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PRETREATMENT OF WOOD CHIPS AND PULPS WITH *FOMES LIVIDUS* AND *TRAMETES VERSICOLOR* TO REDUCE CHEMICAL CONSUMPTION IN PAPER INDUSTRIES

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Key words: *Fomes lividus*, *Trametes versicolor*, biobleaching, kappa point, ISO brightness, ligninolytic enzymes

Abstract – Two white rot fungi *Fomes lividus* and *Trametes versicolor*, isolated from the Western Ghats region of Tamilnadu, India, were used to treat *Eucalyptus grandis* wood chips and hard wood Kraft pulp. Hand sheets that were prepared by conventional chemicals methods and had 20 kappa points and 14.0 ISO brightness were used as a control. Hand sheets prepared by fungal pretreatment followed by treatment with 50% Na_2O chemicals (as that of control) had 15 and 14 kappa points 16 and 17 ISO brightness, *of F.lividus* and *T.versicolor* respectively. Biobleaching and delignification of hard wood and kraft pulp (HWKP) by pretreatment with *F.lividus* and *T.versicolor* in shaking culture reduced the kappa number by 38.7 and 67.7% and increased ISO brightness by 29.8 and 29.9% of on 6th and on 5th day, respectively. Partially purified enzymes of these fungal lignin peroxidase (LiP), manganese dependent peroxidase (MnP) laccase and mixture of these enzymes were treated to hard wood kraft pulp. Laccase at 15U/ml of both fungi reduced the kappa number by 36.1% and increased ISO brightness in *T. versicolor* mixture enzymes by 36.1%. These results revealed and *F.lividus* and *T.versicolor* pretreatment could reduce the chemical usage in paper industries without affecting the paper quality.

INTRODUCTION

Removal of lignin from wood is a key operation in manufacturing of high - value paper products. Although most lignins are removed from wood during pulping, the last vestiges must be removed by a series of oxidation bleaching reactions. This was accomplished by using hyphochlorous acid or chlorine (Reeves, 1992). Pulping and bleaching of Kraft pulp uses large amounts of chlorine and chloride chemicals. Products of these pulping and bleaching are chlorinated organic substances, some of which are toxic, mutagenic, persistent, and bio-accumulating, and cause numerous harmful disturbances in biological systems (Bajpai and Bajpai, 1997). Pretreatment of wood chips with appropriate fungi results in significant energy and chemical-savings and allows an improved paper quality (Paice *et al.*, 1989; Moreia *et al.*, 2001). Vares *et al.*, (1995) reported that biopulping of straw or the treatment of semi chemical straw pulp by lignin modifying enzymes would decrease the energy requirement and the consumption of cooking chemicals to remove lignin during pulping. The enzymes responsible for biobleaching are laccase, manganese-dependent peroxidase (MnP) and xylanases (Nerud *et al.*, 1991; Paice *et al.*, 1993; Gubitz *et al.*, 1997; Vicuna *et al.*, 1997; Haarhoff *et al.*, 1999; Madlala *et al.*, 2001; Pfabigan *et al.*, 2002). Environments friendly enzymatic pulp bleaching techniques have been intensively investigated in recent years. The first enzyme used for pulp bleaching was xylanase (Viikari *et al.*, 1986). Manganese dependent peroxidase (MnP), a ubiquitous peroxidase among white rot fungi (Eggert *et al.*, 1996) is another lignin- degrading enzyme that can be used for pulp bleaching. The activity of MnP is dependent on Mn^{2+}. It has been proposed that MnP oxidizes Mn^{2+} to Mn^{3+}, which in turn oxidizes lignin (Wariishi *et al.*, 1988). Laccase are copper-containing phenol oxidases that require mediator compounds

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such 2,2 -azino-bis (3-ethylbenzthiazoline-6- sulfonic acid) (ABTS) or 1-hydroxybenzotriazole, and of questionable environmental acceptability to effectively delignify kraft pulps (Bourbonnais and Paice, 1992; Sealey *et al.*, 1999). The biobleaching system oxidizes the phenolic compound of lignin, and the residual lignin is demethylated and significantly enriched in carboxylic acid groups (Sealy and Ragauskas, 1998). In this work, two newly isolated white rot fungi *F.lividus* and *T.versicolor* and its ligninolytic enzymes have been used for pretreatment of *E.grandis* wood chips and pulp. The effect of reduced chemical dosage on the quality of paper obtained from these pretreated pulps was analysed .

