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Evaluation of colony characteristics and enzymatic activity of some fungi for potential use in co-culture for bio pulping

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Abstract

The mycelial growth and colony characteristics of ten fungal isolates grown on Potato Dextrose Agar (PDA) medium were observed after seven days of inoculation at 25 ± 1 ^{0}C. The colony characteristic features appeared to be typical and varied with the species which is presented in the present study. The ten fungal isolates have been screened for its ligninolytic; cellulolytic and xylanolytic activity. Results show 4 of the fungal isolates to be lignin degraders as well as xylanase producers indicating its potential applications in paper industry for bio-bleaching process. Three species of *Pleurotus and Phellinus pectinatus* are selective lignin degraders having its application in bio-pulping. The two species of *Trichoderma* are ligninolytic as well as cellulolytic and xylanolytic. *Pleurotus sajorcaju* showing ligninolytic and xylanolytic activity it was selected for co-culture experiments and found to be compatible with *Daedaleopsis confragrosa and Phellinus pectinatus.*

INTRODUCTION

Fungi grow in diverse habitats in nature and are cosmopolitan in distribution requiring several specific elements for growth and reproduction. A wide range of media are used for isolation of different groups of fungi that influence the vegetative growth and colony morphology, pigmentation and sporulation depending upon the composition of specific cultures medium, PH, temperature, light, water, availability and surrounding atmospheric gas mixtures [1-4]. Different concepts have been used by the mycologists to characterize the fungal species out of which morphological (Phenetic or Phenotypic) and reproductive stages are the classic approaches and baseline of fungal taxonomy and nomenclature are still valid [5-8]. Potato dextrose agar medium (PDA) is one of the most commonly used culture media because of its simple formulation and its ability to support mycelia growth of a wide range of fungi [9-10].

Recently, extensive research on fungi has been conducted with an aim to isolate the organism with tremendously secretion of ligninolytic enzymes as well as enzymes with potential industrial applications [11-13].

In the Paper production process, pulping is the step where cellulose fibers are separated by removal of lignin by using chloride. This step even though necessary, is the prime cause of pollution as elemental chlorine reacts with lignin to form chlorinated lignin derivatives such as chlorolignols, dioxins and sulfur compounds [14-15]. Owing to this problem, more companies are investigating alternative methods such as biopulping, hydrogen peroxide or oxygen based delignification.

Paper obtained by biopulping appears to be yellowing so for brightening of cellulose fibers xylanase enzyme needs to be produced. A combination of two fungi which grows together would help in obtaining cellulose fibers of high quality by degradation of lignin and brightening of cellulose fibers gives rise to the hypothesis of Co-culture. In the present study two ascomycetes and 8 basidiomycetes white rot fungi have been screened to its ligninolytic, cellulolytic and xylanolytic activity.

MATERIALS AND METHODS

Isolation of fungi

Fruiting bodies were surface sterilized with 0.1% $Hgcl_2$ and inoculated in petriplate containing PDA medium under asceptic condition and incubated for 7 days at 25±2 Ċ. After development of colony these are sub cultured in slants. Cultures of all fungi except *Daedaleopsis confragrosa* and *Phellinus pectinatus* were obtained from Forest Research Institute, Dehradun.

Subculture of fungi

After 7days growth of sub cultured fungi on slant containing potato dextrose agar (PDA) medium. Disc of agar containing fungus was subcultured on Petriplates containing PDA medium and incubate for 7days at 25±2Ċ. Two replicates were maintained for each fungal subculture. The mycelia disc was inoculated in the centre of the petriplate (Fig.1 A) and also on the margin of the petriplate (Fig.1 B). These cultures were used for analyzing the cultural characteristics.

