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Mechano-Biological Operation of *Dendrocalamus strictus* for better delignification by *Trametes versicolor*

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Abstract : Global concerns about elimination of pollution from pulp and paper mills, has lead us to explore alternative processes for better delignification. In an attempt to address this issue rate of delignification was increased by applying *Trametes versicolor* in destructured samples of *Dendrocalamus stictus,* which was destructured by Impressafiner (compression-cum dewatering processes). The extent of delignification was determined and compared between the non-destructured and destructured samples. It was found that rate of delignification was significantly distinct between the two samples. In *Dendrocalamus strictus*, fungi *Trametes versicolor* shows 15.02% and 22.14% lignin loss in non-destructured and destructured samples respectively with in 21 days. The influence of physical parameters like pH, temperature, media concentration, moisture and incubation time were also examined during the study. It was found that in *Dendrocalamus strictus* lignin degradation by *Trametes versicolor* in destructured sample was approximately 7.12% more than in non-destructured. Scanning Electron Microscopy (SEM) was used to investigate the growth of bamboo colonizing and decay fungi into the tissue structure. Kraft pulping of destructured treated sample shows 5 point reduction in kappa no. than untreated non destructured sample. Thus this paper provides an insight of the delignification extent in *Dendrocalamus strictus* after mechanical operation at varying physical parameters.
Key Words: Biopulping, Delignification, White Rot Fungi, Lignin, Cellulose.

1 INTRODUCTION

Pulp and paper industries use different environmentally hazardous chemicals to separate cellulose fibers from lignin [1]. To surmount these problems, biological processing, offers potential opportunities for making the industry more environment friendly. In the biological pulping process, 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 delignification [2, 3, 4, 5]. 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 [6, 7, 8]. In this type of degradation lignin in the secondary wall and middle lamella is almost removed leaving behind large proportion of cellulose in S2 layer of the cell wall [9, 10].

The main biological challenge in biological delignification is that fungal hyphae can not penetrate to the core of chips, only surface phenomenon occurs during treatment stage. In view of this bamboo (*Dendrocalamus strictus*) chips were mechanically destructured for opening the compact fibers, thereby increasing the surface area and reducing the density. Destructured samples with large surface area and low density allowed the mycelia to penetrate into the core of the fiber on pre-treatment with fungal culture. Selection of process parameters and their optimization are other key aspects of solid state fermentation. This part includes physiochemical and biochemical parameters such as initial moisture content, initial pH, incubation temperature, supplementation of nutrients and incubation period [11].

Scanning electron microscopy was used to investigate the growth of bamboo colonizing and decay fungi into the tissue structure and the modes of attack on bamboo cell walls. SEM offers advantage over most other types of microscopy for such studies in that a wide range of magnification is available, great depth of field and fully three dimentional image is obtained and specimen preparation is relatively straightforward [12].

In this study, the biological pretreatment was performed using *Trametes versicolor*. This fungi was selected because of its effective degradation of lignin and lignin-like compounds.

2 MATERIALS AND METHODS

2.1 Fungal Culture

The freeze dried white rot fungi *Trametes versicolor* was obtained from Forest Pathology Division, Forest Research Institute, Dehradun. The cultures were maintained on potato dextrose agar media (PDA) slants and kept refrigerated until used. PDA plate cultures were inoculated from the slants and incubated at 27 ± 1°C for 7 days.

2.2 Suspension Culture Preparation

Active inocula from these plates were grown in a 250 ml Erlenmeyer flask containing 100 ml malt extract broth. The inoculated flasks were incubated without agitation in an incubator at 25^0C for 7 days. The surface of the medium got covered with the fungus in the form of mat. The fungal mat was removed from the medium and suspended in distilled and sterilized water. The fungal mat was converted into uniform suspension by using magnetic stirrer at high speed. This suspension was used to inoculate the wood samples.

