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Original Research Article

Pattern of bamboo culm degradation by *Daedaleopsis confragrosa* when co-cultured with selected fungi

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Abstract: This study investigates the anatomical and biochemical alterations in bamboo culm when co-cultured with two different fungi. Compatibility of fungi was analyzed by paired interaction test which revealed that *Daedaleopsis confragrosa* was found to be compatible with *Pycnoporus sanguineus* and *Irpex lacteus*. In vitro decay test was carried out to understand the pattern of delignification in bamboo culm by *D. confragrosa*, *P. sanguineus* and *I.lacteus.* Culm blocks inoculated with these fungi were analyzed for different time periods viz. 10, 20 and 30 days after fungal inoculation. Culm blocks inoculated with *D.confragrosa*, *I. lacteus* showed both the pattern of degradation i.e. selective delignification in the initial stage followed by simultaneous degradation during later stage of decay. Both fungal strains initially showed cell separation due to dissolution of middle lamella in early stage of decay and in later stage of decay *D. confragrosa* produced bore holes and *I. lacteus* showed erosion troughs. *P. sanguineus* produced selective delignification in initial stage delignification was observed in fibers and fungal hyphae colonize vessel elements and later on removal of middle lamella was clearly observed. *D. confragrosa* When co-cultured with *P. sanguineus* both characteristic features of the decay pattern could be observed i.e. loosening of cells and presence of bore holes as the pattern was significantly different. The weight loss found in 30 days incubation period was more in co cultured bamboo culms than in monocultured bamboo culms and Bio chemical analysis clearly indicated that lignin loss was also more in co-culture than in the monocultures. Results reveal that rate of degradation was more which could be very well depicted by the bio chemical analysis of lignin and cellulose. The anatomical observations also supported the result.

Keywords: Co- culture*, Daedaleopsis confragrosa, Irpex lacteus, Pycnoporus sanguineus,* Culm decay

Introduction

In the process of pulping in the paper production cellulose fibers are extracted while lignin is removed from the wood material. Treatment of wood chips with lignin-degrading fungi prior to pulping has been shown to have great potential for improvements in mechanical and chemical pulping [1, 2, 3, 4].

Isolation and screening of fungal strains suitable for bio pulping have been performed in many laboratories since the 1970s. The benefits of pretreating wood chips with white-rot fungi, which degrade wood lignin during incubation time 2-4 weeks have been described by several authors [5, 6, 7, 8, 9].

Xylanase enzyme treatment improves the chemical liberation of lignin by hydrolyzing residual xylan. This reduces the need for chlorine based bleaching chemicals required to brighten the cellulosic pulp in subsequent bleaching stages in the paper industry [10, 11, 12]. Many fungi are known to produce xylanase [13, 14, 15].

Trichoderma spp.*; Aspergilus* spp. and *I. lacteus* are the best known fungi which produce enzymes with high xylanolytic activity [16, 17, 18, 19, 20]. For pulp treatment xylanase must be free of cellulase.

Cellulase-free xylanase preparations have been tested successfully in industrial applications such as the prebleaching of kraft pulp in the pulp and paper industry [21, 22]. Study of [23] showed the fungal treatment of cornstalks with *I. 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

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increased loss of lignin (from 75.67% to 80%) and xylan (from 40.68% to 51.37%).

Mixed fungal cultures could lead to a higher enzyme production through synergistic interactions but the final result seems to depend on the particular species combination or on the mode of interactions between species, and on the micro environmental or nutritional conditions in the substrate under colonization [24]. Dual cultures are known to degrade wood more than monocultures [25]. According to [26] enhancement in bleaching of pulp could be obtained with mixed enzyme produced through co-cultivation of *Penicillium oxalicum* and *Pleurotus ostreatus*, which in fact resulted in decrease in kappa number. Antagonism a partial or complete suppression of activity or effect of other isolate by particular one is an important aspect to be considered for the co culturing experiments. Antagonist means one suppresses the effect of another. Antagonistic effect of fungi is the consequence of one fungus counteracting the effects of another fungus. Antagonistic property of fungi can be as the control of wood attacking fungi with fungal biocontrol agent. Most widely studied genera of fungal biocontrol agent *Trichoderma*, which has controlled several soil-borne pathogens under experimental conditions [27]. On a malt agar medium, an isolate of *Trichoderma virens* completely inhibited growth of several white and brown rot fungi [28]. 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 biopulping by degrading lignin in an eco friendly manner and also would help in brightening the cellulosic fibers preventing yellowing of paper. Synergistic interaction is a phenomenon in which interaction of two or more organisms resulting in overall effect which is greater than the sum of individual effect of any of them. Simultaneous action of xylanase and lignin degrading enzymes may prove to be a promising strategy to achieve higher degree of pulp bleaching. The action of xylanase would expose lignin which will be degraded and removed due to presence of lignin degrading enzymes, therefore leading in to improved level of delignification.

Dual culture or co-culturing of fungi means oxidative stress to both fungal partners and acceleration of fungal metabolic switch to secondary metabolism stimulating wood decay and production of lignin degrading enzymes.

The effects of co-culturing two white rot fungi *Ceriporiopsis subvermispora* and *Physosporinus rivulosus* on the production of lignin degrading enzyme activities were investigated [9] which was the first report on the effects of co-culturing of potential biopulping fungi on wood degradation and gives basic knowledge on fungal interactions during wood decay that can be utilized in practical applications.

The chemical alteration following inoculation of *Acacia mearnsii, Eucalyptus dunii, Eucalyptus grandis*, and *Eucalyptus macarthurii* with *Pycnosporus sanguineus / Aspergillus flavipes* co-culture in which cellulose degradation was less in co culture compared to its mono culture of white rot fungi [29]. Evaluation of the effects of co-culturing of four different white rot fungi promising in Biopulping *Ceriporiopsis subvermispora Phenerochaete chrysosporium* and *Pleurotus ostreatus*. Chemical analysis of decayed aspen wood blocks showed that co-culturing of fungi could stimulate wood decay depending on interacting species. Lignin degradation pattern during a two-week incubation period was also altered especially in co-cultures where *P. ostreatus* was included as a partner [9].