Screening of the fungal isolates for cellulolytic/ ligninolytic enzyme by Bavendamn test

Screening was performed by [16]. Fungal isolates were cultured on malt extract agar medium (MEA). For screening purposes malt extract agar medium (3%) was substituted with respective enzyme substrates viz., tannic acid (TA) for ligninases and carboxy methyl cellulose (CMC) for cellulases at pH 5.8 [17]. The Petriplates were incubated at 28±1 Ċ for 7 days. Three sets of replicates were maintained for all the selected fungi. The cellulolytic activity was evaluated by observing the zone of clearance if any, formed by flooding the plates with visualizing dye congo red for 15 min. ligninolytic activity was assessed by observing the dark brown colored zone around the fungal colony.

Screening of the fungal isolates for xylanolytic enzyme

Fungal isolates were screened for their abilities to produce extracellular xylanase during their growth in enriched malt extract agar medium (MEA) containing Xylan as the sole carbon source [18]. The composition of the medium was (g/l): birch wood xylan, 1.0; peptone, 5.0; yeast extract, 5.0; K_2HPO_4, 0.2 and agar

20.0. The inoculated plates were incubated for 5 days at 28 ± 1°C. Three sets of replicates were maintained for all the selected fungi. Positive xylanolytic isolates were detected based on the clear zones of hydrolysis after folding the plates with 0.1% aqueous Congo red followed by repeated washing with 1 M NaCl [19].

Paired interaction tests to detect the antagonistic effect of selected fungi

The isolates were maintained on potato dextrose agar (PDA) medium. In vitro antagonistic potential of *Pleurotus sajorcaju* was evaluated through co-culture technique in which the antagonistic effect of fungi was carried out on 3% malt extract agar (MEA). The isolates were screened for their antagonistic potential against the other fungal isolates by measuring the relative growth rates as a function of the incubation period. Five mm mycelial discs by the help of borer was taken from the margin of young vigorously growing 7 days old culture of *Pleurotus sajorcaju* was inoculated at the margin of the petridish (90mm) containing 20ml sterilized MEA medium at opposite sides of it other fungi was inoculated and then incubated in dark at 25±2˚c with 70% relative humidity for 4 weeks. Petri dishes inoculated with individual fungi were used as controls. Three replicates were used for each experiment. Photographs were taken on digital Sony Cybershot model no. DSC-H2O.

RESULT AND DISCUSSION

In the present study it could be distinctly observed that the mycelial growth and colony characteristics of the fungi studied were typical and showed great variation when grown on PDA medium. The characteristic colony features (form, surface, texture, color, elevation and margin) of the ten fungal isolates analyzed are presented in table 1 and Colony characters are presented in Fig. 1 (C-L). Among the 4 different spp. of *Pleurotus*, ***Pleurotus sajorcaju and Pleurotus eryngii*** show similar culture characteristics. *Trichoderma viride* and *Trichoderma harzianum* could be clearly differentiated from the colour of the mycelial mat and sporulation. In *Trichoderma viride* the mycelium appear light green but in *Trichoderma harzianum* dark green zone appears with sporulation. (C, D).

Trichoderma viride Pres. (Fig.1-C)

Rapidly growing colony very, initially hyaline, soon becoming whitish green with tufted conidial areas in dark-green shades, first in the middle and later the entire colony.

Trichoderma harzianum Rifai. (Fig.1-D)

Rapidly growing colony, initially glassy white, soon with dull green tufts of sporulation, first in the middle, later the entire colony. Sporulation compacting in the center but undulating concentric rings towards the edges.

Pleurotus sajorcaju Fr. (Fig.1-E)

Colony growth moderate. The plate covered in a 1 week. Advancing zone even, with flat white cottony surface and dense marginal hyphae. Growth of hyphae near the innoculum with arachnoid zones.

Pleurotus ostreatus Jasq. (Fig.1-F)

Colony growth moderately slow. The plate covered in 2-3 weeks. Advancing zone even with raised aerial mycelium extending to growth. Surface downy and radially grooved. Mycelium hyaline, growth feathery with whitish zones near the innoculum. Increased aerial mycelium and more cottony at second week.