2.3 Sample Preparation

Two different forms of bamboo sample were taken for the experiments i.e. non-destructured (chips) and destructured. The bamboos (*Dendrocalamus strictus*) were chipped in pilot plant chipper. The chips so obtained were dried in sunlight to the normal moisture content. Destructured sample was obtained by passing the chips in a device called Impressafiner (**Fig.1**). This unit completely compress the chips and squeezes out soluble material along with water. The unit was designed and locally fabricated for the capacity of handling 2-5 kg at one time. For the experiments about 10 kg raw material was taken on oven dry basis. The soaking was carried out in water for overnight. The soaked chips after draining were dewatered in compression-cum dewatering unit (Impressafiner) at 8 r.p.m and 6000 psi so that desirable fibers efficiency could be achieved. The unit is therefore designed to make the material spongy without damaging the fibers. Then the spongy bamboo sample was dried in sunlight to the normal moisture content and packed in poly bags for further experiment. Both chips and destructured sample were analysed for various chemical properties.

2.4 Inoculation Procedure

The biodelignification of bamboo samples was performed in petri plates, containing 50 gm (O.D. basis) destructured and non-destructured samples separately. Distilled water was added to the samples in sufficient quantity to increase the moisture content to 60% - 100% on a dry weight basis for optimum growth of the fungi. The nutrients malt extract broth and molasses solutions having different initial pH values were added at different dose rate to the raw material and mixed well. Petri plates were autoclaved for 20 min at 121°C. The autoclaved destructured and non-destructured samples were inoculated with mycelium suspension of *T. versicolor*. The rate of mycelium application was 0.003 gm (O.D. basis). Each petri plate with sufficient aeration was placed in an incubator at different temperatures for successive time periods. The treated petri plates were harvested at the interval of 7 days for a total incubation period of 42 days. Effect of one variable on delignification was studied after keeping the other variables constant. Further experiments were conducted on the best conditions obtained after a trial of experiments at different variables. For the purpose of analytical studies, petri plates without inoculum were used as control. Each experiment was done in triplicate. The biologically delignified samples obtained after

specified biodelignification time were thoroughly washed with distilled water. The washed samples thus obtained were dried in oven at 50-60°C for 48 hrs. The dried samples were analysed for chemical properties. Dried samples of *Dendrocalamus strictus* were milled in a laboratory disintegrator to produce dust and the dust passing through 40 mesh and retained over 60 mesh was used for all subsequent analytical studies. TAPPI standard methods were adopted for proximate chemical analysis.

2.5 Cooking Process and Conditions

The treated and untreated bamboo destructured and non destructured samples were cooked by kraft pulping process in a laboratory digester consisting of six autoclaves rotating in an electrically heated poly-ethylene glycol (PEG) bath. Before cooking the moisture content of the wood chips was carefully determined using a representative sample. A known weight of chips (200 g O.D.) was charged in each autoclave with appropriate amount of white liquor of 25% sulfidity and 16% active alkalinity at a liquid to raw material ratio of 1:4. The schedule of digester heating consisted of 30 min for heating from ambient temperature to 100°C, 90 min for heating from 100°C to 160°C. The cooking time at 160°C was 90 minutes. Washing was carried out with warm water and followed by mechanical disintegrator to disintegrate the pulp samples. The washing process continued until the color of the water remained unchanged. After washing the pulp yield was determined. After calculating yield, pulp was screened on flat 0.20 mm slotted screen, in order to separate the undesired materials (reject%) from the pulp. The kappa number of pulp was measured by using TAPPI standard method T236 cm-76.