With this background, the aim of the study was to analyze and ascertain the impact of co-culturing *D. confragrosa* with *I. lacteus* and *P. sanguineus* on the degradation of Bamboo culm which is used as raw material commonly used in the paper & pulp industry. An anatomical approach was used to identify the mode of fungal movement, infection in the wood elements and the extent of alterations in the cellwall of various wood elements.

Material and Methods

Isolation of fungi:

Fruiting bodies naturally growing on the trunk of growing *Peltophorum* in the 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 of 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 fungal isolate was identified as *D. confragrosa* from the Forest Research Institute, Dehradun and pure cultures of *I. lacteus* and *P. sanguineus* were obtained from Forest Research Institute, Dehradun.

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 and the culture isolates were also deposited in BARO culture collection centre of The Maharaja Sayajirao University of Baroda and coded *I. lacteus* as SA 101, *D. confragosa* as SA 102, *P. sanguineus* as SA 103. For further studies petridish containing potato dextrose agar medium were inoculated with 0.5 cm diameter agar plug, cut from the growing edge of colonies of the isolates and incubated in incubator at $25(\pm1)^0$C in dark with 70% relative humidity.

Screening of fungal isolates for ligninolytic, cellulolytic and xylanolytic test:

Ten fungal isolates were screened in our laboratory according to [30] to check ligninolytic and cellulolytic enzyme activity of fungi and xylan-agar diffusion method was used to confirm the ability of fungi to produce extracellular cellulase free xylanase during their growth [31].

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, cut from the growing edge of 10 days old culture of selected fungal isolates and inoculated at the margin of the petriplate containing 20 ml sterilized Malt Extract Agar medium at opposite sides of each other and incubated at 25±2°C with 70% humidity for 4 weeks. Petriplate with individually inoculated fungal culture (Mono cultures) isolate was 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.

Wood block decay experiments:

The fungi for culm decay were selected on the basis of the results from the paired interaction test. Three monocultures viz. *D. confragosa, P.sanguineus* and *I. lacteus* were taken and two co-cultures viz. *D. confragosa* with *P. sanguineus* and *D. confragosa* with *I. lacteus* were taken for the experiment. Test cultures for mono cultures were prepared by inoculating pieces of agar from pure culture plates in 250 ml of decay chambers containing 50 ml of Malt Extract Agar (MEA) medium and in case of co-culture/dual culture agar piece of both the selected fungal mycelium were placed opposite to each other in the decay chamber and incubated for 2 weeks under controlled conditions (25°C, 70% Relative humidity and dark) prior to inoculation of bamboo culm in to decay chambers.

Bamboo culm were cut into blocks (1cm × 3cm size), dried at 72 °C for 24-48 hrs until constant weight, marked and dry weight was recorded for each block. The blocks were then soaked in distilled water for 24hrs to get enough humidity and autoclaved for 1hr at 121°C. Three sterile blocks were then inoculated on the distributed actively growing two week old cultures of a single species and a mixed culture in each decay chamber asceptically. Three Sterile wood blocks inoculated in a decay chamber without fungus culture was kept as control (Fig.1A) for each incubation period. Five replicates were prepared for each isolate (Fig.1B) and kept for three different incubation periods i.e, 10, 20 and 30 days at 25±1 °C and 70% relative humidity. After each incubation period the wood blocks were removed from the glass jars and cleaned properly to remove mycelia. The marked blocks were weighed after oven drying to determine final volume and some of the blocks were fixed in FAA [32] and further analysis was conducted. To monitor the progress of the wood degradation, the percentage weight loss due to degradation was calculated as per the ASTM standard [33].

Bio chemical analysis of Wood components:

The control and decayed culm blocks were ground and passed through a 40-mesh-sized screen, prior to chemical analysis of the wood. Lignin content was estimated by using the method suggested by [34]. Flasks containing 1 g of ethanol-benzene extracted wood meal and 20 ml of H_2SO_4 (72%) were gently shaken in a water bath at 30◦C for 1 h. The acid was then diluted with H_2O to 4% (w/v), and the samples were autoclaved at 121◦C for 30 min. The lignin that settled overnight was quantitatively collected by filtration through a crucible, washed free of acid with hot water, and dried. The lignin content was calculated as a percentage of oven-dried, non-extracted wood meal.

Estimation of cellulose was carried out by using the method suggested by [35]. Acetic-nitric reagent (3 ml) was added to 0.5 gram of the sample in a test tube and mix well. The test tubes were placed in a water bath at 100℃ for 30 min and then cooled. The samples were centrifuged for 15-20min at 5000 rpm after cooling and then the supernatant was discarded. The obtained residues were washed with distilled water and 10ml of 67% sulphuric acid was added and allowed to stand for 1 hour. One ml of the above solution was diluted to 100 ml with distilled water and from this 1 ml solution was taken in to three test tubes and to this solution 10ml of anthrone reagent was added and mixed well. The tubes were then heated in boiling water bath for 10 minutes, Cooled and absorbance was measured at 630nm. A blank was prepared with anthrone reagent and distilled water. For the standard graph the D-glucose is taken in 1 mg/ml and different aliquots were treated with anthrone reagent.

Anatomical alteration in fungal pretreated bamboo culm blocks:

Culm blocks of uninoculated bamboo and treated bamboo of 10, 20, 30 days incubation period, were removed, freed of mycelium and fixed in FAA Formaldehyde: acetic acid: alcohol (9:5:5).

15-20 µm thick transverse, radial and tangential sections of the block were cut on sliding microtome and firmly tied onto the slide and then after the slides were passed through alcohol-xylene series for dehydration, stained with toluidene blue and saffranin fast green and mounted in DPX. The sections were observed under Leica DME 750 and important results were photographed at different magnifications.

Statistical data Analysis:

Five replicates were kept for all the set of experiments. The mean values are shown in the tables. Data were expressed as mean ± standard deviation for all experiments and statistical significance was calculated according to Two-way Anova in Microsoft Excel. Values corresponding to $p<0.05$ were considered statistically significant.

Results

From the fungal isolates screened in the laboratory it was found that *P. sanguineus* and *D. confragrosa* were lignin degraders and *P. sanguineus* and *I. lacteus* were xylanase producers [36].

