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Evaluation of *Trichoderma reesei* as a compatible partner with some white rot fungi for potential bio-bleaching in paper industry

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ABSTRACT

Trichoderma species a common filamentous fungi found in the environment and abundantly in soil, because of its antagonistic activity against plant pathogens allows its potential use as a biological control agent. The use of biological agents is becoming an increasingly important alternative to chemical agent in Pulping and Bleaching process of paper industry. Trichoderma reesei is known to produce xylanase which is an enzyme very important for brightening of paper in the pulp and paper industry. In the present study the compatibility of Trichoderma reesei with some ligninolytic white rot fungi has been evaluated to identify a potential compatible ligninolytic white rot fungi with Trichoderma reesei that can be utilized in practical application of co-culture in bio pulping and bio bleaching of pulp and paper industry. Results showed that the six ligninolytic white rot fungi were compatible with Trichoderma reesei proving its potential to be used as compatible fungal partners in co-culturing for efficient bio bleaching.

Key words: *Trichoderma reesei*, ligninolytic, compatibility, biopulping

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INTRODUCTION

The paper and pulp industry is a potential source of major pollution, generating large volumes of intensely colored effluent for each metric ton of paper produced. During the pulp processing, approximately 5-10% of the lignin of the raw materials remains in the pulp which is responsible for the brown colour of the pulp and the last step of the pulping process, Bleaching needs to be carried out in which remaining fraction of lignin is being removed and whitening and brightening of the pulp occurs [1]. The residual lignin from wood pulp is chemically liberated by using chlorine bleaching. Elemental chlorine reacts with lignin and other organic matter in the pulp forming chlorinated compounds that are extracted with alkali. Only about 40-45% of the original weight of the wood goes into paper production and therefore the effluent that comes out is rich in organic matter [2].

Bio pulping and bio bleaching are two alternative technologies which can reduce the chemical pollution. Isolation and screening of fungal strains suitable for biopulping have been performed in many laboratories since the 1970s. The benefits of pre-treating wood chips with white-rot fungi, which degrade wood lignin during incubation time 2-4 weeks have been described by several authors [3, 4, 5]. Lignocellulolytic enzymes-producing fungi are widespread, and include species from the ascomycetes (e.g. *Trichoderma reesei*) and basidiomycetes such as white-rot (e.g. *Phanerochaete chrysosporium)* and brown-rot fungi (e.g. *Fomitopsis palustris).*

Biobleaching which is considered to be effective biological methods as promising alternative to the alkali and chemical bleaches and reduces the utilization of chemicals and energy , pollutants and increase the yield and strength of pulp [6] Many microorganisms, including fungi and bacteria, have been found to be capable of degrading plant cell wall fibers [7].

Biobleaching is an important alternative to reduce the use of chlorine and chlorine compounds in the bleaching process. Biological bleaching is carried out using white-rot fungi to degrade residual lignin in the pulp by using ligninolytic enzymes such as manganese peroxidase and laccase or by using hemicellulolytic enzymes such as xylanases [8].

Fungal treatment decreases mechanical pulping energy, significantly increases paper strength properties, and lowers brightness, but has minimal effect on other optical properties. Paper obtained by fungal treatment appears to be yellowing. For brightening of cellulosic fibers xylanase enzyme is used. There are many fungi which are known to produce xylanase. *Trichoderma* is one of the best known fungi which produce enzymes with high xylanolytic activity. Cellulase-free xylanase preparations have been tested successfully in industrial applications such as the prebleaching of kraft pulp in the pulp and paper industry. Fungal treatment of cornstalks with *Irpex lacteus* is known to have enhanced the delignification and xylan loss during mild alkaline pretreatment and enzymatic digestibility of glucan. The 15 day biotreatment modified the lignin structure and increased loss of lignin (from 75.67% to 80%) and xylan (from 40.68% to 51.37%) [9].

Two biopotential fungi which are not antagonist but compatible/synergistic of which one or both are selective in lignin degradation and producing xylanase enzyme would help in increasing the efficiency of biobleaching by degrading lignin in an ecofriendly manner and also would help in brightening the cellulosic fibers preventing yellowing of paper. Dual culture means combination of two fungi which can grow together without harming each other's growth and are compatible with each other. Dual culture can enhance the rate of degradation too.