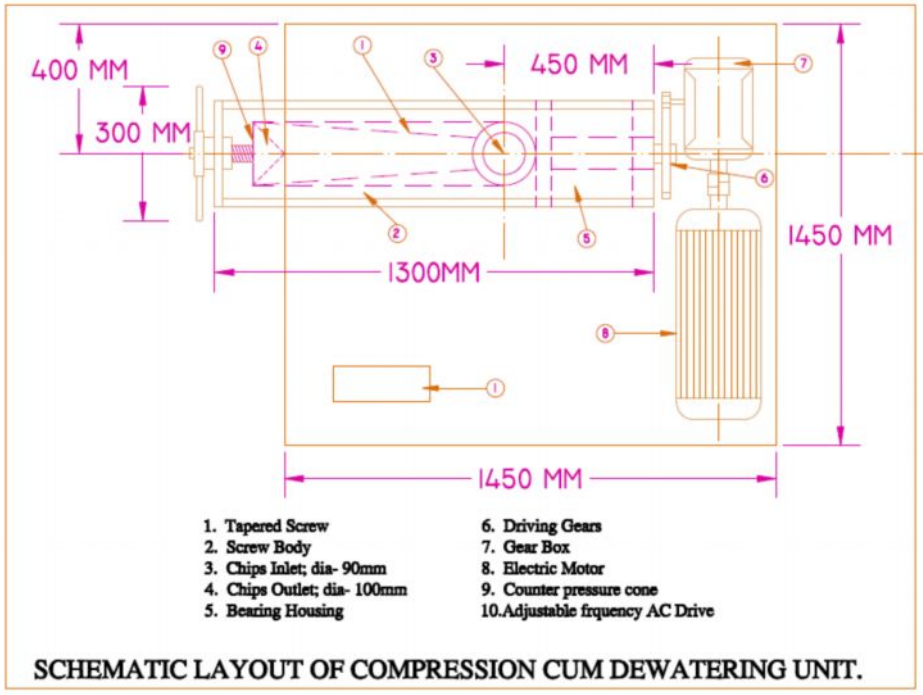

Fig. 1: Schematic layout of compression cum dewatering unit (Impressafiner).

Table 1: Effect of incubation period on lignin degradation in destructured.

S.No	Days	Lignin%	Lignin loss %	Difference in loss %	Holocellulose %	Holocellulose loss %	Difference in loss%
1	Control	27.50	-	-	69.53	-	-
2	7	26.80	2.55	-	68.45	1.55	-
3	14	25.55	7.09	4.54	67.28	3.23	1.68
4	**21**	**22.95**	**16.55**	**9.46**	**65.93**	**5.17**	**1.94**
5	28	22.17	19.39	2.84	64.70	6.95	1.78
6	35	21.50	21.82	2.43	63.60	8.53	1.58
7	42	21.00	23.64	1.82	62.68	9.85	1.32

Table 2: Effect of incubation period on lignin degradation in non-destructured.

S. No	Days	Lignin%	Lignin Loss %	Difference in loss %	Holocellulose %	Holocellulose Loss%	Difference in Loss%
1	Control	28.15	-	-	67.85	-	-
2	7	27.73	1.48	-	67.50	0.52	-
3	14	26.68	5.21	3.73	66.87	1.45	0.93
4	**21**	**24.87**	**11.66**	**6.45**	**65.70**	**3.17**	**1.72**
5	28	24.23	13.91	2.25	64.60	4.79	1.62
6	35	23.85	15.28	1.37	63.68	6.14	1.35
7	42	23.58	16.22	0.94	63.00	7.15	1.01

3 RESULTS AND DISCUSSION

3.1 Chemical Analysis

The chemical analysis of both bamboo (non-destructured and destructured) samples was done by Tappi Useful Method 249 and T 222 om-88 for holocellulose and Klason lignin respectively. The lignin content of non-destructured and destructured was found to be 28.15% and 27.50% respectively. The total holocellulose content was 67.85% and 69.53% respectively.

3.2 Effects of Varying Physical Parameters on Rates of Biodelignification by the *Trametes versicolor*

3.2.1 Effect of Time on Biodelignification

The effect of time on maximum biodelignification of bamboo (non-destructured and destructured) with *Trametes versicolor* was observed at 7, 14, 21, 28, 35 and 42 days of incubation periods. Keeping the other conditions as 2% molasses, 60% moisture, 6 pH and 25^{O}C temperature.

