycelia with different weight was tested for their effect in release of protoplast. Mycelia were taken in different dry weight (0.25g, 0.5g, 1g and 1.5g) in addition to osmotic stabilizer and lytic enzyme.

2.8. Purification of Protoplast:

The mycelial remnants were removed by filtration through a sintered glass filter and the suspended protoplasts were precipitated at 1000xg for 10 min. The protoplasts were then washed twice with the osmotic stabilizer in 1 ml of the osmotic stabilizer which contained 0.6 M MgSO4·7H2O in 0.05 M Sodium Maleate buffer, pH 5. And they were finally suspended in 5 ml of the osmotic stabilizer. Protoplasts obtained from *Ganoderma lucidum* and *Pleurotus florida* were counted using a haemocytometer.

2.9. Mutagenic procedure:

Protoplast suspension (ca. 1.2 x 106/ml) was treated with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) for mutagenesis. The protoplasts were exposed to the action of NTG for varied period of time from 15 to 120 seconds. NTG was used at two concentrations, 15 and 30µg/ml in osmotic stabilizer solution.

2.10. Regeneration:

The protoplasts were immediately washed thrice with osmotic stabilizer to remove NTG and diluted to $1x10^4$ protoplasts /ml. 0.1 ml from the dilution was used for protoplast regeneration on a plate with regeneration medium. The plate was incubated at 25ºC until colonies occurred. Each colony was isolated and inoculated in Malt extract agar slants.

2.11. Selection of mutants:

To select the mutants, all the colonies from regenerating medium were transferred to minimal medium (MM) without amino acids. Regenerated colonies those did not grow on MM were tested for their requirements by further transfer onto MM containing different amino acids (20µg/ml). Genotypes were considered to be stable after five successive subcultures on appropriately supplemented MM.

2.12. Protoplast Fusion:

Freshly prepared protoplasts (diluted to be $1x10^6$ protoplasts/ml) of *Ganoderma lucidum* and *Pleurotus fusion* were mixed and centrifuged at 1000x g for 10 min. The supernatant was rinsed off and 1 ml of sterilized PEG (6000) (40 g PEG in 100 ml 0.05 M $CaCl_2 \cdot 2H_2O$) was added to the mixture and incubated at room temperature for 20 minutes. The tubes were intermittently shaken for every 5 min and then centrifuged at 1000x g for 10 minutes. The supernatant was discarded and the protoplast mixture was taken for hybrid screening.

2.13. Decolourisation procedure:

Decolorization flasks were prepared in triplicates each containing 50 ml of nutrient media for three days observation. The medium was sterilized (121^{o}C) in an autoclave for 15 min. Each flask was inoculated with 5 numbers of agar plug cut from PDA plates, and allowed to grow for four days in 30^{o}C. Fourth day three dye stuffs were added in a concentration of 100 mg/l separately in 3 sets. Biotic control (with inoculum and without dye) and abiotic control (with dyestuff and nutrient, but received no inoculum) were maintained. For every 24 hours samples were removed, centrifuged at 8,000x g for 20 minutes and the supernatant obtained was used for the determination of residual dyestuff concentration.

2, 14.Dyestuff analysis:

Absorbance measurements were done with UV/ Visible spectrometer. When the wavelength with maximum absorbance (λmax) was measured, it was 440, 590 and 420 nm for the dyes Reactive Red 2, Reactive Black 5 and Reactive Yellow 44 respectively. Respective blanks (containing only medium, omitting the respective dye) were used to calculate the absorbance values for supernatants collected at time intervals. The absorbance values were used to know the percentage of decolorization.

$$\text{Decolorisation \%} = \frac{\text{Initial absorbance} - \text{observed absorbance}}{\text{Initial absorbance}} \times 100$$

Result and Discussion

The study is based on the formation, fusion, regeneration of *Ganoderma lucidum* and *Pleurotus florida* protoplasts and application of the hybrid in textile dye decolorization.