The main objective of present study was to identify two compatible biopotential fungi so that it can be used in dual culture to increase the efficiency of bio bleaching. The fungal combination has to be such that one is ligninolytic and the other helps to improve the quality of the cellulose fiber, especially the brightening property of the paper.

MATERIALS AND METHODS

Source of fungal isolates:
Daedaleopsis confragosa used in the present study was isolated from fruiting bodies growing on the dead/living wooden logs collected from Pavagadh forest near Vadodara in Gujarat state and fruiting bodies of *Phellinus pectinatus* was collected from Arboretum of The Maharaja Sayajirao University of Baroda, Gujarat, India. The fruiting bodies were excised, packed in sterile poly ethylene bags and brought to the laboratory. Fruiting bodies were surface sterilized by 0.1% $HgCl_2$ for 60 seconds and washed thoroughly with distilled water followed by 70% ethanol for few seconds and inoculated on Potato Dextrose Agar (PDA) Medium. Pure cultures were established by routine methods and maintained at 4 $(+1)^0$C. *Daedaleopsis confragosa* (DC) and *Phellinus pectinatus* (PHE) were identified from the Forest Research Institute, Dehradun. The fungal isolates of *Trichoderma viride* (TV), *Trichoderma harzianum* (TH), *Pleurotus eryngii* (PE), *Pleurotus florida* (PF) , *Pleurotus ostreatus* (PO), *Pleurotus sajorcaju* (PS), *Irpex lacteus* (IL) and *Pycnoporus sanguineus*(PYS) were procured from Forest Research Institute, Dehradun. The fungal isolate of *Trichoderma reesei* 4876 (TR) and *Phanerochaete chrysosporium*787(PC) were procured from MTCC culture collection centre, Chandigadh.

All the cultures were maintained on Potato dextrose agar (PDA) at 4 $(+1)^0$C in Seed Anatomy laboratory of Department of Botany at The Maharaja Sayajirao University of Baroda, Gujarat, India. For further studies petridish containing potato dextrose agar medium were inoculated with 0.5 cm diameter agar plug, cut from the growing edges of colonies of the isolates and incubated in incubator at 25($\pm$1)^{0}C in dark with 70% relative humidity.

Screening of fungal isolates for ligninolytic, cellulolytic and xylanolytic test: All fungal isolates were screened in our laboratory to check ligninolytic and cellulolytic enzyme activity of fungi [10]. Xylan-agar diffusion method [11] was used to confirm the ability of fungi to produce extracellular cellulase free xylanase during their growth. Ligninolytic activity was assessed by observing the dark brown colored zone around the respective fungal colonies

[10] and Cellulolytic and xylanolytic activities were analysed by observing zone of clearance if any formed surrounding the fungal colonies.