Table 1 and 2 shows that with increase in incubation time the lignin content decreases. But a sharp increase in lignin loss was observed between the incubation period of 14 to 21 days when it went up from 7.09% to 16.55% i.e. an increase of 9.46% in destructured samples, whereas the value was only 6.45% when it went up from 5.21% to 11.66% in case of non-destructured samples at the same incubation period. After increasing the incubation period above 21 days, rate of delignification starts decreasing. At the same time holocellulose loss was 5.17% and 3.17% for destructured and non-destructured respectively. On the basis of above observation 21 days were taken as optimum incubation period for both the samples to conduct further experiments.

3.2.2 Effect of Moisture on Biodelignification

Effect of different initial moisture content on biodelignification was observed by adding distilled water at the rate of 60%, 80% and 100% for both non-destructured and destructured samples. Keeping the other conditions as 2% molasses dose with pH 6 kept at 25^{O}C temperature for 21 days.

Table 3 depicts that the loss in lignin content under different moisture conditions show an increase in percent lignin loss upto 80% moisture in case of destructured samples however in case of non destructured samples maximum loss was noted at 60% moisture. At higher moisture content (100% moisture) lignin loss was reduced from 18.30% to 15.39% in case of destructured samples and 11.66% to 9.00% in non destructured samples. At the same time yield loss percent of holocellulose were 5.96% and 3.17% in destructured and non-destructured. On the basis of above observation 60% and 80% initial moisture content was taken as optimum for non-destructured and destructured samples respectively to conduct further experiments. The difference in the optimum conditions might be due to the open structure of the wood which results in more water absorbance in destructured sample than non destructured.

Table 3: Effect of moisture on lignin degradation in destructured and non-destructured.

S. No	Moisture %	Destructured				Non-Destructured			
		Lignin%	Lignin loss %	Holo cellulose %	Holo cellulose loss%	Lignin%	Lignin loss %	Holo cellulose %	Holo cellulose loss %
1	Control	27.50	-	69.53	-	28.15	-	67.85	-
2	60	22.95	16.55	65.93	5.17	**24.87**	**11.66**	**65.70**	**3.17**
3	80	**22.47**	**18.30**	**65.38**	**5.96**	25.12	10.78	65.85	2.95
4	100	23.27	15.39	66.17	4.84	25.62	9.00	65.97	2.78

Table 4: Effect of media and media dose percent on lignin degradation in fibers and non-destructured.

S. No	Media	Media Dose	Fibers				Non-Destructured			
			Lignin%	Lignin loss %	Holocellulose %	Holocellulose loss %	Lignin%	Lignin loss %	Holo -cellulose %	Holo-cellulose Loss %
1	Malt Extract Broth	2	22.13	19.52	64.08	7.83	24.5	12.97	65.02	4.18
2		**4**	**21.17**	**23.03**	**66.12**	**4.91**	**23.75**	**15.63**	**66.05**	**2.65**
3		6	21.48	21.88	65.6	5.65	23.98	14.8	65.67	3.22
4		8	22.03	19.88	65.02	6.49	24.25	13.85	65.15	3.98
5		10	22.75	17.27	64.17	7.71	24.57	12.72	64.6	4.79
1	Molasses	2	22.47	18.3	65.38	5.96	24.87	11.66	65.7	3.17
2		**4**	**21.53**	**21.7**	**67.37**	**3.11**	**24.1**	**14.39**	**66.53**	**1.94**
3		6	21.9	20.36	67	3.64	24.33	13.55	66.25	2.36
4		8	22.48	18.24	66.4	4.5	24.6	12.61	65.8	3.02
5		10	23.17	15.76	65.62	5.63	25.05	11.01	65.22	3.88

3.2.3 Effect of Media and Media dose on Biodelignification

Experiments were performed using malt extract broth and molasses separately with various doses i.e. 2%, 4%, 6%, 8% and 10%. The initial conditions maintained were pH 6, 60% moisture content for non-destructured and 80% moisture content for destructured kept for an incubation period of 21 days at 25^{O}C temperature.

