Evaluation Of White Rot Fungi, Trametes Versicolor Induced Biodelignification In Wood Plant Eucalyptus Tereticornis

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ABSTRACT

Biopulping offers an environment friendly alternative to remove lignin by treatment of lignocellulosic materials with white rot fungi. In the present study biological pre-treatment of Eucalyptus chips was carried out with a fungal species, Trametes versicolor. The influence of growth parameters like incubation period, moisture level, media, media dose, pH and temperature were also optimized. During the study it was found that white rot fungi, Trametes versicolor shows 19.88% lignin loss within 21 days at optimum conditions i.e. pH (5.5), temperature (25^{0}C), moisture (60%) and molasses dose (4%). The fungal pre-treatment decreased the kappa number from 28.92 to 24.10 at 60 minutes optimum cooking time period with 3.41% reasonable yield loss from 46.50 to 43.09%. Thus, the study has provided an insight to find economically feasible conditions to reduce pollution load.

Introduction

Biodelignification, an extracellular oxidative process has been largely investigated by using various types of fungi and their extracellular enzymes. It is an alternative way to save the environment from hazardous chemicals (1-2). White-rot fungi are the most efficient degraders of lignin and are probably also the most suitable organisms to be utilized in an industrial process that requires lignin degradation (3-6). They are not only capable of producing lignin-degrading enzymes such as peroxidases and laccases, but are also able to penetrate the substrate to transport these enzymes into materials such as wood chips (7-9). The genus Trametes is one of the most actively investigated species in the phylum of Basidiomycota for lignolytic enzyme formation and applications. A large number of reasons account for the attractiveness of Trametes. Major reasons among them are the constitutive, extracellular secretion and the non-specific nature of the lignolytic enzymes, which obviate the need for adaptation to the target molecule. The lignolytic enzymes that make Trametes very attractive are laccase and manganese peroxidase, while lignin peroxidase has only rarely been reported in Trametes (10).

The activity of micro-organisms and wood degrading fungi depends on a number of physical, chemical and biological factors. These factors can act as inhibitors to hinder the activity of these micro-organisms and fungi leading to a complete stop of growth. These factors are therefore considered as important elements in the degradation activity of wood degrading fungi. The knowledge and use of these factors in order to influence the fungal growth is one of the keys in wood lignin degradation. In present paper extent of lignin degradation by Trametes versicolor in Eucalyptus tereticornis was evaluated under various conditions (incubation period, moisture level, media, media dose, initial pH and temperature). Scanning Electron Microscopic examinations were used to observe the comparative growth of fungi on the substrate. The treated and untreated chips were subjected to kraft pulping under standard cooking conditions to obtain pulp of required kappa number and to understand the impact of lignin degradation in treated chips.

Experimental

Materials and Methods

The freeze dried white rot fungi, Trametes versicolor, obtained from Forest Pathology Division, Forest Research Institute (FRI), Dehradun was inoculated in Potato Dextrose Agar plates and incubated at 27 ± 1°C for 7 days. Active inocula from these plates were grown in a 250 ml Erlenmeyer flask containing 100 ml malt extract broth. The fungal mat was removed from the medium, suspended in sterilized distilled water and was converted into uniform suspension by using magnetic stirrer at high speed (1500 rpm). This suspension was used to treat the wood chips.

Logs of Eucalyptus tereticornis were collected from preserved forest of FRI, Dehradun and chipped in a laboratory chipper to obtain 2.0 mm 2.3 mm thick chips for the study. The chips were dried in sunlight for 15 days and analysed for lignin and holocellulose contents using standard TAPPI test methods (11-13).

The biodelignification of eucalyptus chips was performed in petri plates, containing 50 g chips (Oven Dry basis). Distilled water was added to the samples in sufficient quantity to increase the moisture levels from 60 to 100% on a dry weight basis for optimum growth of the fungi. Chips were thoroughly mixed with 2, 4, 6, 8 and 10% w/v nutrients, malt extract broth and molasses solutions having different initial pH values ranging from 4.5 to 7.0. The wood chips were autoclaved for 20 min at 121 °C and treated with mycelium suspension of T. versicolor. The dose of mycelium application was 0.003 gm (O.D.).