Paired interaction test/Hyphal interactions on agar plate:

D. confragosa was found to be highly compatible/synergistic when co-cultured with *P. sanguineus* and *I. lacteus* (Fig.1C, D) which was clearly indicated by the overlapping zone formed between the two inoculated fungi. *D. confragosa* is a lignin degrader while *I. lacteus* and *P. sanguineus* are xylanase producers. These fungi were selected for the further wood decay test.

Weight loss of culm blocks by mono and co cultures:

Weight loss in the mono and co-culture fungal treated wood block after the specified incubation periods has been represented in (Table 1). All the monocultured and cocultured fungi begin to degrade the wood within 10 days of incubation. The monocultures and co-cultures showed different rates of wood degradation after 30 days of incubation period. The monocultures caused 18.17% to 24.41% weight loss, amongst which highest weight loss was observed in bamboo culms exposed to *P. sanguineus* and the lowest weight loss occurred in bamboo culms exposed to *D. confragosa* i.e. 18.17% respectively. Decay caused by dual / co-culture ranged from 22.77 to 25.91 % weight loss in which *D. confragosa* and *I. lacteus* caused 22.77% weight loss and *D. confragosa* and *P. sanguineus* caused 25.91% weight loss.

Table.1: percent weight loss during different incubation time

Sr. No	Days of incubation	*D. confragosa*	*I. lacteus*	*P. sanguineus*	*D. confragosa + I. lacteus*	*D. confragosa + P.sanguineus*
1.	10 days	4.24±0.82	3.23±0.86	8.12±1.99	4.76±1.26	9.35±1.08
2.	20 days	10.96±1.28	12.78±1.07	20.72±1.35	14.58±4.72	23.85±2.98
3.	30 days	18.17±2.22	21.23±1.63	24.41±3.85	22.77±3.95	25.91±2.32

±Average percentage weight loss determined from three replicates after respective incubation period.
± Results were significant at p <0.05 level by Two way ANOVA

Bio chemical analysis of the bamboo culm blocks showed change in lignin and cellulose content as represented in (Table 2). The lignin loss was more in co-cultured blocks compared to monocultured blocks. Among monocultures, in 30 days incubation period the percentage lignin loss was more in *P. sanguineus* treated samples (22.48%), *D. confragosa* and *I. lacteus* caused 19.41% and 20.16% loss of lignin respectively.

In the initial 10 days incubation period the bamboo culm blocks exposed to co-culture of *D. confragosa* and *I. lacteus* showed an increase of 5% loss of lignin i.e., monoculture of *D. confragosa* in 10 days caused (7.10%) and *I. lacteus* caused (5.13%) but their co culture caused (12.76%) which clearly indicated 5% more lignin loss in co culture of *D. confragosa* and *I. lacteus* and after 30 days of incubation period the loss of lignin content increased by 6% in co culture of *D. confragosa* and *I. lacteus*.

In initial 10 days *P. sanguineus* infected culm blocks showed maximum lignin loss (10.41%), and its co-culture with *D. confragosa* showed only (8.31%) but in 20 days incubation period the loss of lignin was more in the co culture compared to mono cultures. Similarly co-culture of *D. confragosa* and *P. sanguineus* showed an increase of 8% lignin loss after 30 days of incubation period.

Table.2: percent lignin and cellulose loss during different incubation time

Decay fungi	Incubation period	Loss of KL (%)	Loss of CHC (%)
	10 days	7.10±0.30	5.75±0.83
D. confragosa	20 days	12.26±0.84	11.56±0.80
	30 days	19.41±1.69	19.00±0.57
	10 days	5.13±0.66	5.10±0.68
I. lacteus	20 days	14.47±0.73	11.19±0.70
	30 days	20.16±0.99	16.03±0.84
	10 days	10.41±0.24	2.51±0.51
P. sanguineus	20 days	16.19±0.84	8.22±0.40
	30 days	22.48±0.89	11.50±0.34
	10 days	8.38±0.36	11.20±0.68
D. confragosa + P. sanguineus	20 days	17.22±0.73	16.14±0.32
	30 days	23.23±0.67	22.28±0.82
	10 days	12.76±0.23	8.31±0.67
D. confragosa + I. lacteus	20 days	20.57±0.35	13.42±0.19
	30 days	26.13±0.86	25.13±0.91

±Percentage loss of klason lignin, chlorite holocellulose, and ratios of the percent of each component are of the three replicates. Uninoculated wooden blocks were incubated for 30 days to act as a control.
± Results were significant at p <0.05 level by Two way ANOVA.

The percentage lignin loss was maximum in the wood blocks incubated for 30 days with co-culture *D. confragosa* and *I. lacteus* (26.13%). *D. confragosa* when co-cultured with *I. lacteus* showed a loss of (23.23%) lignin content which is higher than obtained in mono cultures indicating that co-culturing of two white-rot fungi increase lignin degradation.

The period of exposition of the bamboo culm blocks was significant for monocultures as well as co cultures. For *D.confragosa* loss of cellulose increased from 0% to 5.75% between 0 day after inoculation and 10 days after inoculation this later increased to 19.00% at 30 days after inoculation. Similarly for the co-culture of *D. confragosa* with *I. lacteus* the cellulose increased from 0% to 8.31% between 0 day after inoculation and

10 days after inoculation this later increased to 25.13% at 30 days after inoculation. Loss of cellulose content was significant with co-culture of *D. confragosa* and *I.lacteus*.

Similarly monoculture of and *P. sanguineus* loss of cellulose increased from 0% to 2.51% between 0 day after inoculation and 10 days after inoculation this later increased to 11.50% at 30 days after inoculation. For the co-culture of *D. confragosa* with *P. sanguineus* the cellulose increased from 0% to 11.20% between 0 day after inoculation and 10 days after inoculation this later increased to 22.28% at 30 days after inoculation.

The increase in the amount of cellulose could not be ascertained but probably it may be as a result of additional sugar to the total polysaccharide due to hydrolysis of esterified substances found in the wood. The main constituents of bamboo culm are holocellulose (60-70%), pentosans (20-25%), hemicelluloses and lignin (each amounted to about 20-30%) and minor constituents like resins, tannin, wax &inorganic salts. A similar kind of observation has been noted by [37] in *Pleurotus tuber-regium* incubated woodchips of *Sterculia setigera.* Cellulose increased from 45.9 to 52.8% between 0 week after incubation and 6 week after incubation which later decreased to 48.8% at nine weeks after incubation.