Pleurotus florida Mont. (Fig.1-G)

Colony growth moderate. Mycelium hyaline, with arachnoid zones near the inoculum initially. But with an increased aerial cottony mycelium with irregular margin at a later stage. Mycelial mat produces a yellow volatile compound in the medium. Advancing zone initially cottony but towards margin becomes slimy or buttery.

Pleurotus eryngii Dc. (Fig.1-H)

Colony growth moderately rapid. The characteristic feature of the colony appears almost similar to *Pleurotus sajorcaju*. The plate covered in a 1 week. Advancing zone even with dense marginal hyphae, raised aerial, mycelium extending to growth.

Irpex lacteus Fr. (Fig.1-I)

Colony growth moderately rapid. Mats white, margins even, slightly raised, isolates around inoculums thin, then becoming moderately thin to thick, raised loosely, cottony towards margins. Advancing zone even, hyaline and slightly raised. Hyphae distant and branched. Aerial mycelium at first downy, becoming thinly cottony white.

Pycnoporus sanguineus L. (Fig.1-J)

Colony growth moderately rapid. The plates usually been covered in 4 days. The advancing zone is even. The mats are white at first, slightly raised, plain to fine woolly-floccose, thin and translucent, with some appressed opaque white areas, usually beginning as complete zone surrounding the inoculum. Color begins to appear after 3 to 4 days, as granules of moderate orange over the inoculum. In 2 weeks individual mats show intensification of color or increase in extent of colored areas or in thickness of raised mycelium.

Daedaleopsis confragosa Boltom. (Fig.1-K)

Growth moderately rapid, the plates get covered in a 1 week. The surface is with smooth texture. Appearing as a light white translucent cottony mass showing a dendroid (tree like) margin. No sporulation.

Phellinus pectinatus Kl. (Fig.1-L)

Growth slow, plates covered in 1 to 2 weeks. Advancing zone even with white, slightly raised aerial mycelium. Mycelium mat initially cottony, later becoming fully white. Mycelial mat raised to the top of the petri plate in the vicinity of innoculum and gradually sloping to level of agar. Margin thick and woolly.

Screening of ligninolytic, cellulolytic and xylanolytic activity

It is known for a long time that the mycelia of certain higher fungi contains enzymes which catalyze the oxidation of phenols and related compounds [20]. Bavendamn, was the first to point out the difference between the white rot and brown rot fungi with respect to their oxidative enzymes. When cultivated on nutrient agar containing certain phenolic compounds as gallic acid or tannic acid, the white rot fungi produce a deeply brown colored zone around the mycelium while the fungi causing brown rot do not.

The production of strongly oxidizing enzymes acting on certain phenolic compounds is a constant characteristic of a number of wood destroying fungi. The production of different oxidizing enzymes by the mycelium was tested by the addition to the mycelium, drops of phenolic compound (Congo red) in