Table 1: Experimental Conditions at Various Parameters for Delignification by *Trametes versicolor*.

Experiment No.	Conditions		
1	**Time**	Upto 42 days	
	Media	Malt Extract Broth	
	Media Dose	2%	
	Initial Moisture	60%	
	pH	6.0	
	Temperature	25°C	
2	**Time**	21 days	
	Media	Molasses and MEB	
	Media Dose	2%-10%	
	Initial Moisture	60%	
	pH	6.0	
	Temperature	25°C	
3	**Time**	21 days	
	Media	Molasses	
	Media Dose	4%	
	Initial Moisture	60-100%	
	pH	6.0	
	Temperature	25°C	
4	**Time**	21 days	
	Media	Molasses	
	Media Dose	4%	
	Initial Moisture	60%	
	pH	4.5-7.0	
	Temperature	25°C	
5	**Time**	28 days	
	Initial Moisture	60%	
	Media	Molasses	
	Media Dose	4%	
	pH	5.5	
	Temperature	20°C- 35°C	

Inoculated chips were incubated for different time periods (7, 14, 21, 28, 35 and 42 days) with different moisture ratio (60, 80 and 100%) and temperature (20, 25, 30 and 35^{0}C). Various experiments were conducted to study effect of one variable on lignin degradation while keeping the other variables constant (Table 1).

Petri plates without inoculum were used as control (untreated). All treated chip samples were oven dried at 60^{0}C for 48 hours and used for proximate analysis. Dried samples of eucalyptus were converted into dust by Willy Mill and dust passing through 40 mesh and retained over 60 mesh was used for all subsequent analytical studies. TAPPI T 222 om-88 and Useful Method 249 were used for determination of klason lignin and holocellulose respectively.

nning Electron Microscopy

a 200 FEG, Type FP 2032/11 scanning electron microscope, ed in a secondary electron mode at an accelerating voltage of 15 s used to obtain SEM micrographs of fungal treated and ted eucalyptus chips. Sections of the sample were sputter l with gold after uniform cutting.

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eated and untreated eucalyptus chips were cooked by kraft g process in a laboratory digester consisting of six autoclaves g in an electrically heated poly-ethylene glycol (PEG) bath. A weight of chips (200 g Oven Dry Basis) was charged in each ave with appropriate amount of white liquor of 25% sulfidity and ctive alkalinity at a liquid to raw material ratio of 4:1. The aves were then tightly closed and placed into the heated glycol nd the rotation was started. The schedule of digester heating ted of 30 minutes for heating from ambient temperature to , 90 minutes for heating from 100°C to 160°C. The cooking time °C was 30 minutes, 60 minutes, 90 minutes and 120 minutes tively. Calculation of H-factor was started when the content of aves heated up. The calculated H factors were 315.65, 514.55, 5 and 912.35 respectively. After pulping, the contents were d until the color of the water remained unchanged. After washing ulps were centrifuged until water came out and then genized. The samples were weighed and the total yield was determined. Kappa number was estimated by following TAPPI standard method, T236 om-76, 2008c.

TABLE 2
Effect Of Incubation Period On Lignin And Holocellulose Degradation by *Trametes Versicolor* in Eucalyptus Chips.

Samples	Incubation periods (Days)	Lignin %	Lignin Loss %	Holocellulose (%)	Holocellulose Loss (%)
Untreated	Nil	34.20 ± 0.18	Nil	64.38 ± 0.16	Nil
Treated	7	33.28 ± 0.12	2.68 (2.68)	63.90 ± 0.20	0.75 (0.75)
	14	31.97 ± 0.15	6.53 (3.85)	63.15 ± 0.15	1.91 (1.16)
	21	29.32 ± 0.13	14.28 (7.75)	62.02 ± 0.19	3.67 (1.76)
	28	28.30 ± 0.17	17.25 (2.97)	60.95 ± 0.16	5.33 (1.66)
	35	27.78 ± 0.19	18.76 (1.51)	59.97 ± 0.16	6.86 (1.53)
	42	27.33±0.22	20.08 (1.32)	59.25 ± 0.19	7.97 (1.11)

Values are mean ± SD of 6 analysis. Difference of percent loss is given in parenthesis.