Anatomical characterization of mono and co cultured decayed wood blocks Normal anatomical structure of the bamboo culm:

Vascular bundles are peripherally arranged/internal and are of the first order [38] i.e., protoxylem and metaxylem cells are well developed. The vascular bundles are larger in the inner parts, becoming smaller and denser towards the periphery of the culm wall. They are elongated and the vascular elements are surrounded by very thick walled sclerenchyma. Vascular bundles consist of xylem with one small protoxylem elements and two large metaxylem elements. Towards the central portion opposite to the xylem elements oval to round lignified parenchyma cells are present (Fig.1E). Fibers constitute the sclerenchymatous tissue and occur as caps of vascular bundles and it sheaths around the vessels. Surrounding the vascular bundles elongated parenchymatous ground tissue is present.

Epidermis of the bamboo culm is continous and singe layered composed of square cells surrounded by a cutin layer. Parenchyma cells appear square, thin walled and with floral shaped silica bodies intermittently (Fig.1F). All the cells of the epidermis show presence of silica bodies.

Longitudinal sections of culm show presence of opposite bordered pitting in metaxylem vessel elements (Fig.1G). Longitudinally when observed larger amount of parenchyma cells are observed (Fig.1H). Protoxylem elements show annular type of thickening.

Decay by *P. sanguineus:*

Fungal mycelia begin to grow and completely cover the culm within 10-15 days. After 30 days of inoculation a selective delignification occurs. In transverse sections delignification was easily observed in fibers. Erosion troughs were observed between parenchyma cells (Fig.1I) and fiber cells. Fungal hyphae colonize the vessel elements (Fig.1J) and the fungal hyphae branch within the lumen penetrating further in to adjacent cells through the pits. Sporulation was also distinctly observed within the lumen (Fig.1K). Advanced thinning resulted in the localized removal of cell wall and the middle lamella.

Decay by *D. confragosa:*

Decay by *D. confragosa* appears to be conspicuously different from the pattern of decay caused by *I.lacteus* and *P. sanguineus.* It resembles selective delignification which was prominent during the early stage of decay. Degradation commenced in the corners of fibers, along the middle lamella without any effect on the primary or the secondary wall layers. The fiber cells and parenchyma cells are penetrated by fungi through bore holes (Fig.1L) which gradually degrading it leaves behind a wide space. With the progress in decay, localised degradation of lignin, hemicelluloses and cellulose resulted in the formation of cavities within the wall layers and the adjacent cavities fused to form a relatively larger one and irregular in shape (Fig.1M).

Longitudinal view clearly depicted the bore holes in fibers and vessel elements. The hyphae penetrate through the pit openings of the vessels and then exude ecto-enzymes on the hyphal surface. This leads to an enlargement of pit opening, transforming

them into long elliptical openings. Pits of ray and axial parenchyma cells become more prominent, larger in size and irregular in shape. By the same process the pit openings change into large round holes which often merge together to form very large holes. These further decay and bore holes join into cavities decaying the whole part of the cell wall.

Decay by *Irpex lacteus*:

In transverse section delignification was early observed in fibers. Many of them showed presence of erosion troughs across the cell walls and loosening of parenchyma cells because of the degradation of middle lamella (fig.2A). The middle lamellae loses its integrity and the fiber cells get separated from each other. The S_2 layer of the secondary wall is broken down separating out the S_3 layer (Fig.2B). In many of the parenchyma cells the secondary walls stained green instead of the compound middle lamella and the cell corners stained red with saffranin fast green (Fig.2C). Penetration of the fungal hyphae appears to be through bordered pits of xylem vessel elements and traversed in to the neighboring cells from one pit apertures to the other of the parenchyma cells (Fig.2 D). Dissolution of middle lamella appears to begin from the corners of the parenchyma cells (Fig.2E). Section dissolution of the middle lamella between two adjacent cells placed side by side and one above the other in radial rows were observed (Fig.2F). As middle lamella degrades loosening of parenchyma cells was clearly observed (Fig. 2G). The preferential degradation of the compound middle lamella results in the parenchyma cells becoming separated from their matrix and individual cells occur completely isolated from one another. In longitudinal section cells appeared almost completely detached from each other and many cells were deformed or destroyed due to loss of rigidity of their walls. Troughs formed were irregular in shape and size. Many of them also showed cell wall thinning, a concentric delignification starting in S_2 layer of the fiber wall (Fig.2H). The secondary walls of the fibers exhibit numerous cracks and splits. Further the wall gets completely degraded. Fiber wall separation is very well distinct. Erosion channels were observed distinctly in the vessel elements.

Decay in co-culture:

D. confragosa when co-cultured with *P. sanguineus* both characteristic features of the decay pattern could be observed i.e. loosening of cells and presence of bore holes as the pattern was significantly different. Chemical analysis clearly indicated an increase in lignin and cellulose loss in co-culture than in the monocultures.

D. confragosa when co-cultured with *I. lacteus* anatomically no specific characteristic pattern was observed as the pattern of decay by the mono cultures appeared more or less similar. But the rate of degradation was more which could be very well depicted by the chemical analysis of lignin and cellulose.