Table 4 shows loss in lignin percent is increased with increase in media dose from 2% to 4%. Decrease in lignin degradation was observed after increasing the media dose from 4% to 10%, in both media. Maximum lignin loss was observed at 4% media doses, either molasses or malt extract broth, in comparison to the control samples. In comparison to the control sample, the loss in lignin was 23.03% in destructured and 15.63% in non destructured samples using malt extract broth. However with molasses loss in lignin was 21.70% in destructured and 14.39% in non destructured samples.

A slight difference in the behaviour of holocellulose degradation was observed in the presence of any of the two media. Degradation was minimum at 4% media dosage but on increasing the dose percent to 10%, the loss in holocellulose also increased with both the media in destructured and non destructured samples. The minimum loss observed was 4.91% in destructured and 2.65% in non destructured samples using malt extract broth. The holocellulose loss observed in destructured samples was 3.11% and 1.94% in non destructured samples using molasses.

On the basis of above observation more delignification was found with malt extract broth but if we consider the commercial aspect the molasses are economically 10 times cheaper compared to malt extract broth and saves holocellulose yield. In this view molasses was used to conduct further experiments.

3.2.4 Effect of pH on Biodelignification

The effect of different pH values of media were investigated by adjusting initial pH of media from 4.5 -7.0 at an interval of 0.5. The initial conditions maintained were 4% molasses, 25^{O}C temperature, 60% moisture content for non-destructured and 80% moisture content for destructured kept for an incubation period of 21 days.

Table 5 show the effect of pH on lignin and holocellulose degradation by *Trametes versicolor* in destructured and non destructured samples. The maximum lignin loss was observed when initial pH was adjusted to 5.5 in the case of both the samples. The lignin losses were observed to decrease with increase in pH from 5.5 to 7.0 in both the samples. Similar trend was observed by decreasing the pH of the media below 5.5. The holocellulose loss was found to increase with increase in pH from 6.0 to 7.0 in both the samples. However below pH 6.0 loss in holocellulose starts decreasing to pH 4.5.

From the results it is evident that biodegradation of lignin in the two samples with *Trametes versicolor* keeping in view the saving of holocellulose content, optimum initial pH value for the further experiments was taken as pH 5.5.

Table 5: Effect of pH on lignin degradation in destructured and non-destructured.

S. No	pH	Destructured				Non-Destructured			
		Lignin%	Lignin loss %	Holocellulose %	Holocellulose loss %	Lignin%	Lignin loss %	Holocellulose %	Holocellulose loss %
1	Control	27.50	-	69.53	-	28.15	-	67.85	-
2	4.5	22.97	16.48	68.2	1.91	25.02	11.13	67.13	1.06
3	5	21.9	20.36	67.83	2.44	24.23	13.91	66.45	2.06
4	**5.5**	**21.27**	**22.67**	**67.55**	**2.85**	**23.87**	**15.22**	**66.7**	**1.69**
5	6	21.53	21.70	67.37	3.11	24.1	14.39	66.53	1.94
6	6.5	22.01	19.94	67.18	3.38	24.37	13.44	66.28	2.3
7	7	22.6	17.82	66.95	3.71	24.7	12.26	65.95	2.8

Table 6: Effect of temperature on lignin degradation in destructured and non-destructured.