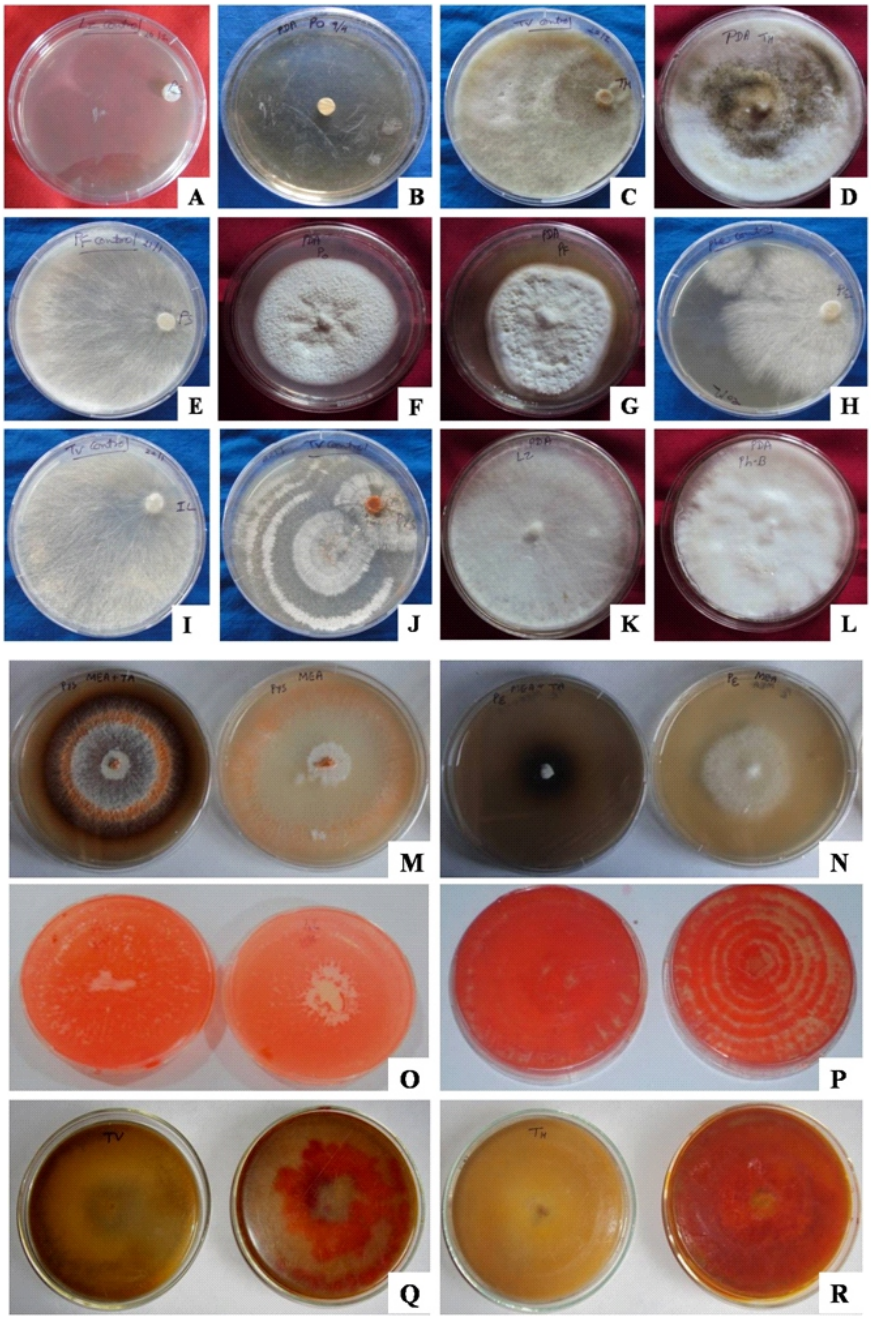

Fig. 1 (A-L) Colony characteristics features of fungal isolates on PDA medium

A: Inoculation of fungal isolate at the edge of the petriplate
B: Inoculation of fungal isolate in the centre of the petriplate
C: *Trichoderma viride*
D: *Trichoderma harzianum*
E: *Pleurotus sajorcaju*
F: *Pleurotus ostreatus*
G: *Pleurotus florida*
H: *Pleurotus eryngii*
I: *Irpex lacteus*
J: *Pycnoporus sanguineus*
K: *Daedaleopsis confragosa*
L: *Phellinus pectinatus*

Fig. 1 (M-R) Ligninolytic, cellulolytic, xylanolytic activity of fungal isolates

M: Ligninolytic activity of *Pycnoporus sanguineus:* upper view of the petriplate showing brown colour in the whole area of colony
N: Ligninolytic activity of *Pleurotus eryngii*: upper view of the petriplate showing brown colour in the whole area of colony
O: Xylanolytic activity of *Irpex lacteus:* lower view of the petriplate showing zone of clearance surrounding the colony
P: Xylanolytic activity of *Pycnoporus sanguineus:* lower view of the petriplate showing zone of clearance surrounding the colony
Q: Cellulolytic activity of *Trichoderma viride*: lower view of petriplate showing zone of clearance surrounding the colony
R: Cellulolytic activity of *Trichoderma harzianum*: lower view of petriplate showing zone of clearance surrounding the colony

alcoholic solution. If the phenolic compounds in the presence of certain oxidative enzymes were oxidized a marked color reaction specific to the substances tested appeared immediately or after some hours.