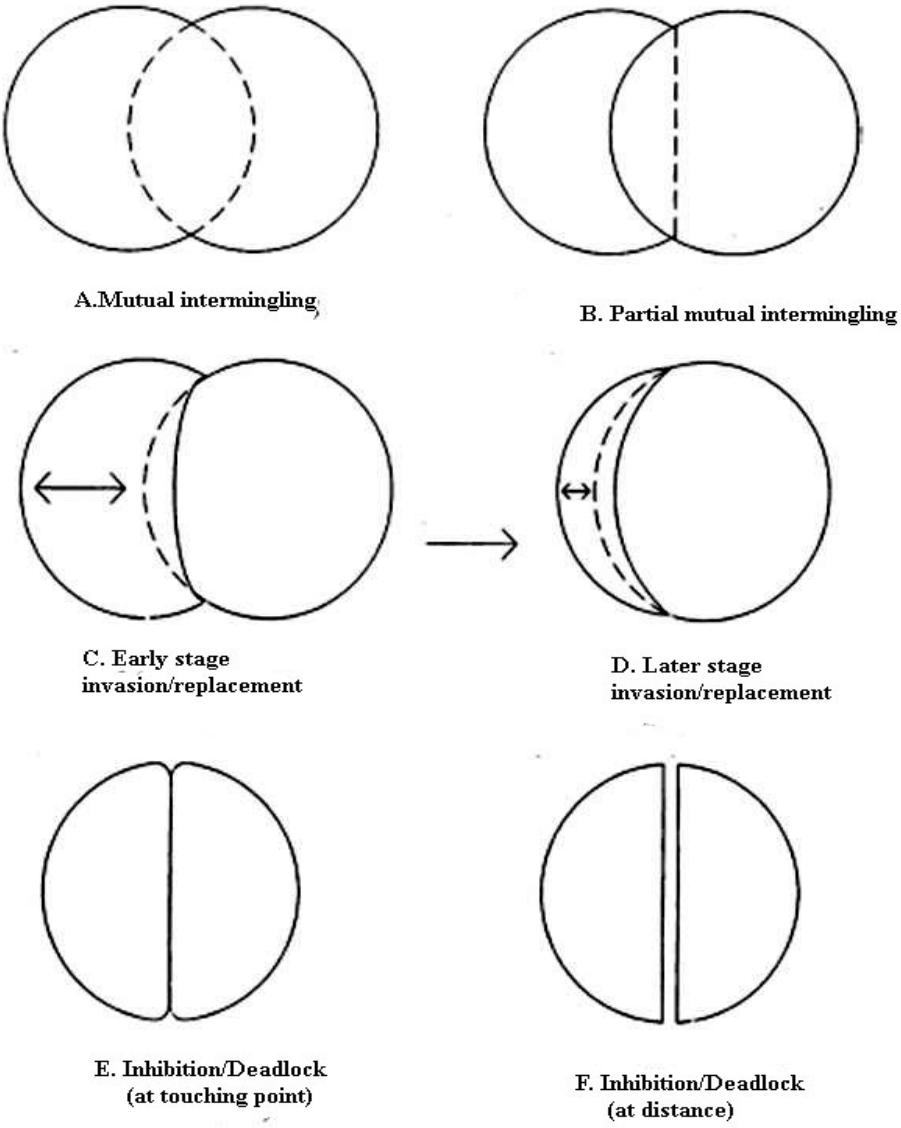

Fig. 1 Schematic diagram representing interactions between two different fungal strains grown adjacently (4 cm apart) on MEA culture media observed after one week incubation period (based on the observations of Porter (1924) [12] and cited by Skidmore & Dickinson (1976)[13], and Stahl & Christensen [14](1992))

Paired interaction test on agar plates: In vitro antagonistic effect of the fungi was evaluated through paired interaction test. This method was used to check compatibility of two fungi to grow together. Five mm diameter agar plug was cut from the growing edge of 10 days old culture of the fungal isolate and inoculated at the margin of the petriplate containing 20 ml sterilized Malt Extract Agar medium. The paired test fungi (agar plug) were placed on opposite sides of petriplate and incubated at 25±2°C maintaining 70% humidity for 4 weeks. Petriplate with

individually inoculated fungal culture (Mono cultures) isolates were kept as control. Three replicates were kept for each set of experiment.

The isolates were screened for their compatibility/antagonistic potential against the other fungal isolates by observing the relative growth of both fungi till one fungus inhibits the growth of other or one kills the other or they both overgrow each other and form overlapping zone. The compatibility was assessed by observing the interaction between the two test fungi at the zone of contact as per Porter 1924. A schematic diagram of the interaction proposed by Porter 1924 [12] is represented in Fig. 1.

According to Porter (1924) the five different modes of interaction are as follows.
1. Mutually intermingling growth where both fungi grew into one another without any macroscopic signs of interactions. (Fig.1 A)
2 (i) Intermingling growth where the fungus being observed is growing into the opposed fungus either above or below or above and below its colony, and its corollary. (Fig.1 B)
(ii) Intermingling growth where the fungus under observation has ceased growth and is being overgrowth by another colony. (Fig.1 B)
3. Slight inhibition where the fungal growth approach each other until almost in contact and a narrow demarcation line, 1–2 mm, between the two colonies is clearly visible. (Fig.1 E)
4. Mutual inhibition at a distance of >2 mm. (Fig.1 F)
According to Stahl & Christensen (1992) [14] the mode of interactions can be categorized as
1. Neutral intermingling – one or both colonies grow into the other with no apparent adverse effect on the mycelium of either. (Fig. 1 A)
2. Replacement – one mycelium grows into the other and begins to consume and replace it. (Fig.1 C & D)
3. Deadlock – neither mycelium can enter territory occupied by the other. (Fig. 1 E)
All the important results were photographed with Sony cybershot model no. DSC-H2O.