Results And Discussion

Studies on effect of variables on bio-delignification of eucalyptus chips by T. versicolor:

The effect of treatment of T. versicolor on eucalyptus chips after incubation for different time periods was evaluated by analysing the lignin and holocellulose content in untreated and treated chips. Table-2 shows the

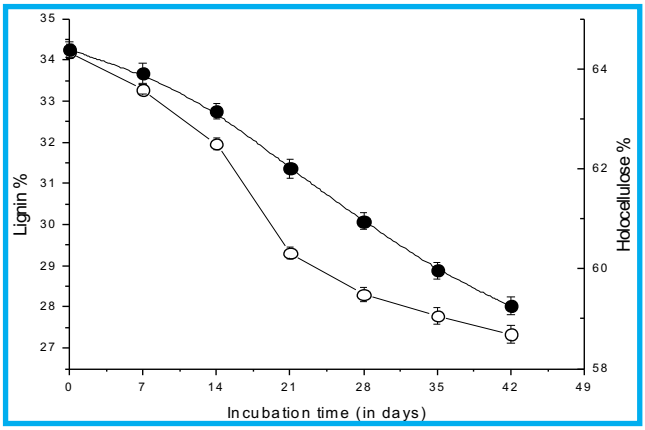

Fig 1: Effect of Incubation time on Lignin on Holocellulose Degradation

results of analysis of lignin and holocellulose in untreated and treated eucalyptus chips with fungi after different periods of incubation.

Results show that lignin content in eucalyptus chips dropped from 34.20 to 27.33% after incubation over a period of 42 days. On a periodic basis, loss in lignin observed from 7 to 42 days was 2.68 to 20.08%. A sharp increase in lignin loss was observed between the incubation periods of 14 to 21 days when it went up from 6.53 to 14.28% i.e. an increase of 7.75%. After increasing the incubation period above 21 days, rate of lignin degradation was found to decrease. Loss in holocellulose after different incubation periods of 7 to 42 days was 0.75 – 7.97%. T. versicolor is basically a ligninolytic organism **(9),** therefore cellulose was only sparingly attacked over a period of 42 days. On the basis of maximum rate of lignin degradation, 21 days was considered as optimum incubation period.

Malt extract broth and molasses were used as media for the inoculation of fungi on eucalyptus chips. Effect of both these media and their dosages on lignin and holocellulose degradation during treatment of T. versicolor on eucalyptus chips was also studied. Table-3 shows the loss in lignin and holocellulose in the untreated and treated eucalyptus chips after incubation with T. versicolor in different media dosages for a period of 21 days.

Table-3 shows that maximum lignin content reduction compared to untreated chips was observed at 4% media dosage using any of the media either molasses or malt extract broth.

For chips treated with fungi in 4% malt extract broth, lignin content was found to be 27.2% which corresponds to loss of 20.47%. Similarly the lignin content in case of 4% molasses was 27.83% corresponding to 18.62% lignin loss. Thus lignin degradation with malt extract broth is 1.85% higher than the molasses, however considering the holocellulose yield, it is found that its yield is

TABLE - 3

Effect of Media and Media Dose Percent on Lignin and Holocellulose Degradation by *Trametes Versicolor* In Eucalyptus Chips.

Media	Samples	Media Dose (%)	Lignin (%)	Lignin Loss (%)	Holo cellulose (%)	Holo cellulose Loss (%)
Malt Extract Broth	Untreated	Nil	34.20± 0.18	Nil	64.38 ± 0.16	Nil
	Treated	2	28.85 ± 0.38	15.64	61.33 ± 0.14	4.73
		4	27.20 ± 0.10	20.47	62.47 ± 0.26	2.97
		6	27.62 ± 0.55	19.25	62.03 ± 0.14	3.65
		8	28.25 ± 0.20	17.40	61.50 ± 0.14	4.47
		10	29.18 ± 0.16	14.67	60.95 ± 0.13	5.33
Molasses	Untreated	Nil	34.20± 0.18	Nil	64.38 ± 0.16	Nil
	Treated	2	29.32 ± 0.13	14.28	62.02 ± 0.19	3.87
		4	27.83 ± 0.08	18.62	62.92 ± 0.18	2.27
		6	28.15 ± 0.26	17.69	62.58 ± 0.19	2.79
		8	28.53 ± 0.08	16.57	62.10 ± 0.24	3.54
		10	29.37 ± 0.21	14.13	61.55 ± 0.37	4.40

Values are mean ± SD of 6 analysis.