3.1. Isolation of Protoplast:

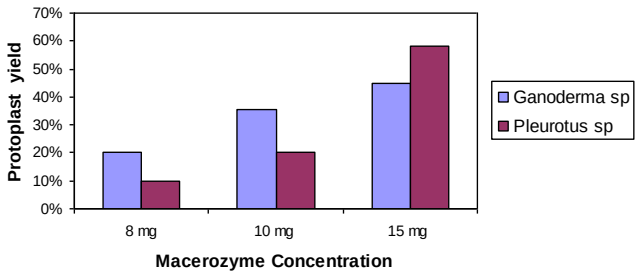
The commercially prepared enzymes Novozyme 234 and Cellulase CP have been used previously to effect protoplast release from a variety of fungal species (Hamlyn, et al.,1981). Our results extend the use of macerozyme enzyme to release protoplasts from *Ganoderma lucidum* and *Pleurotus florida*. Although pretreatment with thiols was required for efficient protoplast release in some fungal agents (Brown, 1971; Fincham, and Day, 1971.), no such pretreatment was necessary for *Ganoderma lucidum* and *Pleurotus florida.* The amount of protoplasts obtained in *Ganoderma lucidum* and *Pleurotus florida* were 3x 10^{5} and $1.8x10^{5}$ protoplasts/ml, respectively. The results achieved correlate to some extent with those published by Dhitaphichit and Pornsuriya (2005).

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 (Peberdy, 1989) and of the osmotic stabilizer (Peberdy and Fox, 1993) and complex interactions exist between them.

3.2. Effect of lytic enzyme:

Among the various concentrations (8mg, 10mg, and 15mg) of lytic enzyme Macerozyme used, both the strains showed more yield in 15 mg/ml concentration (Fig 1).

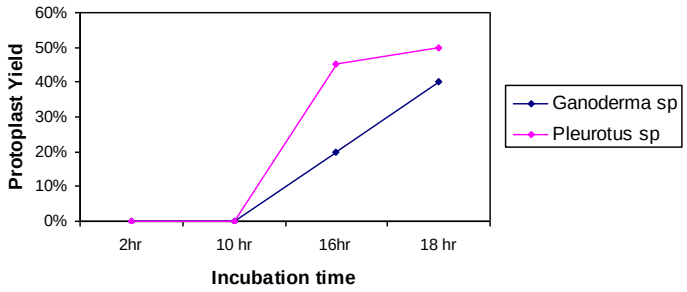
Fig. 1 Effect of enzyme concentration on protoplast yield



3.3. Effect of incubation time:

Release of protoplasts was found to begin after 10h of incubation and attained maximum at 18h (Fig 2).

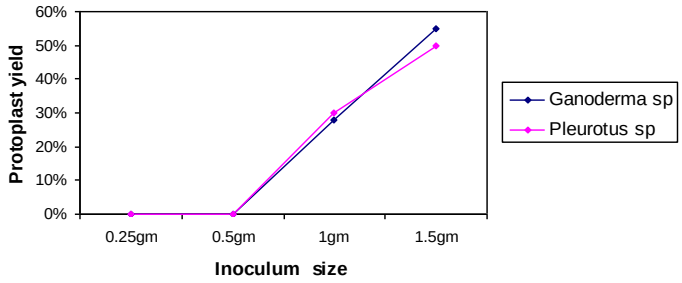
Fig. 2 Effect of incubation time on protoplast yield



3.4. Effect of Inoculum size:

When freeze dried mycelia were taken in different weight (0.25g, 0.5g, 1g and1.5g), the highest percentage of protoplast release was observed in 1.5g of inoculum (Fig 3).