D. confragosa is found to be compatible and growing well with two other white rot fungi *P. sanguineus* and *I. lacteus* and so for co-culturing *D. confragosa* can be co-cultured with *P. sanguineus* and *I. lacteus.*

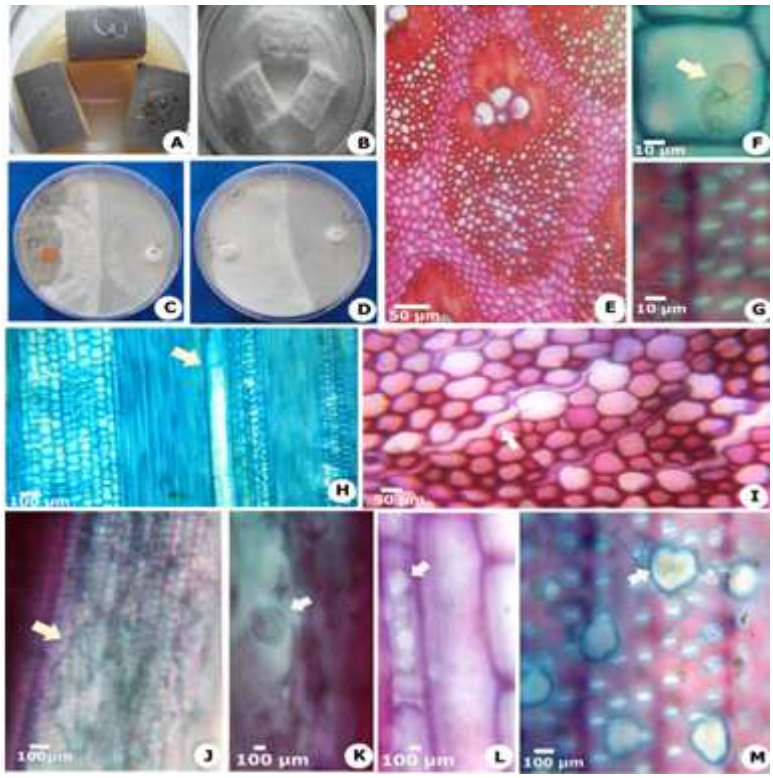

Fig.1:
A: Uninfected Bamboo blocks (control)
B: Bamboo blocks inoculated on the *Daedaleopsis confragosa* culture
C: Paired interaction test of *Daedaleopsis confragosa* and *Pycnoporus sanguineus*
D: Paired interaction test of *Daedaleopsis confragosa* and *Irpex lacteus*
E: Large inner vascular bundle embedded in elongated parenchymatous ground tissue
F: Parenchyma cells with silica body (arrow)

G: Metaxylem vessel element with opposite bordered pitting
H: Parenchyma cells and xylem elements with annular thickening (arrow)
I: Erosion channels formed between parenchyma cells (arrow)
J: Fungal hyphae colonizing vessel element (arrow)
K: Sporulation of *Pycnoporus sanguineus* in vessel element (arrow)
L: parenchyma cells with prominent bore holes (arrow)
M: Bore holes in vessel elements which coalesce increasing the size of Pits (arrow)

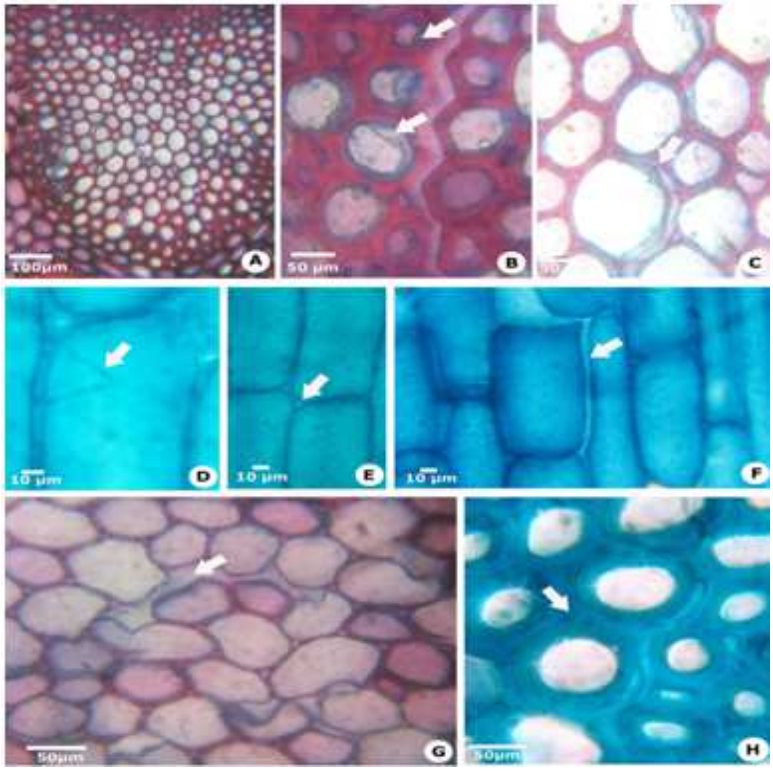

Fig.2:
A: Transverse section of bamboo culm decayed by *Irpex lacteus*
B: Trough / Channel formed in fibres (arrow)
C: Dissolution of middle lamella (arrow)
D: Fungal hyphae penetrating through pits in parenchyma cells (arrow)
E: Dissolution of middle lamella at the corners of the cells (arrow)
F: Dissolution of middle lamella between tangential wall of adjacent parenchyma cells (arrow)
G: Loosening of lignified parenchyma cells (arrow)
H: Concentric delignification in S_2 layer separating the micro fibrils (arrow)

Discussion

Many microorganisms are likely to degrade wood. The most relevant of them are fungi because they produce important changes on the anatomical, physical and mechanical wood properties [39, 40]. Among fungi, Basidiomycetes are of interest as they are able to grow on lignocellulosic substrates and to exploit all wood components due to secretion of cellulases, xylanases and laccases. White rot fungi are of interest because they are one of the few groups of microorganisms that can selectively degrade lignin [41]. Some of the white-rot fungi have the potency to degrade all cell wall components simultaneously while others are selective of which material to degrade.

Screening of white-rot fungi for potential bio pulping is important to select efficient lignin degraders which are capable of accomplishing delignification in a reasonable period of time and which are also capable of performing selective delignification which leaves the cellulose fibers intact. In the present study *D. confragosa, P. sanguineus* and *I. lacteus* were found to be efficient lignin degrader and *P. sanguineus* and *I. lacteus* also produce xylanase enzyme.

It is typical for many fungi to live and grow near close proximity to each other in microenvironments. These fungi may display antagonistic interaction resulting in faster nutrition exploitation or in parasitism or may form deadlock interactions where no hyphae of one species can enter the territory occupied by the other. The interactions may be synergistic i.e. species can act in co ordination to degrade the substrate [42]. A typical result of pathogenic and antagonistic interactions is oxidative stress [43] creating reaction oxygen species (ROS) which in turn play a role in fungal decay [44]. *D. confragosa* was found to be compatible with *P. sanguineus* and *I. lacteus.* Fungal lignin degrading systems can be stimulated by interspecific interactions with other white rot fungi. The understanding of this process encourages the use of co-cultivation of white rot fungi as an improved method for bio pulping.