TABLE - 4

Effect of Moisture on Lignin and Holocellulose Degradation by *Trametes Versicolor* In Eucalyptus Chips.

Samples	Moisture (%)	Lignin (%)	Lignin Loss (%)	Holocellulose (%)	Holocellulose Loss (%)
Untreated	Nil	34.20 ± 0.18	Nil	64.38 ± 0.16	Nil
Treated	60	29.32 ± 0.13	14.28	62.02 ± 0.19	3.67
	80	29.82 ± 0.25	12.82	62.20 ± 0.24	3.39
	100	30.25 ± 0.14	11.55	62.47 ± 0.18	2.97

Values are mean ± SD of 6 analysis.

TABLE -5

Effect of pH on Lignin and Holocellulose Degradation by *Trametes Versicolor* in Eucalyptus Chips.

Samples	pH	Lignin (%)	Lignin Loss (%)	Holocellulose (%)	Holocellulose Loss (%)
Untreated	Nil	34.20 ± 0.18	Nil	64.38 ± 0.16	Nil
Treated	4.5	29.32 ± 0.18	14.28	63.65 ± 0.12	1.13
	5.0	28.10 ± 0.20	17.84	63.28 ± 0.19	1.70
	5.5	27.40 ± 0.20	19.88	63.07 ± 0.12	2.04
	6.0	27.83 ± 0.82	18.62	62.92 ± 0.18	2.27
	6.5	28.30 ± 0.09	17.25	62.83 ± 0.35	2.40
	7.0	29.15 ± 0.19	14.77	62.72 ± 0.11	2.58

Values are mean ± SD of 6 analysis.

TABLE - 6

Effect of Temperature on Lignin and Holocellulose Degradation by *Trametes Versicolor* in Eucalyptus Chips.

Samples	Temperature	Lignin (%)	Lignin Loss (%)	Holocellulose (%)	Holocellulose Loss (%)
Untreated	Nil	34.20 ± 0.18	Nil	64.38 ± 0.16	Nil
Treated	20	33.27 ± 0.12	2.73	64.13 ± 0.17	0.38
	25	27.40 ± 0.2	19.88	63.07 ± 0.12	2.04
	30	27.98 ± 0.18	18.18	63.30 ± 0.27	1.68
	35	31.65 ± 0.10	7.46	63.75 ± 0.28	0.98

Values are mean ± SD of 6 analysis.

0.7% less with malt extract broth than molasses. Since molasses are economically 10 times cheaper compared to malt extract broth therefore it was used to conduct further experiments.

To study the effect of moisture, pH and temperature on lignin and holocellulose degradation by treatment of T. versicolor using molasses as media for 21 days incubation, the samples were exposed to different moisture levels, pH & temperatures. The lignin and holocellulose content in the chips was observed with variation in moisture, pH & temperature and is depicted in Tables 4-6 for the untreated and treated eucalyptus chips.

Table-4 depicts that the lignin loss percent decreased with increase in moisture levels from 60 to 100%. On the basis of these observations 60% initial moisture level was considered as optimum for treatment of chips for obtaining best results. At the same moisture content, the observed loss in percent of holocellulose was only 3.67.

From Table-5 it is evident that highest lignin loss is observed at pH 5.5. At this pH there is 19.88% loss in lignin whereas the corresponding holocellulose loss is only 2.04% . Therefore, according to the analysis pH 5.5 was considered as optimum and used as pH for all further studies.