RESULTS AND DISCUSSION

The objective of the present study was to screen a fungal co culture that produce ligninolytic enzymes along with xylanase and absence or scanty production of cellulolytic enzymes. In identifying an unknown fungus for its potential use in paper industry one of the first questions is whether this fungus is ligninolytic or celulolytic. This is usually determined by the well known Bavendamn test. It is known since long that mycelia of certain higher fungi contain enzymes which catalyze the oxidation of phenols and related compounds. Bavendamn (1928) [15] was the first to point out the difference between the white rot and brown rot fungi with respect to their oxidative enzymes. When white rot fungi cultivated on nutrient agar containing certain phenolic compounds as gallic acid or tannic acid, the white rot fungi produce a deeply coloured zone around the mycelium while the fungi brown rot do not.

In the present study enzymatic activities of 12 fungal isolates have been screened by using solid media containing coloured indicator compound which enables visual detection of enzyme production and darkbrown colour zone and its intensity indicating positive extracellular enzyme production and Results obtained by Bavendamn's test has been represented in Table 1.

Out of 12 fungi all fungi except *Trichoderma reesei* showed positive reactions to tannic acid, indicating them to release lignin degrading enzymes and hence are potential lignin degraders. After seven days of incubation the medium which is substituted with Tannic acid showed the culture with brown coloration zone when viewed from the lower side of the petridish indicating presence of ligninolytic enzymes. Petridish substituted with CMC (carboxy methyl cellulose) after one week incubation period when flooded with Congo red did not show clear zone except TV and TH confirming the presence of ligninolytic enzymes and absence of cellulolytic enzymes.

Xylanases in combination with cellulases have applications in food processing [16] whereas, xylanases without cellulases are important in paper industry as the quality of paper depends upon the amount of cellulose present in the paper. Therefore, to select potential cellulase-free xylanase producing fungi, rapid plate technique was carried out and enzyme activities (xylanase and cellulase) were measured. Interestingly, *Trichoderma* species, popular in biological control of plant diseases showed its broad range of xylanase activity.

One of the major problems in screening large number of microbial strains for their xylanase producing ability, is the lack of single rapid reliable screening technique. Hence, solid agar screening method was used for screening. Initially, solid screening medium containing xylan as the sole carbon source developed for this purpose was employed [17]. Xylanase producing organisms were identified on the basis of the clearing zone formed around the colonies. Petriplates substituted with 1% xylan after one week incubation period when flooded with congo red and washed by 1% NaCl solution, all the fungi except PO, DC and PC showed zone of clearance showing positive reaction for xylanase enzyme activity (Table1). All the screened fungi except TR produces ligninolytic enzymes. TV and TH showed positive reaction for ligninolytic, cellulolytic and xylanolytic activity. Except TV and TH all the test fungi shows negative reaction to celluloytic activity. TR shows an absence of ligninolytic and xylanolytic activity but positive xylanolytic activity.

Table: 1 Ligninolytic, Cellulolytic and Xylanolytic activity of different fungi

Fungal isolates	Ligninolytic	Cellulolytic	Xylanolytic
TV	Positive	Positive	Positive
TH	Positive	Positive	Positive
TR	Negative	Negative	Positive
PS	Positive	Negative	Positive
PO	Positive	Negative	Negative
PF	Positive	Negative	Positive
PE	Positive	Negative	Positive
IL	Positive	Negative	Positive
PYS	Positive	Negative	Positive
DC	Positive	Negative	Negative
PHE	Positive	Negative	Positive
PC	Positive	Negative	Negative

Table: 2 Characteristic features observed on 3rd, 6th and 9th day of incubation of different fungal isolates grown with its co culture partner *Trichoderma reesei*