Among the three selected white rot fungi monocultures of *P. sanguineus* produced a higher percentage of weight loss compared to *D. confragosa* and *I. lacteus*. Wood samples co cultured with *D. confragosa* and *P. sanguineus* showed slightly elevated percentage of weight loss in 30 days. Visually the amount of decay appeared to be qualitatively more in co cultured wood blocks. Based on percent weight loss by fungal attack bamboo resistance was classified by [45, 46]. Highly resistant bamboo culm showed none or negligible weight loss, resistant bamboo culm

shows 0 to 5% weight loss, moderately resistant bamboo culm showed 5 to 10% weight loss, nonresistant bamboo culm shows 10 to 30 % weight loss and bamboo culm shows 30 % or greater weight loss considered to be Perishable. According to this classification, the wood blocks of Bamboo culm tested in the laboratory showed nonresistance to white-rot-causing fungi at 30 days of incubation, as incubation period increases the percentage weight loss also increases in both the monocultures and cocultures.

Fungi capabilities to decay bamboo vary in accordance with bamboo species. Weight loss is affected by the amount of chemical components in bamboo species [46]. The durability of bamboo against fungal attack was strongly associated with chemical composition, among others cellulose, lignin and pentosan [47]. Hence the selection of bamboo species for paper production has to be considered. Bamboo culm showed nonresistance is appropriate for biopulping and paper production.

Bamboo wood blocks decayed by *I. lacteus* and *D. confragosa* shows loosening of cells due to the degradation of middle lamella, *I. lacteus* and *P. sanguineus* produced a selective delignification of the tissue with mainly the loosening and separation of cells and further concentric delignification of wall layers.

Based on the degradation pattern fungal infection is categorized in to white rot, brown rot and soft rot [48, 49, 50]. The white rot is further classified in to selective rot and simultaneous rot depending upon their degradation pattern. In selective delignification the most remarkable anatomical characteristic is the separation of cells by dissolution of middle lamella and in the simultaneous rot formation of erosion troughs, bore holes etc considered as major characteristics. Earlier studies had shown that *P. sanguineus* produced a selective delignification along with other signs of degradation like boreholes and erosion channels features similar to simultaneous decayers [51].

However in the present study *P. sanguineus* produced no boreholes, according to our findings in bamboo culm as clearly manifested by cell separation, a feature considered as the best indicator of the selective type of decay as agreed by [52].

The enzymatic activity of the hyphae affects the inner S_2 layer of the secondary wall. The degradation begins with the diffusion of enzymes from the lumen through the S_3 layer. The latter appears largely resistant to enzymatic destruction and is often still present even with advanced degradation of S_2 layer. Within the S_2 layer the enzymes advanced preferentially between the lamellae, following the direction of the fibrils, the cell wall collapsing into sub microscopic layers. This leads to individual lamellae of the inner secondary wall often dissolving inwards into the lumen.

Pattern of degradation by *D.confragosa* distinctly differed from *I. lacteus* and *P.sanguineus*. *D.confragosa* produced features similar to simultaneous degraders. Formation of boreholes, erosion troughs and erosion channels are considered to be characteristic features of simultaneous rot [53, 54].

D. confragosa when co-cultured with *I.lacteus* produced anatomically no specific pattern of degradation both anatomically signs of lignin degraders and simultaneous degraders were observed. Chemical analysis indicated a significant increase in the degradation. Synergistic effect on wood degradation was noted when *D. confragosa* when co-cultured with *P.sanguineus* and *I.lacteus*.

During the maturation of woody cells, middle lamella with all the layers of wall impregnated with lignin. Lignification is especially pronounced in compound idle lamella i.e, between the rounded corners of fibers [55, 56]. In *D.confragrosa* degradation commenced in the corners of fibers along with middle lamella, in *I. lacteus* middle lamella degrades and fiber cells get separated so loosening of parenchyma cells takes place and in *Pycnoporus sanguineus* also cell separation occurs and as the hyphae penetrates the cell wall and delignify the middle lamella, all of the these changes indicates clear degradation of lignin and cellulose was left relatively during this kind of selective delignification [57,58] which is most important for the paper making process. The lignin degradation occurred by these fungi in the fiber cells and

middle lamella regions suggest the use of fungi in the bio pulping process.

Current bio pulping practice requires a two week long incubation period which may be shortened by implementation of fungal co cultivation. Also chlorinated chemicals required for brightening of the fibers leading to toxic polluted effluents can be done away with the use of these xylanase producing co cultures.

Conclusion

The results of present study allow the following conclusions to be drawn. *D. confragosa* shows both pattern of degradation as degradation starts with the dissolution of middle lamella and later on simultaneously degrade lignin in bamboo culm as formation of boreholes were observed. *I. lacteus* shows both pattern of degradation was observed initially dissolution of middle lamella is seen which is characteristic of selective delignification and erosion troughs were also observed which the characteristic feature of simultaneous degradation is. *P.sanguineus* selectively degrade lignin in bamboo culm as in the initial stage delignification was observed in fibers and later on removal of middle lamella was clearly observed and cell separation is also very clear. *D. confragrosa* when co-cultured with *P. sanguineus* both characteristic features of the decay pattern could be observed i.e. loosening of cells and presence of bore holes as the pattern was significantly different. Based on percent loss and the ratio of percent Klason lignin and percent Chlorite holocellulose the wood decay occurred in co-culture is significantly high as compared to mono cultures indicates co-culture of white rot fungi increase degradation of lignin. The enhancement in selective degradation of wood by the two compatible fungi and the reduction in the amount of lignin in the cell wall appears an ideal pulping method for use in pulp and paper industry.

Interactions with *D. confragosa* appeared to be beneficial with regards to dry weight loss and lignin loss. Higher degradation has been reported for co-culture of *D. confragosa* with *I. lacteus* and *P. sanguineus* compare to the respective monocultures. Thus *D.confragosa* appears to have potential for using as co-cultures for bio pulping and the long incubation period may be shortened by implementation of fungal co cultivation.