With most of the fungi the color produced was stable during a week and more but with some others the colors of several reagent especially of gallic acid, tannin were bleached after 3-5 days. The result of ligninolytic, cellulolytic, xylanolytic activity is shown in Fig.1 (M-R).

In the present study out of 10 fungi, 8 showed positive reactions to tannic acid used in the test, indicating them to release lignin degrading enzymes and hence are potential lignin degraders. The data from the Bavendamn test provided evidence for the presence of laccase activity in those fungi. Out of 10 fungi, 5 showed zone of clearance when flooded with 1% Congo red dye solution indicating production of xylanase enzyme.

The fungal isolates identified for efficient production of ligninolytic enzymes and xylanolytic enzymes having potential application in paper industry because ligninolytic enzymes degrade lignin and leave cellulose and hemicellulose from which xylan is degraded by xylanase enzyme produced by fungi and cellulose remains free and quality of paper depending up on amount of cellulose present in the paper.

Trichoderma viride and *Trichoderma harzianum* showed a positive reaction to both cellulolytic and ligninolytic enzymes indicating it to be ligno-cellulolytic degraders which would cause a simultaneous type of decay. Selective lignin degraders may have significant potential bio technological applications when the removal of lignin is required to obtain intact cellulose such as in bio-pulping process.

Paired interaction test

Compatibility of the fungi in the co-culture / paired interaction tests distinguished into three types.

1. The two fungal isolates in the paired interaction test come in contact and growth of both fungal isolates are inhibited i.e. No further growth occurs once they 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 *Pleurotus sajorcaju* with *Irpex lacteus Daedaleopsis confragosa, Phellinus pectinatus and Pycnoporus sanguineus* represented individually as below in Fig:2(A-L). *Pleurotus sajorcaju* with other 3 species of *pleurotus* namely *Pleurotus eryngii, Pleurotus florida, Pleurotus ostreatus* and 2 species of *Trichoderma* namely *Trichoderma harzianum and Trichoderma viride* represented in Fig:3(A-O).

(1) *Pleurotus sajorcaju Irpex lacteus* (PS-IL) (Fig.2 A-C)

After the inoculation at the 4th day the growth of IL was more than PS and both fungi come in contact with each other. IL and PS equally advance and come in contact. At the 7th day the IL overgrow on PS and overlapping zone was visible and the growth of PS stopped .At 10th day IL growth increase and the overlapping zone was also increased. Overlapping zone of IL on PS is distinct. However PS is not killed.

(2) *Pleurotus sajorcaju- Daedaleopsis confragosa* (PS-DC) (Fig.2 D-F)

After the inoculation at the 4th day the growth of PS was higher than DC. At the 7th day the growth of DC was increased than the PS and contact between both fungi occur and the growth of PS was stops. At the 10th day DC overgrows on PS and further it covers the whole plate. DC goes on advancing over PS and does not kill it but growth is inhibited. Even after 3 weeks the growth of PS is slightly increased. DC grows profusely covering the petriplate. Both are compatible but growth of PS is very slow.

(3) *Pleurotus sajorcaju-Phellinus pectinatus* (PS-PHE) (Fig.2 G-I)

After the inoculation at the 4th day the growth of PS was more than PHE. PHE growth was slow. At the 7th day both fungus comes under contact with each other. At the 10th day the growth of PHE was increase and PHE can overgrow on PS and the overlapping zone was form. The growth of PS does not stop here. PHE over grows PS further and the two continue to grow. The two are compatible and grow together.