Name of fungi	3rd day	6th day	9th day	Nature of interaction
Trichoderma viride (TV) Fig. 1 (A-C)	Both the fungi come in contact with eachother	Sporulation of TR started and TR overgrows TV	TV also overgrows TR but growth of TV was restricted	Partial mutual intermingling
Trichoderma harzianum (TH) Fig. 1 (D-F)	Growth of TR was more than TH	Sporulation of TR started and TR overgrows TH	Growth of both the fungi stopped.	Invasion /replacement
Pleurotus eryngii (PE) Fig. 1 (G-I)	Growth of PE was very slow & TR covered the whole plate	Sporulation of TR started and growth of PE continued to occur on TR	TR grown till the inoculums disc of PE and killed PE	Invasion /replacement
Pleurotus florida (PF) Fig. 1 (J-L)	Growth of TR was more than PF and growth of PF just started.	The growth of PF was inhibited by TR	TR completely grown on the PF & killed PF	Invasion /replacement
Pleurotus ostreatus (PO) Fig. 1 (M-O)	TR grows faster and covered the whole plate	TR overgrows on the inoculums disc of PO but growth of PO also stated	PO continued to grow but later on its growth was stopped	Partial mutual intermingling
Pleurotus sajorkaju (PS) Fig. 1 (P-R)	TR grows faster and covered the whole plate	Growth of PS continued to occur on TR	PS continued to overgrow till the inoculums disc of TR	Mutual intermingling
Irpex lacteus (IL) Fig. 2 (A-C)	Growth of TR was more & both the fungi came in to contact with each other	IL overgrows on TR and almost covered half of the plate	Growth of IL continued up to 3 weeks of incubation period	Mutual intermingling
Daedaleopsis confragrosa (DC) Fig. 2 (D-F)	Growth of TR was more & both the fungi came in to contact with each other	Growth of both the fungi seems to be stopped but DC grows slowly	Growth of DC continued up to 4 weeks of incubation	Mutual intermingling
Phellinus pectinatus (PHE) Fig. 2 (G-I)	Growth of TR was more & both the fungi came in to contact with each other	Both the fungi came in to contact with each other	PHE continued to over grow even after 20 days of incubation period	Mutual intermingling
Pycnoporus sanguineus (PYS) Fig. 2 (J-L)	Growth of TR was more than PYS & TR covered the whole plate	PYS overgrows on TR but sporulation of PYS was not occured	PYS continued to grow and later covered the whole plate	Mutual intermingling
Phanerochaete chrysosporium (PC) Fig. 2 (M-O)	Growth of TR was more & both the fungi came in to contact with each other	PC overgrows on TR and almost covered half of the plate	PC continued to grow and completely overgrown on TR	Mutual intermingling

Observations on the compatibility of the fungi in the co-culture / paired interaction tests could be distinguished into three categories.

1. Both fungi come in contact on the PDA medium and growth of both fungal isolates are inhibited i.e. No further growth occurs once the two come in contact.
2. The two fungal isolates in the paired interaction test come in contact and growth of one is inhibited by the other but it is not killed. The fungal isolate grows on the counterpart.
3. The two fungal isolates in paired interaction test come in contact, one overgrows over the other and kills it.

A paired fungi was considered compatible once they come in contact and still each one grows over the other at its own pace with the formation of an overlapping zone which increases / advance towards both the sides. The growth of the fungal isolates with its counterpart has been represented individually in figure 2 and 3. Characteristic features of co cultures observed after the different incubation period have been represented in Table 2.

Based on the observations of fungal isolates with its counterpart on 3rd, 6th and 9th day indicated in Table 2 it could be depicted that *Trichoderma reesei* was found to be compatible with six fungal isolates PS, IL, DC, PHE, PYS and PC.