Fig. 3 Effect of inoculum size in the release of protoplast



3.5. Effect of osmotic stabilizer:

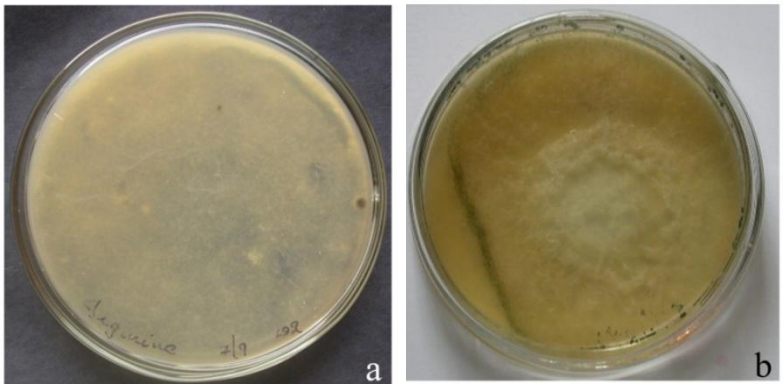
Protoplasts released from *Ganoderma lucidum* and *Pleurotus florida* were found to be osmotically fragile spherical bodies, mostly of uniform size, accompanied by a few relatively bigger ones. Among different concentrations of lysing enzymes tried, we optimized that 15mg/ml with 0.6M MgSo4 and Sodium Maleate buffer as osmotic stabilizer to release higher number of protoplast from *Ganoderma* and *Pleurotus sp.* However, Pe'er and Chet (1990) obtained highest protoplasts from *T. harzianum* using Novozym 234 at 10 mg/ ml with 0.6MKCl and Tschen and Li used 15 mg/ ml of Novozym with 0.6M sucrose to isolate maximum protoplasts from *T. harzianum* and*T. Koningii*. Further, Balasubramanian et al., (2003) reported maximum number of protoplasts from *Trichothecium roseum* using Novozym 234 in combination with chitinase and cellulase each at 5mg/ml.

3.6. Regeneration:

In the process of protoplast regeneration, the critical element required for stabilizing is the hypertonic solution. It was observed that the choice of a suitable osmotic stabilizer solution has a pronounced effect on regeneration. Maleic acid – NaoH buffer (pH- 5.0) with 0.6$MgSO_4$ was the osmotic stabilizer reported for *Schizophyllum commune, Coprinus pellucidus* and *Coprinus cinereus* (De Varies et al., 1972) and the same was found to be most suitable in the present study too.

Protoplasts that survived and regenerated after various exposures were screened for any auxotrophy in minimal medium with aminoacids. It should be mentioned that although a number of colonies were identified as auxotroph on initial screening, most of them quickly reverted back to the wild type on subsequent subculturing. Among the mutants, those did not revert back for successive five generations were selected for the further study. arg^- auxotrophic mutant was identified in *Ganoderma lucidum* and phe^- auxotrophic mutant was identified in *Pleurotus florida* (fig 4).

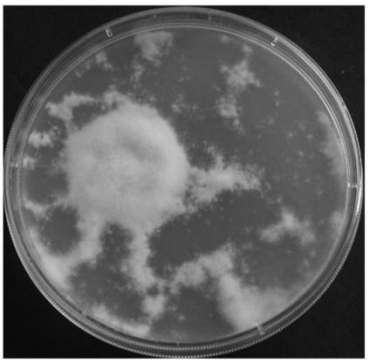
Fig: 4 a. arg^- Auxotroph- *Ganoderma lucidum* b. phe^- Auxotroph- *Pleurotus florida*



3.7. Protoplast fusion:

The molecular weight of the PEG and concentration of the fusogen employed seem to be critical to obtain optimal fusion. Inter-generic protoplasts fusion between *Ganoderma lucidum* and *Pleurotus florida* was achieved by employing 40% PEG (600) similar to that reported by Mrinalini et al., (1996). Regeneration of mycelia happened when inoculated on regeneration media (fig 5).