(4) *Pleurotus sajorcaju-Pycnoporus sanguineus* (PS-PYS) (Fig.2 J-L)

After the inoculation at the 4th day the growth of PYS was more than PS. At the 7th day both fungus comes under contact with each other. At the zone of contact PYS sporulation gets covered by PS. At the 10th day both fungi can overgrow on each other and overlapping zone is visible and at particular stage. Further the growth of PS first stops and then PYS stop. PS advances 0.6 cm over PYS and then growth of both fungi stops.

(5) *Pleurotus sajorcaju- Pleurotus eryngii* (PS-PE) (Fig.3 A-C)

After the inoculation at the 4th day the growth of PS was more than PE. At the 7th day both fungus comes under contact with each other and PS can overgrow on PE and overlapping zone of 1.3cm was clearly visible. At the 10th day the PS almost cover the whole plate and the growth of PE was inhibited. PS does not kill.

(6) *Pleurotus sajorcaju- Pleurotus florida* (PS-PF) (Fig.3 D-F)

After the inoculation at the 4th day the growth of PS was more than PF. Growth of PF is very slow. At the 7th day both fungus comes under contact with each other PS advances faster than PF and reaches almost 3/4th of petriplate where it comes in contact with PF and the growth of PF is inhibited. At the 10th day PS can overgrow on PF and it covers the whole plate. Slowly advancing even towards the cover/ lid of the petriplate.

(7) *Pleurotus sajorcaju- Pleurotus ostreatus* (PS-PO) (Fig.3 G-I)

PO is also a very slow growing fungi. After the inoculation at the 4th day the growth of PS was more than PO. At the 7th day the growth of PS was very higher than PO reaching almost 3/4th of petriplate and PS overgrows on PO. At the 10th day PS almost cover the whole plate and the growth of PO was inhibited. PO advances with growth of PS but at every slow rate.

(8) *Pleurotus sajorcaju-Trichoderma harzianum* (PS-TH) (Fig.3 J-L)