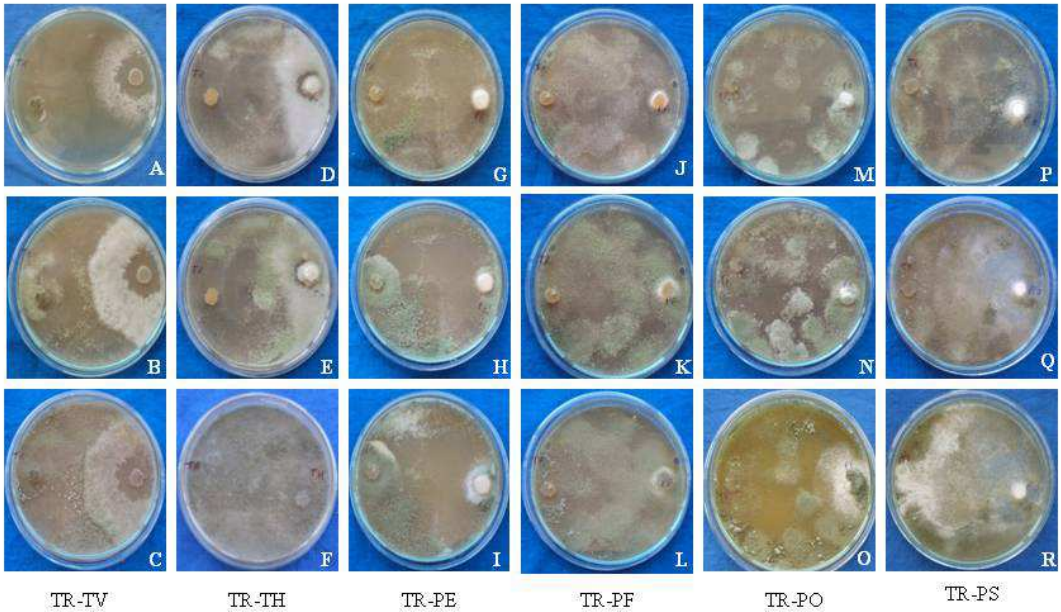

Fig: 2 Paired interaction test of *Trichoderma reesei* with other fungal isolates

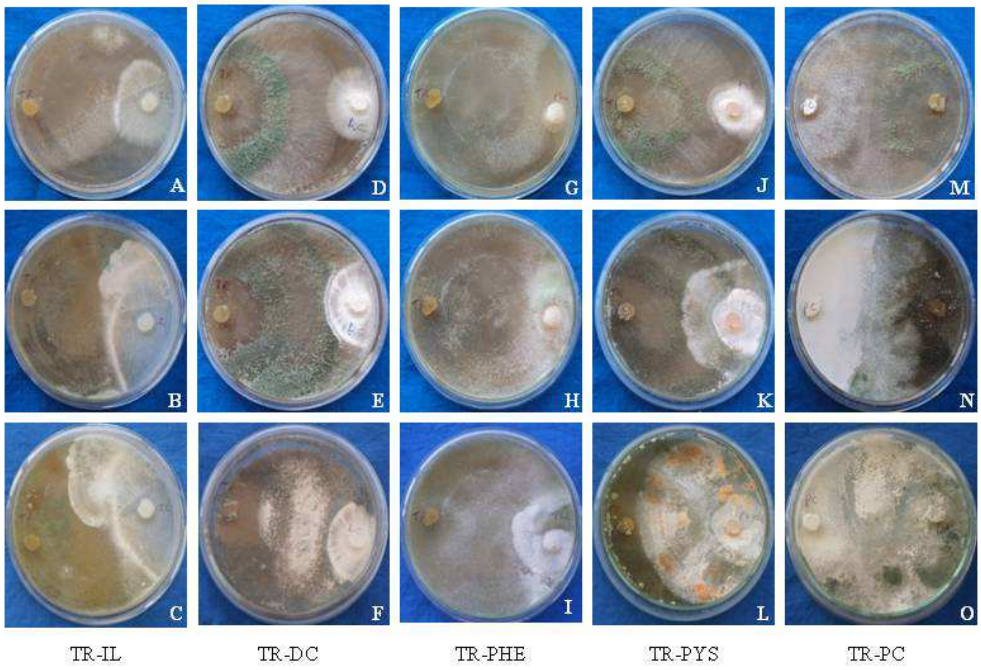

Fig. 3

Fig: 3 Paired interaction test of *Trichoderma reesei* with other fungal isolates

Fig. 2, 3

Paired interaction test of TR with the other 9 fungal isolates

Fig. 2
A-C: *TR-TV*
D-F: *TR -TH*
G-I: *TR -PE*
J-L: *TR -PF*
M-O: *TR-PO*
P-R: *TR-PS*

Fig. 3
A-C: *TR -IL*
D-F: *TR -DC*
G-I: *TR -PHE*
J-L: *TR -PYS*
M-O: *TV-PC*

A total of 11 interactions of different fungal isolates with *Trichoderma reesei* (Table 2) were studied at three different incubation periods. In all these 11 combinations, growh of TR was very fast compared to other fungal isolates and growth of the four *Pleurotus* sp. were found to be slower. The growth of other fungal isolates studied was relatively slower than TR but faster than the *Pleurotus* sp.

