Evaluation of potential compatible co-partner for lignin degrader *Irpex lacteus*

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Biological agents are important alternative to chemical agents in pulping and bleaching process of paper industry. *Irpex lacteus*, a white rot fungus with a great biotechnological potential, is currently considered the most important lignocellulose degrading organism because of its potential to degrade lignin and bio-remEDIATE other lignin related pollutants. Experimental evidences of mixed cultures/co-cultures of fungal isolates suggest improved ligninolytic activity compared to monocultures. Here, we explored potential compatible fungal co-partner for *Irpex lacteus* to produce enhanced ligninolytic enzymes. Results of paired interaction test showed that among the eleven fungal isolates evaluated, *I. lacteus* was compatible with three species of *Pleurotus*, *Phellinus* and *Daedaleopsis confragrosa*. The results suggest the above three fungal isolates to be potential fungal partner for *I. lacteus* in co-culturing for efficient lignin degradation and biobleaching in pulp and paper industries.

**Keywords:** Basidiomycetes, Biobleaching, Daedaleopsis confragrosa, Ligninolytic enzymes, Paper industry, Pleurotus, Phellinus, Pulping, White rot fungi

Paper mills are responsible for many environmental problems. Different Biotechnological techniques, such as enzyme engineering has helped in developing economically feasible and well-designed methods in production of pulp & paper sector. Chemical pulping utilizes large amounts of chemicals, such as chlorine and chlorine dioxide to breakdown and also soften the wood, and thereby pollute the environment to great extent. The concept of biopulping process is based on the ability of white rot fungi to colonize and degrade lignin leaving behind cellulose intact. This process has potential and needs to be scaled up for industrial level, with optimization of various steps involved in the process of pulping for economic feasibility. This biobleaching, an effective alternative to the alkali and chemical bleaching, not only reduces the utilization of chemicals and energy, pollutants but also increases the yield and the strength of pulp.

Mixed culture appears to be more successful than using single microorganism cultures, because of the potential to utilize synergisms between the metabolic pathways of the strains involved in co-culture. Mixed culture fermentation process applies two or more organisms and are widely used for many processes including the production of antibiotics, enzymes, single cell protein production and domestic waste water sludge. In co-culture, evaluation of antagonistic effect of the selected fungal co-cultures is important. Gelber et al. demonstrated *Aspergillus niger* and *A. oryzae* mixed cultures to secrete broader range of plant cell wall degrading enzymes than the respective monocultures. Interaction between the two fungal strains were stable and there was no sign of competition which makes the two fungi compatible for co-cultivation.

In the present study, we tried to identify compatibility of potential biobleaching fungal strains for its efficient co-culturing to increase the efficiency of biopulping and thereby provide useful guidelines for further investigations to pursue and develop efficient treatment system for paper industry.

**Materials and Methods**

**Source of Fungal isolates**

Eleven different fungal isolates were evaluated for its compatibility with *I. lacteus*. Fungal isolates of *Phellinus pectinatus* (L.) Quel. (PHE) and *Daedaleopsis confragrosa* J. Schrot. (DC) were isolated from fruiting bodies obtained from naturally growing habitat on the trunk of growing *Peltophorum* in the Arboretum of The Maharaja Sayajirao University of Baroda, Gujarat, India. The fungal isolates of *Trichoderma reesii* Simmons. (TR) 4876 and *Phanerochaete chrysosporium* Burdass. (PC) 787 were procured from MTTC culture collection centre, Chandigarh. and *Irpex lacteus* (Fr.) Fr. (IL), *Trichoderma viride* Pers. (TV), *Trichoderma harzianum* Rifai. (TH), *Pleurotus florida* (Jacq. exfr.) P. Kumm (PF), *Pleurotus ostreus* (Jacq. exfr.) P. Kumm (PO), *Pleurotus eryngii* (DC.) Quel. (PE), *Pleurotus sajorkaju* Fr. (PS) and *Pycnoporus*
sanguineus (L.) Murrill. (PYS) were procured from Forest Research Institute, Dehradun, India.