The effect of temperature on lignin and holocellulose degradation, shown in Table-6, depicts that at optimum temperature of 25^{0}C the lignin loss was highest. The lignin loss observed at 25 ^{0}C was 19.88%, whereas the holocellulose loss was only 2.04%.

Scanning Electron Microscopic Studies

The treated samples were subjected to microscopic evaluation to study the extent of degradation after fungal treatment. Fig.1 shows the Scanning Electron Micrographs of eucalyptus treated and untreated chips, showing wood fibres without fungal hyphae as well as any pores in the untreated chips and the fungal hyphae penetration through the pores on fibres surface after fungal treatment. Observations of SEM micrographs of the treated samples have shown an extensive colonization by the fungi and it is evident that in advanced stages of decay, cell walls are eroded extensively, therefore holes within adjacent cell walls are observed. This confirms the observations of Kleist et al.(14). In general, SEM observations made on the colonization of Trametes versicolor confirmed an extensive hyphae growth in all tissues of the eucalyptus treated samples.

Studies on Pulping Characteristics of fungal treated and untreated eucalyptus chip:

The fungal treated and untreated chips were cooked under alkaline kraft conditions in the laboratory digester to study the effect of fungal treatment on pulping characteristics. Effect of pulping time at top temperature was studied to find out the pulp yield and kappa number of the pulp with and without fungal treatment. Table-7 shows the results of pulp yield and kappa number on treated and untreated chips after pulping for different time periods. The results in Table 7 show that in case of untreated samples the lowest kappa number 24.35 was obtained after pulping upto 120 minutes at top temperature, whereas in

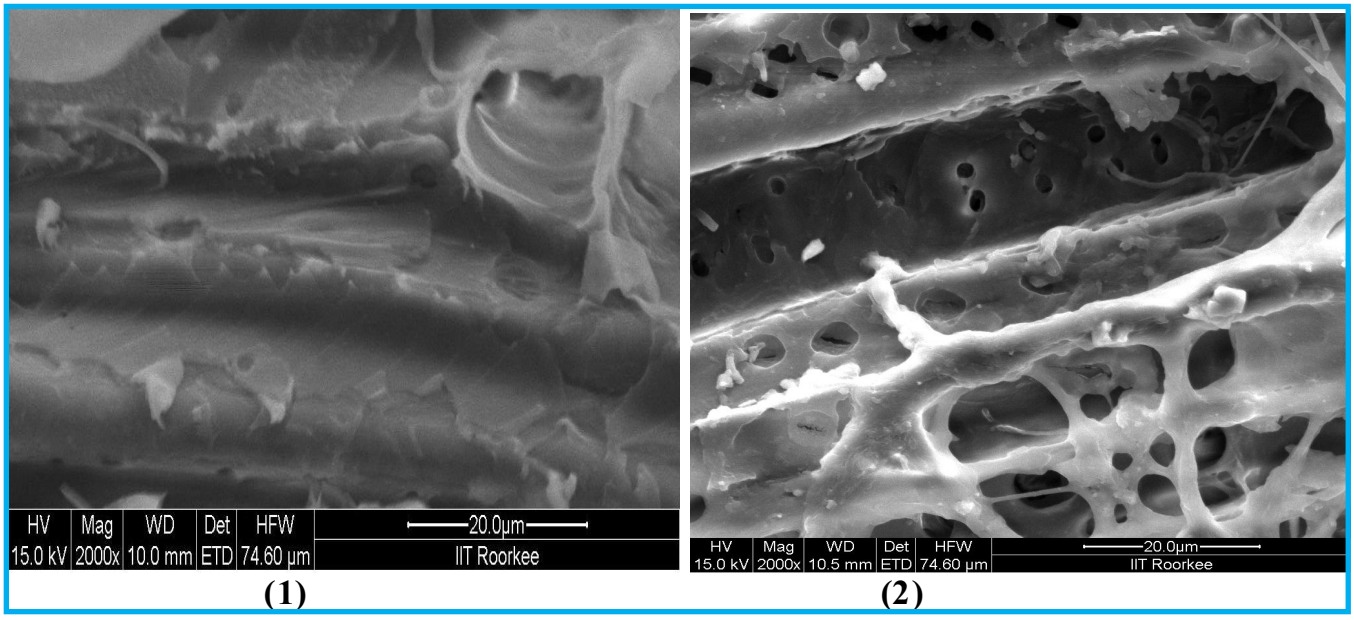

Fig 1: Scanning Electron Micrographs of Eucalyptus treated and untreated chips (1) Untreated chips showing fibres without fungal hyphae and any pore. (2) Treated chips showing fungal hyphae penetration through the pores on chips surface.