S. No	Temperature	Destructured				Non-Destructured			
		Lignin%	Lignin loss %	Holocellulose %	Holocellulose loss %	Lignin%	Lignin loss %	Holo-cellulose %	Holo-cellulose loss %
1	Control	27.50	-	69.53	-	28.15	-	67.85	-
2	20	25.40	7.64	68.95	0.83	26.92	4.38	67.4	0.66
3	**25**	**21.27**	**22.67**	**67.55**	**2.85**	**23.87**	**15.22**	**66.7**	**1.69**
4	30	21.67	21.21	68.00	2.20	24.22	13.97	67.05	1.18
5	35	23.90	13.09	68.58	1.36	25.85	8.17	67.2	0.96

3.2.5 Effect of Temperature on Biodelignification

Temperature is an important factor affecting the performance of fungal cells. Experiments were performed at four different temperatures i.e. 20^0C, 25^0C, 30^0C and 35^0C. Other factors were set initially as 4% molasses with pH 5.5, 60% moisture content for non-destructured and 80% moisture content for destructured kept for an incubation period of 21 days.

Table 6 shows the effect of temperature on lignin and holocellulose degradation by *Trametes versicolor* in destructured and non destructured samples. The maximum lignin and holocellulose losses were observed at 25^0C in the case of both the samples. The lignin and holocellulose losses were observed to decrease with increase in temperature from 25^0C to 35^0C in both the samples. However on decreasing the temperature below 25^0C, lignin and holocellulose losses also decreased. From the results it is evident that optimum biodegradation of lignin and holocellulose in the two samples treated with *Trametes versicolor* is observed at 25^0C.

3.2.6 Scanning Electron Microscopy

Scanning Electron Microscope (SEM) images of treated and untreated samples of *Dendrocalamus strictus* were obtained using Quanta 200 FEG, Type FP 2032/11 scanning electron microscope, operated in a secondary electron mode at an accelerating voltage of 15 kv. The fibers were coated with gold for 120s. Samples were examined at various magnifications.

Observations of SEM micrographs of the treated samples showed an extensive colonization by the fungi and in advanced stages of decay, cell walls are eroded extensively, and holes within adjacent cell walls are frequently observed (Fig 2). In general SEM observations made on the colonization of *Trametes versicolor* indicated an extensive hyphae growth in all tissues of the bamboo treated samples. In non destructured samples, fungal hyphae penetration through holes is observed only on the surface whereas in destructured samples the penetration is observed in all the separated fibres from various directions.