All fungal strains were maintained on Potato Dextrose Agar (PDA) at 4 (±1)°C in Seed Anatomy Laboratory of Department of Botany at The Maharaja Sayajirao University of Baroda, Gujarat, India. Petriplates containing PDA medium were inoculated with 0.5 cm diameter agar plug, cut from growing edges of colonies of the isolates and incubated at 25 (±1)°C in dark.

Screening of fungal isolates for enzyme activity test

Fungal isolates were first screened in laboratory to determine ligninolytic, cellulolytic and xylanolytic enzyme activity\(^\text{10}\). The ability of fungi to produce extracellular cellulose free xylanase during their growth was determined by xylan-agar diffusion method. Fungal inoculum was inoculated on malt extract agar medium (2%) with tannic acid (0.5%) to test ligninolytic enzyme activity. Similarly, for cellulose activity test, carboxymethyl cellulose (0.5%) was added into medium. Ligninolytic enzyme activity was analyzed by observing the dark brown coloured zone around the fungal colonies. Cellulolytic and xylanolytic enzyme activities were analysed by observing zone of clearance around the fungal colonies\(^\text{11}\).

Paired interaction test on agar plates

The paired interaction method was used to determine compatibility of two fungi to grow together. Agar plug containing 5 mm diameter was cut from the growing edge of 10 days old fungal culture and inoculated at the margin of the Petriplate 20 mL autoclaved malt extract agar (MEA) medium. The agar plugs of two different fungi were inoculated on opposite sides of Petriplate and incubated at 25 (±2)°C for 4 weeks. Monocultures of each fungus were kept as control. Three replicates were kept for each set of experiment. A schematic diagram of the interaction given by Porter 1924 is shown in Fig. 1.

The compatibility of two test fungi was analysed by observing the interaction between those two test fungi at the zone of contact as per Porter\(^\text{10}\). According to Porter\(^\text{10}\), there are five different modes of interaction: (1) Mutually intermingling: where both fungi grow into one another without any macroscopic signs of interactions; (2) Intermingling growth: This interaction has been further categorized into (i) where the fungus grow into the opposed fungus either above or below or above and below its colony, and its

Results and Discussion

The purpose of the present study was to screen a fungal co-culture that produce ligninolytic enzymes along with xylanase. Bavendamn (1928) was the first to point out the difference between the white rot and brown rot fungi with respect to their oxidative enzymes\(^\text{11}\).

Bavendamn test was used to determine ligninolytic and cellulolytic enzyme activities of fungal species. When white rot fungi cultivated on nutrient agar containing certain phenolic compounds as gallic acid, the white rot fungi produce a deeply coloured zone around the mycelium while the brown rot fungi do not produce coloured zone. Confirmatory results obtained by Bavendamn’s test earlier in our laboratory is given in Table 1\(^\text{13}\).
other at its own pace with the formation of an overlapping zone which increases towards both the sides.

A total 11 interactions of fungal isolates with IL were studied at three different incubation periods. In all 11 co-cultures, IL was found growing faster compared to other fungal isolates except in combination of IL-TH.

Based on the observations on 5th, 8th and 11th day indicated in Table 2. It could be concluded that IL was found to be compatible with 6 fungi isolates which are DC, PE, PF, PHE, PS and TR. The observations are shown in Figs. 2 and 3.

IL showed partial intermingling with all the three species of Pleurotus except PO, with which it showed invasion. While IL showed mutual (compatibility) only with one species of Trichoderma which is TR and with other two species TH and TV, it showed invasion and deadlock at touching point respectively. Combination of IL and PHE showed mutual intermingling, while with PYS and PC it showed deadlock at touching point. Growth of IL towards all the Pleurotus sp. was very fast and within five days of incubation period completely growing and covering the whole plate. IL overgrows on PC and IL covering the inoculum disc, thus this combination falls under the third category which is called as invasion. DC and PHE overgrows on IL.