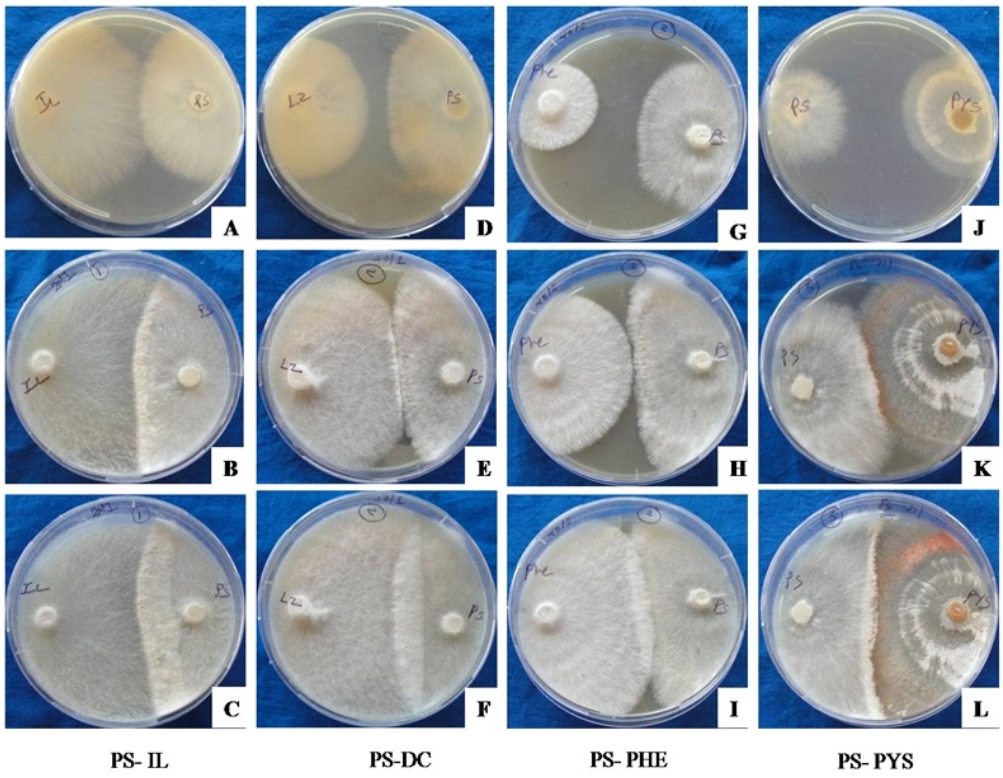

Fig: 2(A-L) Paired interaction test of *Pleurotus sajorcaju* with 4 different fungal isolates
A-C: *Pleurotus sajorcaju Irpex lacteus*
D-F: *Pleurotus sajorcaju- Daedaleopsis confragosa*
G-I: *Pleurotus sajorcaju-Phellinus pectinatus*
J-L: *Pleurotus sajorcaju-Pycnoporus sanguineus*

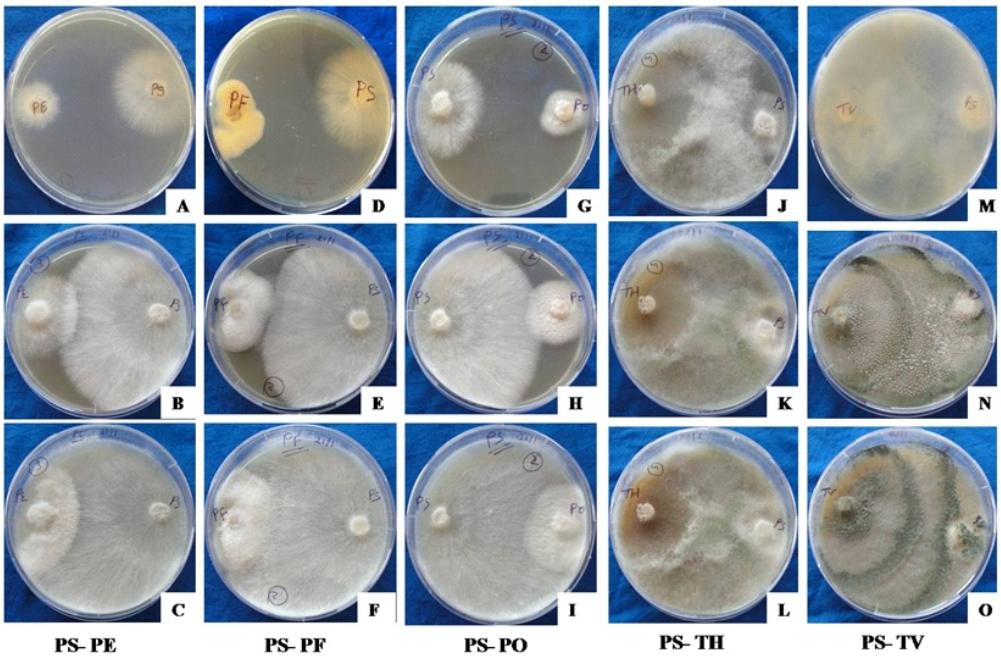

Fig: 3(A-O) Paired interaction test of *Pleurotus sajorcaju* with 5 different fungal isolates
A-C: *Pleurotus sajorcaju- Pleurotus eryngii*
D-F: *Pleurotus sajorcaju- Pleurotus florida*
G-I: *Pleurotus sajorcaju- Pleurotus ostreatus*
J-L: *Pleurotus sajorcaju-Trichoderma harzianum*
M-O: *Pleurotus sajorcaju-Trichoderma viride*