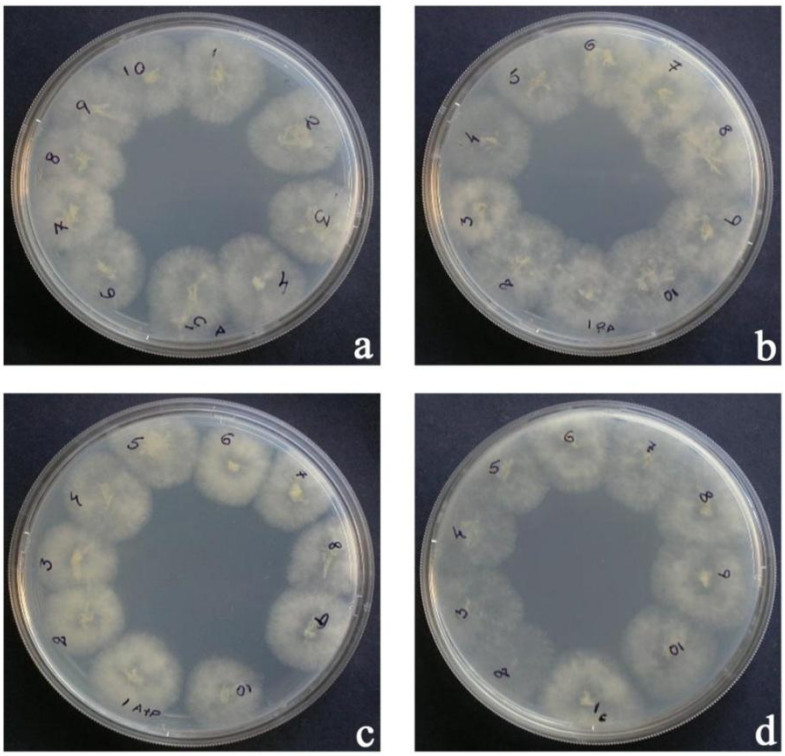
Fig: 5 Regeneration of fused protoplast



3.8. Hybrid selection:

After regeneration, selection of hybrid was carried out by inoculation on different synthetic media. The hybrids that need either arginine or phenylalanine are intraspecific fusion products and they were eliminated. The mutants that were stable, retaining the auxotrophy after several subcultures, did not require arginine and phenylalanine supplementation in the synthetic medium after fusion were considered as the hybrids (fig 6) and used further.

Fig. 6 Confirmation of hybrid grown on minimal medium after protoplast fusion (a. minimal medium with Arginine, b. minimal medium with Phenyl alanine, c. minimal medium with arg+ phe, d. growth on minimal medium.



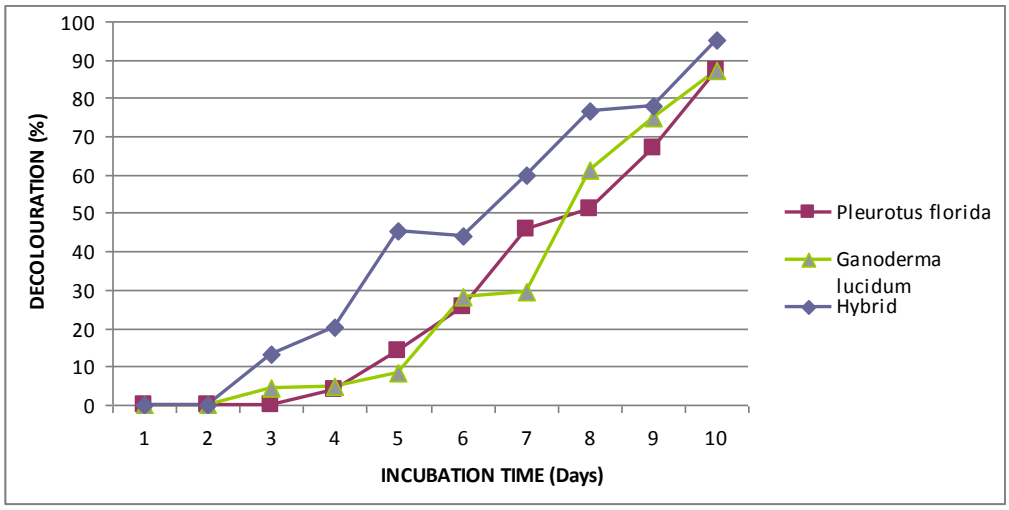
3.9. Dye decolorization and Dyestuff analysis:

Results of time course studies on decolorization of Reactive Black 5 (Black B) are displayed in Table 1, Fig 7. Although the rate of colour removal was nil within the first 3 days for *Pleurotus florida* and 2 days in the case of both the hybrid and *Ganoderma lucidum*, hybrid has brought the highest rate of decolorization (94.94%) than the parent strains. Both *Pleurotus florida* and *Ganoderma lucidum* could bring about 87.37% decolorization after 10 days of incubation.

Table 1 Decolorization of Reactive Black 5 (Black B)

	Decolorization(%)		
Day	*Pleurotus florida*	*Ganoderma lucidum*	Hybrid
1	0	0	0
2	0	0	0
3	0	4.4	13.13
4	4.04	5.05	20.2
5	14	8.58	45.5
6	25.75	28.3	44.14
7	46	29.3	59.75
8	51.01	61.25	76.76
9	67.17	74.75	77.78
10	87.37	87.37	94.94

Fig.7 Decolorization of Reactive Black 5 (Black B)



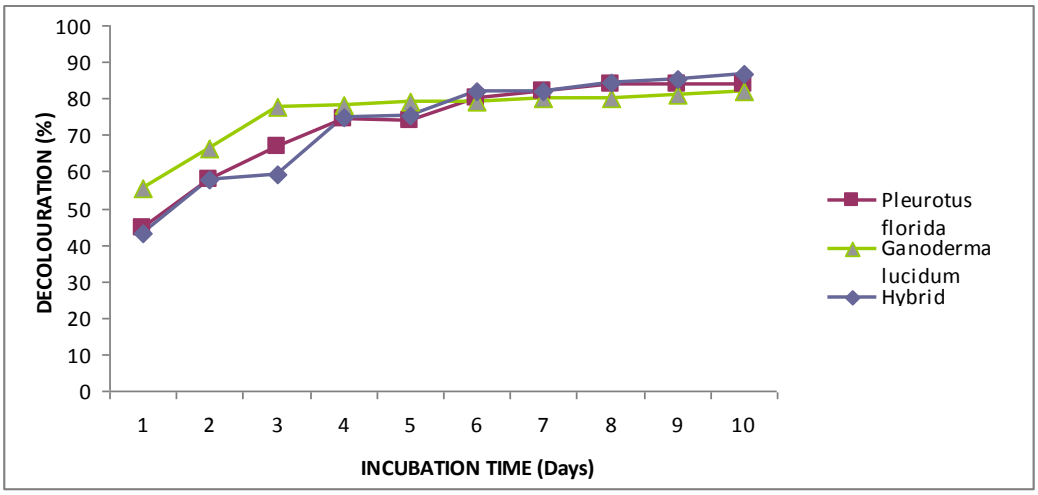
A time course study on decolorization of Reactive Yellow 44 (Yellow MR) is shown in the Table 2, Fig 8. Even though Reactive Yellow 44 (Yellow MR) was decolorized effectively upto 84% in 8 days by *Pleurotus florida* and there was no further change till 10 days of incubation. 82.2% of decolorization was observed by *Ganoderma lucidum*. The hybrid showed the highest rate of decolourisation (86.7%) when compared to the parents.