The first report on the effects of fungal co-culturing was reported by Chi et al.2. They investigated the effects of co-culturing two white rot fungi Ceriporiopsis subvermispora and Physosporinus rivulosus on the production of lignin degrading enzyme activities. The effects of co-culturing of three different white rot fungi promising in biopulping Ceriporiopsis subvermispora, Phanerochaete chrysosporium and Pleurotus ostreatus was evaluated by Chi et al.2 who found Pleurotus ostreus to be promising fungal partner in cultures aimed for biopulping. Co-culture of Clostridium acetobutylicum and Saccharomyces cerevisiae was had shown improvement of butanol production in study driven by Hongzhen Luo et al.14. The study reveals that fungal strain could secrete amino acids under stress environment which may be desirable for butanol synthesis14. Eka metreveli et al.15 found 11% increased lignocellulose saccharification potential compared to enzymes produced by I. lacteus monoculture. In their co-culture study, increase in cellulase and xylanase through compatible

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Table 1 — Ligninolytic, cellulolytic and xylanolytic activity of different fungi

<table>
<thead>
<tr>
<th>Fungal isolates</th>
<th>Ligninolytic</th>
<th>Cellulolytic</th>
<th>Xylanolytic</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trichoderma virida</em> (TV)</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td><em>Trichoderma hazarium</em> (TH)</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td><em>Trichoderma reesei</em> (TR)</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td><em>Pleurotus sajorjka</em> (PS)</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td><em>Pleurotus ostreatus</em> (PO)</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td><em>Pleurotus floria</em> (PF)</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td><em>Pleurotus eryngii</em> (PE)</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td><em>Irpex lacteus</em> (IL)</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td><em>Pycnoporus sanguineus</em> (PYS)</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td><em>Daedaleopsis confragosa</em> (DC)</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td><em>Phellinus pectinatus</em> (PHE)</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td><em>Phanerochaete chrysosporium</em> (PC)</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

All fungal species except *Trichoderma reesei* showed positive reactions to tannic acid, indicating them to be potential lignin degrader. The colour reactions were checked after 5th, 8th and 11th days. The medium showed brown coloured zone when viewed from the lower side of Petridish indicating the 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 the absence of cellulolytic enzyme.

The quality of paper depends upon the amount of cellulose present in the paper. The enzyme activities (xylanase and cellulase) were carried out, measured and rapid plate technique was carried out for selection of potential cellulase-free xylanase producing fungi, because xylanase without cellulase are important in paper industry. IL showed its broad range of xylanase activity.

Compatibility of fungi in the conducted co-culture tests could be distinguished into three categories: (i) Both fungi come in contact on the PDA medium and growth of both fungal isolates are inhibited i.e. No further growth occurs once they come in contact; (ii) The two fungal isolates in the paired interaction tests come in contact and growth of one is inhibited by the other but it is not killed; and (iii) 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 when they come in contact and still each one grows over the
interactions between *I. lacteus* and *Schizophyllum commune* has been reported.

*Trichoderma viride* was tested for its antagonistic potential against white rot wood decay fungi *Ganoderma adspersum, G. lipsiense, Inonotus liespidus, Polyporus squamosus* and ascomycete *Kretzschmara*. *Trichoderma reesei* was observed to be a compatible fungal partner with *T. viride, Pleurotus ostreatus, P. sajorkaju, Irpex lacteus, Daedaleopsis confragosa, Pycnoporus sanguineus* and *Phanerochaete cryosophorium*. In Highley’s study, co-cultures of *I. lacteus* and *T. viride* were found to...
overgrow and kill the fungi. Fungal isolates of *T. virens*, *T. harzianum* and *T. polysporum* completely inhibited the growth of white and brown rot.17

Hiscox and Boddy18 indicated that such interactions could be beneficial for novel antibiotics with different combinations of fungal competitors stimulation production of specific compounds. Co-cultivation of *Ganoderma lucidum* and *Trametes versicolor* showed remarkable enhancement of laccase activity. Faster decolonization of malachite green was observed in co-cultivation than in monocultures19. The co-culturing of fungal strains could sustain more changeable environments; hence, stability of co-culture system would be higher than monoculture. Inoculation ratio should be maintained in co-existence of fungal strains; it may stimulate the desired output20.

The present study indicates that *Irpex lacteus* is a compatible fungal partner of *Trichoderma reesei*, *Pleurotus ostreatus*, *P. sajor-caju*, *Daedaleopsis confragosa*, *Pycnoporus sanguineus* and *Phanerochaete chryosporium* which can be used for efficient production of lignin degrading enzymes and brightening of cellulose fibres in pulp and paper industries.

References