TABLE - 7
Pulping Time, Pulp Yield and KAPPA Number of Eucalyptus Treated and Untreated Pulp Samples.

Pulp Samples	Cooking Time at 160°C (H factor)	Un-screened Pulp Yield (%)	Kappa Number	Rejects (%)
UTS * 1	30 (315.65)	48.89	43.11	16.79
UTS 2	60 (514.55)	46.50	28.92	7.231
UTS 3	90 (713.45)	46.15	25.68	5.217
UTS 4	120 (912.35)	45.08	24.35	3.813
TS #1	30 (315.65)	45.55	39.25	13.65
TS 2	60 (514.55)	43.09	24.10	4.341
TS 3	90 (713.45)	41.72	21.27	2.298
TS 4	120 (912.35)	39.71	19.77	1.421

UTS*- Untreated Samples TS# - Treated Samples

treated samples it reduced to 19.77 kappa number after 120 minutes of cooking.

The properties of pulp sheets for untreated and treated samples were compared to find out the variation in Freeness, Tear Index, Tensile Index and Burst Index of unbleached sheets. These properties are also analyzed keeping in consideration the drop in Kappa number in the respective sheets. Table- 8 lists the basis weight (g/m^2), freeness CSF (ml), Tensile index (N.m/g), Tear Index (mNm^2/g) & Burst Index ($K.Pa.m^2/g$) and the corresponding Kappa number of the sheets prepared from treated and untreated pulp samples of 24.3 and 24.1 kappa number respectively.

The results show that tensile and tear index of the treated samples are marginally lower than the untreated samples. However, the burst index is higher in case of treated samples.

TABLE 8
Strength Properties of The Sheets Made From Unbleached Pulp of Treated and Untreated Eucalyptus Chips.

Pulp Samples	Kappa Number	Basis Weight, g/m2	Freeness, CSF,ml	Tensile Index, N.m/g	Tear Index, mNm2/g	Burst Index, $K.Pa.m^2/g$
Untreated pulp	24.3	73.80	300	102.1	8.26	7.01
Treated pulp	24.1	63.10	310	101.1	8.02	8.31

Conclusions

The studies conducted on Eucalyptus tereticornis for assessment of lignin degradation by Trametes versicolor have revealed the optimum experimental conditions such as incubation period, moisture level, media, media dose, initial pH and temperature etc., for most effective fungal treatment of chips. The effectiveness of fungal treatment was ensured by SEM examinations and observing the growth of fungi on the chips. The kraft pulp obtained from pulping of these treated chips were tested for the kappa number and yield and compared with the conventional kraft pulping used by the paper mills.

The kappa number of the fungal treated chips when compared with the conventional kraft pulp, were found to be lower for fungal pre-treated chips by 4.59 points, without affecting the yield at the same chemical charge and cooking time. The studies on strength characteristics of the pulp have also confirmed that the treated pulp has higher strength properties than the conventional cooked pulp. The investigations confirm that rate of lignin degradation of treated samples is higher than the untreated sample, and this is presented by decrease in kappa number of the treated chip samples. Determination of unbleached sheet weight, strength, tear and tensile properties tests showed that, the strength properties of the pulp samples did not show much difference among treated and untreated samples. A significant decrease in kappa number signifies reduced bleach chemical demand in successive bleaching stages. This would result in minimum pollution from pulp mill due to lesser amount of harsh chemical treatments given for bleaching purpose.

Acknowledgment

This study is the part of research work done at Forest Research Institute, Dehradun. We thank Mr. Samit Kumar, IIT Roorkee for providing SEM photographs of the samples.

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