All the combinations of fast growing fungal cultures with TR showed mutual intermingling except the interaction with TV and TH which appeared to be a partial mutual intermingling (Fig. 1 B) and invasion/ replacement of early stage (Fig. 1 C) respectively. This interaction of TV and TH in the combination with TR was distinct on the 6th day when sporulation of both fungi started. At a later stage (9th day) the co culture showed clear overlapping zone followed by the growth of TV invasion/replacement and growth of both the fungi TR and TH further stopped.

Growth of TR towards all the *Pleurotus* sp. was very fast and within three days of incubation period completely growing and covering the whole culture plate. Growth of all *Pleurotus* sp. started on 6th day except PF which started to grow on the 3rd day itself. But on the 9th day TR over grows on PE and PF and TR covering the inoculum disc. These two combinations thus fall under the category invasion/replacement of later stage (Fig.1 D). Growth of PO

stopped on the 9th day is categorized to be invasion/replacement of later stage (Fig.1 D) and combination of TR with PS is mutual intermingling as PS also grows along with TR till the inoculums disc[13,14].

In the interaction of the two different fungi the pattern of hyphal growth (one below the other) was observed by Skidmore & Dickinson (1976),[13] and they explained the hyphal mode of growth by interacting fungi such as either above or below or above and below its colony, and its corollary. The partial mutual intermingling (represented diagrammatically in Figure 1), was recognized by Skidmore & Dickinson (1976),[13] as simply 'intermingling' and 'neutral intermingling' by Stahl & Christensen (1992)[14]. Among all mutual intermingling interactions of TR with fast-growing fungi, the interaction of all fungi with TR were observed quite clear, and showed overlapped hyphal growth without any kind of changes and abnormalities.

A total of three different modes of interacting fungi were observed from the present study which could be compared with the citations of Porter (1924) [12] and Stahl & Christensen (1992)[14]. Porter (1924)[12]described four interactions, but did not mention invasion/replacement. Stahl & Christensen (1992)[14] combined mutual and partial intermingling into one 'neutral intermingling', which was described in separate events (basically in three steps) by Porter (1924)[12]. They also combined the interactions *viz*. inhibition at contact point as well at a distance into deadlock and introduced the category of 'replacement'.

Trichoderma belonging to class *Ascomycetes* is one of the most widely studied genera for biocontrol agents [18]. On Malt agar medium, isolates of *Trichoderma viren, T. harzianum, T. polysporum* completely inhibited the growth of several white rot fungi [19]. Several attempts have been made to enhance enzyme production by the fungi co-cultivation strategy. Paranthaman *et al* 2009 [20] studied the effect of fungal co culture for production of tannic acid and gallic acid from grape waste under solid state fermentation. The co culture of *Penicillium chrysogenum* and *T. viride* produced highest activity than other organisms. Laccase activity in *Tremetes versicolor* was increased several times when this fungus was cocultivated with *Trichoderma harzianum* and other soil fungi [21]. Moreover, the induction of new isozymes was also observed in dual cultures of *Pleurotus ostreatus* with *Trichoderma longibrachiatum* [22] and *Trametes* sp. AH28-2 with a *Trichoderma* strain [23]. In the present study *Trichoderma resei* though very rapidly grow as compared to other *basidiomycetes* fungi but six of them are compatible with TR. *Trichoderma reesei* is known for producing xylanase enzyme and other fungi PS, IL, DC, PHE, PYS, PC are well known selective delignifying white rot wood decaying fungi interact synergistically in mixed culture and together helps in increase the process of delignification as well as brightness of cellulose fibers. The present study evaluated only three different interaction *viz*. mutual intermingling, partial mutual intermingling and invasion/replacement. Inhibition/deadlock at touching point and at a distance was not observed. From the present study it could be conferred that TR interact synergistically in mixed culture and is compatible with six white rot fungi (PS, IL, DC, PHE, PYS, PC) indicating its potential use for co culture in industrial applications.

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