Table 2 Decolorization of Reactive Yellow 44 (Yellow MR)

	Decolorization(%)		
Day	*Pleurotus florida*	*Ganoderma lucidum*	Hybrid

1	44.4	55.45	43.3
2	57.8	66.4	57.8
3	66.7	77.8	59.3
4	74.3	78	75
5	74	79	75.5
6	80	79.3	82.22
7	82.2	80	82.22
8	84	80	84.4
9	84	81.2	85.3
10	84.1	82.22	86.66

Fig.8 Decolorization of Reactive Yellow 44 (Yellow MR)



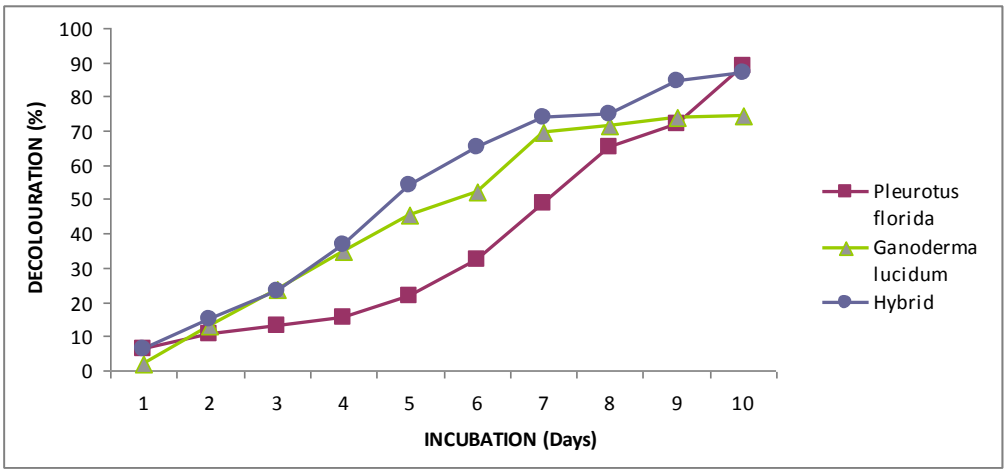
Time course studies on decolorization of reactive red 2 (Red M_5B) is exhibited in the Table 3, Fig 9. *Ganoderma lucidum* showed the least rate of decolorization (74.2%) and *Pleurotus florida* exhibited the highest rate of decolorization (89.13%) in 10 days. But more than 50% decolorization was obtained only after 7 days of incubation. Even though the decolorization rate showed by the hybrid was comparatively lesser than that of *Pleurotus florida*, it pronounced more than 50% decolourisation within 5 days of incubation.

Table 3 Decolorization of reactive red 2 (Red M_5B)

	Decolorization(%)		
Day	*Pleurotus florida*	*Ganoderma lucidum*	Hybrid
1	6.5	2.1	6.5
2	10.8	13	15
3	13	23.9	23.2
4	15.5	34.7	36.5
5	21.7	45.6	54.3
6	32.6	52.3	65
7	49	69.5	73.9
8	65.2	71.7	74.78
9	72	73.91	84.78

10	89.13	74.2	86.9

Fig.9 Decolorization of reactive red 2 (Red M_5B)



It has been reported that azo dyes are recalcitrant to decolourisation and could only be decolourised to a limited extent (Nyanhongo et al., 2002). But *Ganoderma lucidum* and *Pleurotus florida* had efficiently degrade Reactive red 2(red M5 B), Reactive Black 5 (Black B) and Reactive Yellow 44 (Yellow MR). As a highlight the hybrid has shown more efficiency in decolorizing all the dyestuffs than the parent strains.

Physiological differences among *Ganoderma lucidum*, *Pleurotus florida* and the hybrid may account for difference in their decolorization abilities. The complex enzymatic system responsible for dye degradation and pattern of its expression may also vary. Intergeneric hybrid of *Ganoderma lucidum* and *Pleurotus florida* had exhibited good potential for decolorization of relatively less studied reactive textile dyestuffs. It may be due to enhancement in the enzymatic system occurred by fusion.

Conclusion

The results of the study will form the basis for development of cost effective and robust indigenous technology for bioremediation of these reactive dyes and dye based effluents.

Acknowledgement

The authors thank the University Grants Commission (UGC), India for their financial support to carry out this study.

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