References

1. Breen A, Singleton FL, Fungi in lignocellulose breakdown and biopulping, *Current Opinion in Biotechnology,* 10, 1999, 425-444.
2. Perez LM, Besoain X, Reyes M, Pardo G, Montealegre J, The expression of extracellular fungal cell wall hydrolytic enzymes in different *Trichoderma harzianum* isolates correlate with their ability to control *Pyrenochaeta lycopersici*, *Biological Research*, 35, 2002, 401-410.
3. Martinez AT, Mariela S, Francisco JR, Patricia F, Susana C, Francisco G, Matinez MJ, Ana G, Jose CD, Biodegradation of lignocellulosics: microbial, chemical and enzymatic aspects of the fungal attack of lignin*, International Microbiology*, 8, 2005, 195-20.
4. Elisashvili V, Kachlishvili E, Tsiklauri N, Metreveli E, Khardziani T, Agathos S, Lignocellulose-degrading enzyme production by white-rot Basidiomycetes isolated from the forests of Georgia, *World Journal of Microbiology and Biotechnology*, 25, 2009, 331-339.
5. Eriksson KEL, Blanchette RA, Ander P, Microbial and enzymatic degradation of wood components, Springer-verlag, Berlin, 1990.
6. Kashino X, Nishida T, Takahara Y, Fujita K, Kondo R, Sakai K, Biomechanical pulping using white-rot fungus, *Tappi Journal*, 76, 1993, 167-171.
7. Messner K, Srebotnik E, Biopulping: An overview of developments in an environmentally safe paper-making technology, *FEMS Microbiology Review*, 13, 1994, 351-364.
8. Akhtar M, Blanchette RA, Myers G, and Kirk TK, An overview of biomechanical pulping research (ed) Young RA, Akhtar M Environmental Friendly Technologies for the Pulp and Paper Industry, Wiley, New York, pp. 1998, 309–340.
9. Chi Y, Hatakka A, Maijala P, Can co-culturing of two white-rot fungi increase lignin degradation and the production of lignin degrading enzymes, *International Biodeterioation Biodegradation*, 59(1), 2007, 32-39.
10. Atik C, Imamoglu S, Bermek, H, Impact of xylanase pretreatment on peroxide bleaching stage of biokraft pulp, *International Biodeterioration and Biodegradation*, 58, 2006, 22-26.
11. Zhao J, Li X, Qu Y, Application of enzymes in producing bleached pulp from wheat straw, *Bio resource technology*, 97, 2006, 1470-1476.
12. Savitha S, Adhasivam ES, Swaminathan, EK, Application of *Aspergillus fumigatus* Xylanase for quality improvement of waste paper pulp, *Bulletin of Environmental Contamination and toxicology*, 78 (3-4), 2007, 217-221.
13. Maheshwari R, Bhardwaj G, Bhatt M K, Thermophilic fungi: Their physiology and enzymes, *Microbiology and molecular Biology Reviews*, 63, 2000, 461-488.
14. Singh S, Madlala AM, Prior BA, *Thermomyces lanuginosus*: Properties of strains and their

hemicellulases, *FEMS Microbiology Reviews*, 27, 2003, 3-16.

15. Yang SQ, Yan QJ, Jiang ZQ, Li LT, Tian HM, Wang YZ, High level of xylanase production by the thermophilic *Paecilomyces themophila* J18 on wheat straw in solid-state fermentation, *Bioresource Technology*, 97, 2006, 1794-1800.

16. Anthony T, Chandra Raj K, Rajendran A, Gunasekaran P, High molecular weight cellulase-free xylanase from alkali-tolerant *Aspergillus fumigatus* AR1, *Enzyme Microbial Technology*, 32 (6), 2003, 647- 654.

17. Raghukumar C, Muraleedharan U, Gaud VR, Mishra R, Xylanases of marine fungi of potential use for biobleaching of paper pulp, *Journal of Industrial Microbiology and Biotechnology*, 31, 2004, 433-41.

18. Betini JHA, Michelin M, Peixoto-Nogueira SC, Jorge JA, Terenzi H F, Polizeli MLTM, Xylanases from *Aspergillus niger*, *Aspergillus niveus* and *Aspergillus ochraceus* Produced under solid-state fermentation and their application in cellulose pulp bleaching, *Bioprocess and Biosystems Engineering*, 32 (6), 2009, 819-824.

19. Sridevi B, Singara Charya MA, Isolation, identification and screening of potential cellulase-free xylanase producing fungi, *African Journal of Biotechnology*, 10(22), 2011, 4624-4630.

20. Tallapragada P, Venkatesh K, Isolation, identification and optimization of xylanase enzyme produced by *Aspergillus niger* under submerged fermentation, *Journal of Microbiology and Bio technology Research*, 1(4), 2011, 137-147.

21. Durate MCT, Silva EC, Gomes IMB, Ponezi AN, Portugal EP, Vieente JR, Davanzo E, Xylan-hydrolysing enzyme system from *Bacillus pumilus* CBMAI 0008 and its effects on *Eucalyptus grandis* kraft pulp for bleaching improvement, *Bio Resource Technology*, 88, 2003, 9-15.

22. Srinivasan MD, Rele MV, Microbial xylanases for paper industry. *Current Science*, 77, 1999, 137-142.

23. Yu H, Du W, Zhang J, Ma F, Zhang X, Zhang W, Fungal treatment of cornstalks enhances the delignification and xylan loss during mild alkaline pretreatment and enzymatic digestibility of glucan, *Bio resource technology*, 101, (17), 2010, 6728-6734.

24. Gutierrez-Correa M, Tengerdy RP, Production of cellulase on sugar cane bagasse by fungal mixed culture solid substrate fermentation, *Biotechnology Letters*, 19, 1997, 665–667.

25. Watanabe T, Sabrina T, Hattori T, Shimada M, A role of formate dehydrogenase in the oxalate metabolism in the wood-destroying basidiomycetes *Ceriporiopsis subvermispora*,*Wood Research*, 90, 2003, 7-8.