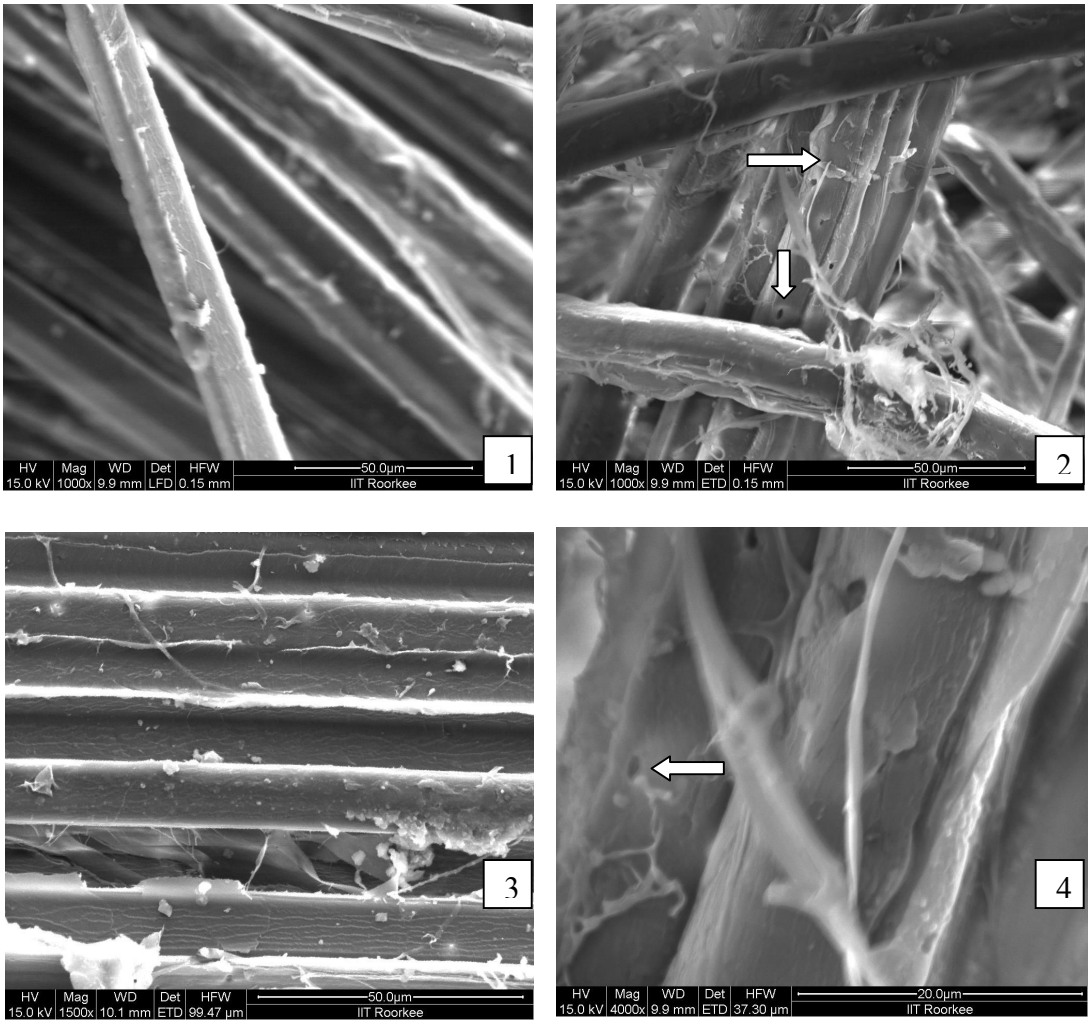

Fig 2: Scanning Electron Micrographs of fibers and chips of *Dendrocalamus strictus* treated by *Trametes versicolor*. (1) Untreated fibers of *Dendrocalamus strictus*. (2) Showing hyphae penetration inside the open wood fibers (3) Untreated chips structure. (4) Showing fungal hyphae on chips surface.

Table 7: Pulp yield and Kappa number of Bamboo treated and non treated Samples.

S.No	Pulp Samples	Pulp Yield (%)	Kappa Number	Rejects (%)
1	NDC*	45.32	15.26	0.082
2	DC **	47.43	14.68	0.0982
3	NDT #	44.96	13.31	0.0221
4	DT##	45.03	10.25	0.0194

*NDC- Non destructured control, **DC- Destructured control, #NDT- Non destructured treated and ##DT- Destructured treated.

3.3 Pulp Characteristics

Table 7 summarizes the results of kraft pulping experiments that were conducted to investigate the effect of fungal treatment on kappa number and pup yield (%).The investigation has shown that delignification rate of destructured treated sample (DT) was high which was represented by decrease in kappa number. After fungal treatment 5 point reduction in kappa number was observed. Whereas a little difference in pulp yield (%) was observed.

4.0 CONCLUSION

Rate of delignification was estimated by applying *Trametes versicolor* and the comparison was made between the non destructured and destructured samples. It was found that extent of delignification was significantly distinct between the two samples. The influence of physical parameters like pH, temperature, media and media concentration, moisture and incubation time were also optimized during the study. The concluded optimized parameters for destructured sample using *Trametes versicolor* were 80% moisture, 4% dose of molasses with pH adjusted to 5.5 at 25^0C temperature for 21 days of incubation. Whereas in case of non destructured samples moisture condition was different i.e. 60%. A significant decrease in kappa number (5 point) in treated destructured bamboo samples would minimizes the amount of harsh chemical treatments given for bleaching purpose,

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