Table 1. : Colony characteristics and enzymatic activity of fungal isolates

Fungal isolate	Form	Surface	Texture	Color	Elevation	Margin	Zonation	Ligninolytic/ Cellulolytic/ Xylanolytic
Trichoderma viride	Irregular	Rough	Dry	Light green cloudy and translucent	Crateriform	Curled	A single peripheral concentric ring with central furrow	Ligninolytic Cellulolytic and xylanolytic
Trichoderma harzianum	Circular	Rough	Moist and cottony	Cloudy and opaque	Crateriform	Entire	A single peripheral concentric ring with dark green sporulation	Ligninolytic Cellulolytic and xylanolytic
Pleurotus sajorcaju	Filamentous	Veined	Dry and fibrous	Cloudy and translucent	Flat	Entire but filiform/ filamentois	Shallow centre and slightly raised margin	Ligninolytic and xylanolytic
Pleurotus ostreatus	Circular	Rough and shrivelled	Moist and britlle	White opaque	Umbonent with knotty protuberance	Entire	Centre with radiating furrows with raised fine margin	Ligninolytic
Pleurotus florida	Circular to slightly irregular	Rough	Moist and butyrous at the margin	White opaque	Umbonent having a knotty protuberance and elevated margin	Entire	Centre with shallow radiating furrows, margin with irregularly distributed pits.	Ligninolytic
Pleurotus eryngii	Filamentous	Veined	Dry and fibrous	White translucent	Flat	Filiform	No zonation	Ligninolytic
Irpex lacteus	Filamentous	Veined	Dry and fibrous	White translucent	Flat	Filiform	With shallow centre and slightly raised margin	Ligninolytic and xylanolytic
Pycnoporus sanguineus	Circular	Dull and shrivelled	Dry, fibrous and powdery	White translucent	Undulating	Curled	With concentric zones of opaque white and translucent regions	Ligninolytic and xylanolytic
Daedaleopsis confragosa	Circular and filamentous	Veined	Dry and fibrous	Translucent	Flat	Filiform	No zonation	Ligninolytic
Phellinus pectinatus	Irregular	Glisternng	Moist and viscous	Opaque	Raised and undulating	Undulated	No zonation but with shallow pits in centre	Ligninolytic and xylanolytic

After the inoculation at the 4th day the growth of TH was more than PS. It covered more than 80% of the petriplate. At the 7th day TH almost cover the whole plate and the growth of PS was inhibited. At the 10th day the situation remains same. The growth of TH further advanced and become dense. PS is killed.

(9) *Pleurotus sajorcaju-Trichoderma viride* (PS-TV) (Fig.3 M-O)

After the inoculation from the 4th day TV almost covers the whole plate and PS just start the growth but after that the growth of PS inhibited. At the 7th day TV start sporulation and green spores were observed in concentric zones. At the 10th day the situation remain same and it killed PS. TV covered the PS inoculums and sporulation was also observed in this zone (fig). PS advances fast and grows up to almost half of the petriplate in 5 days. PS inhibits the growth of PE and overgrows it without killing it. PS when comes in contact with PYS growth of both the fungi is inhibited. The other fungal isolates inhibit growth of PS in time of one week. TV and TH completely over grow PS killing it. TV and TH also produce sporulation and PS and LZ are compatible. PO is also compatible with PS.

CONCLUSION

The digestibility of lignocellulosic materials is very low because of the inherent lignin in cellulose and hemicelluloses matrix. Cell wall lignifications of crop residues have been reported as a major factor that limits the availability of cell wall structural carbohydrate for utilization.

Some species of *Pleurotus* are able to colonize different types of lignocellulosic materials including agricultural wastes and agro industrial products increasing their digestibility [21-23]. *Pleurotus sajorcaju* showing ligninolytic as well as xylanolytic activity selected for the co-culture experiments and found to be compatible with *Daedaleopsis confragosa and Phellinus pectinatus.*

Daedaleopsis confragosa and *Phellinus pectinatus* are White rot fungi which produce several enzymes having ability to degrade lignin selectively. Co-culturing of *Pleurotus sajorcaju* with *Daedaleopsis confragosa* and *Phellinus pectinatus* would be highly potential in increasing the lignin degradability and can be use for the purpose of biopulping.

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