26. Dwivedi P, Vivekanand V, Pareek N, Sharma A, Singh R, Bleached enhancement of mixed wood pulp by xylanase-laccase concoction derived through co-culture strategy, *Applied Biochemistry and Biotechnology*, 160, 2010, 255-268.

27. Cook RJ, Baker KF, The nature and practice of biological control of plant pathogens, American Phytopathogical Society, St paul, MN, 1983, 539.

28. Highley TL, Ricard J, Antagonism of *Trichoderma* spp. and *Gliocladium virens* against wood decay fungi, *Material and Organismen*, 23, 1988, 157-169.

29. Van Heerden A, Le Roux NJ, Swart J, Gardner Lubbe S and Botha A, Assessment of wood degradation by *Pycnoporus sanguineus* when co-cultured with selected fungi, *World Journal of Microbiology and Biotechnology*, 24(11), 2008, 2489-2497.

30. Bains RK, Rahi DK, Hoondal GS, Evaluation of wood degradation enzymes of some indigenous white rot fungi, *Journal of Mycology & Plant pathology*, 36, 2006, 161-164.

31. Teather RM, Wood PJ, Use of congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen, *Applied Environmental Microbiology*, 43 (4), 1982, 777-780.

32. Berlyn GP, Miksche JP, Botanical microtechnique and cytochemistry, The Iowa State University Press, Ames Iowa, 1976, pp. 326.

33. American Society for Testing Materials (ASTM) Standard method of accelerated laboratory test of natural decay resistance of woods, ASTM D-2017-71 (Reapproved 1978), Annual Book of ASTM Standards, 1981, Part 22, Philadelphia, pp 639- 64.

34. Dill I, Kraepelin G, Palo podrio: Model for extensive delignification of wood by *Gandoderma applanatum*, *Applied and environmental microbiology*, 86, 1986, 1305-1312.

35. Yemn EW, Willis AJ, The estimation of carbohydrates in plant extract anthron, *Biochemical Journal*, 57, 1954, 508-514.

36. Albert S, Pandya B, Padhiar A, Evaluation of colony characteristics and enzyme activityof some fungi for potential use in co-culture for bio-pulping, *Asian journal of Biological and Lifesciences*, 1 (2), 2012, 83-89.

37. Oluwadare AO, Asagbara EO, Biodegradation of *sterculia setigera* chips and its effect on wood basic chemical composition*, International journal of Botany.* 4(4), 2008, 461-465.

38. Ellis R P, A procedure for standardizing comparative leaf anatomy in the *Poaceae*. I. The leaf-blade as viewed in transverse section, *Bothalia* 12, 1976, 65–109.

39. Wilcox WW, Review of literature on the effects of early stages of decay on wood strength, *Wood and Fiber*, 9 (4), 1978, 252-257.

40. Highley TL, Clausen CA, Croan SC, Green F, Illman BL, Micales JA, Research on biodeterioration of wood, Decay mechanisms and biocontrol, USDA Forest Service, Research Paper Forest Products Laboratory 529, 1994, 1-20.

41. Otjen L, Blanchette RA, A discussion of microstructural changes in wood during

decomposition by white rot Basidiomycetes, *Canadian journal of botany*, 64 (5), 1985, 905-911.

42. Boddy L, Interspecific combative interactions between wood-decaying basidiomycetes, *FEMS Microbiology Ecology*, 31, 2000, 185–194.

43. Baker CJ, Orlandi EW, Active oxygen in plant pathogenesis, *Annual Review of Phytopathology*, 33, 1995, 299-321.

44. Hammel KE, Kapich AN, Jensen KA, Ryan ZC, Reactive oxygen species as agents of wood decay by fungi, *Enzyme and Microbial Technology*, 30, 2002, 445–453.

45. Martawijaya A, Laboratory Testing on the Resistance of Wood against Fungi, Ministry of Agriculture, Jakarta, 1975.

46. Seng OD, Specific Gravity of Indonesian Woods and its significance for practical use, Communication No.13. Forest Products Research and Development Centre, Bogor, 1990.

47. Li XB, Physical, chemical and mechanical properties of bamboo and its utilization potential for fiber board manufacturing, M.Sc. thesis, Louisiana State University, Baton Rouge, 2004.

48. Blanchette RA, Delignification by wood-decay fungi, *Annual Review in Phytopathology*, 29, 1991, 381–98.

49. Eaton RA, Hale MDC, Wood - decay, pests and protection, Chapman & Hall, London, 1993, 76-110.

50. Schwarze FWMR, Fink S, Host and Cell type affect the mode of degradation by *Meripilus giganteus*, *New Phytologist*, 139, 1998, 721-731.

51. Luna ML, Murace MA, Keil GD, Otan ME, Patterns of decay caused by *Pycnoporus sanguineus* and *Ganoderma lucidum* (Aphyllophorales) in poplar wood, *International Association of Wood Anatomist's Journal*, 25, 2004, 425–433.

52. Anagnost SE, Light microscopic diagnosis of wood decay, *International Association of Wood Anatomist's Journal*, 19, 1998, 141-167.

53. Liese W, Ultrastructural aspects of woody tissues disintegration, *Annual Review of Phytopathology*, 8, 1970, 231-258.

54. Koyani R, Sanghvi G, Bhatt I, Rajput K, Pattern of delignification in *Ailanthus excelsa* Roxb. wood by *Inonotus hispidus* (Bull.: Fr.) Karst, *Mycology*, 1(3), 2011, 204-211.

55. Fergus BJ, Goring DAI, The location of guaiacyl and syringyl lignins in birch xylem tissue, *Holzforschung*, 24, 1970a, 111-117.

56. Fergus BJ, Goring DAI, The distribution of lignin in birch wood as determined byultraviolate microscopy, *Holzforschung*, 24, 1970b, 118-124.

57. Schwarze FWMR, Lonsdale D, Mattheck C, Detectability of wood decay caused by *Ustulina deusta* in comparison with other tree-decay fungi, *European journal of forest pathology*, 25, 1995, 327–341.

58. Schwarze FWMR, Engels J, Mattheck C, Fungal strategies of wood decay in trees, Heidelberg, Springer, Germany, 2004.

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