MATERIALS AND METHODS

Organisms

Fruit bodies of *Fomes lividus* and *Trametes versicolor* were surface-sterilized with 1% mercury chloride solution, repeatedly washed with sterile distilled water and inoculated on 2% malt agar plates. The plates were incubated at 37°C for 6 days for sporulation (Walting 1971).

Enzyme preparation

The spore suspension (10^5 spores/ml) prepared from malt agar plates were inoculated into C-limited medium (10%v/v) of Janshekar and Fiechter (1988) having following composition: D-glucose, 3.0 g, diammonium tartarate, 0.66 g, $MgSO_4.7H_2O$, 0.15 g, $CaCl_2.2H_2O$, 30 mg, $FeSO_4.7H_2O$, 5.55 mg, H_3PO_4 (2N), 3.27 ml, trace element solution, 0.30 ml, vitamin solution, 0.30 ml and distilled water 1000 ml. Trace element solution (g/l): nitrolotriacetate, 1.5, $MnSO_4.H_2O$, 1.0, $CoCl_2.6H_2O$, 1.0, $ZnSO_4.7H_2O$, 3.0, $CuSO_4.5H_2O$, 3.0, Alk $(SO_4)2$, 0.01, H_3BO_3, 0.01, Na_2MoO_4, 0.01, vitamin solution (mg/l): biotin,2.0, folic acid, 2.0, thiamine HCL, 50.0, riboflavin, 5.0, Pyridoxine HCl, 10.0, cyanocobalamine, 0.1, nicotinic acid, 5.0,calcium pantothenate, 5.0, p-amino benzoic acid, 5.0, thioacetic acid 5.0). The pH of the medium was adjusted to 4.5. The culture flasks were incubated at 30 °C for 6 days. After incubation, the fungal biomass was removed by filtration and the culture filtrate was centrifuged at 18,000 X g for 30 min at 4°C. Enzymes in the clear culture filtrate were partially purified by acetone precipitation (66%v/v) and sephadex G100 column chromatography. (Selvam, 2000). The protein-containing fractions were analyzed for lignin peroxidase (LiP) manganese-dependent peroxidase (MnP) and laccase activities.

Enzyme assay

The lignin peroxidase (LiP) activity was assayed by the method of Linko (1988). The assay solution contained the culture filtrate, 1.0 ml; sodium tartarate buffer (pH 3.5), 100 mM; veratryl alcohol, 0.4 mM; freshH_2O_2 0.3mM immediately after adding H_2O_2, the change in absorbance at 310 nm was recorded at 30 sec intervals. The enzyme activity was expressed as U/ml (1U= µmole of vertry1 alcohol oxidized in 1 min). The manganese dependent peroxidase (MnP) activity was assayed by the method of Kuwahara *et al.*, (1984). The reaction mixture consists of culture filtrate, 1.0ml; phenol red-0.01%; lactate- 25 mM $MnSO_4$-100 µM; egg albumin-0.1%, H_2O_2-100 µM in 1ml of 20mM sodium succinate buffer, pH 4.5.Reactions were carried out at 30°C for 5 min and terminated by the addition of NaOH. The absorbance was measured at 610 nm. The enzyme activity was expressed as U/ml (1U=change in OD/min at 610nm). The laccase activity was measured by the method of Evans (1985). The reaction mixture consists of culture filtrate, 0.5ml, guaiacol-0.35µl (0.035%, v/v), sodium acetate buffer 0.1M; (pH 5.0), and 2.0ml. The enzyme activity was expressed as the change absorbance at 440nm for 1 minute.

Pulping and bleaching of wood chips

Eucalyptus grandis wood chips were used for pulp preparation .The wood chips were cooked in Na_2O solution (1:2.8 w/v) at 170°C for 90 min. Before cooking, the wood chips and the chemicals were preheated at 0-100°C for 30 min and 100-170°C for 80 min. After cooking, the pulp was subjected to alkali-chlorine (EDED) bleaching with a chlorine multiple of 0.18 (Buchert *et al.*, 1992).

Biopulping and bleaching of wood chips

The fungus was grown on wood chips moistened with C-limited medium for 40 days. The chips were then washed thoroughly with water to remove the mycelial growth and cooked as in conventional method and with 50% Na_2O concentration, that is, wood chip and Na_2O in the ratio of 1:2.8 and 1:1.4 (w/v) resepestively. The cooked pulp was subjected to EDED process with chlorine multiple of 0.18 and 0.15.

Biobleaching and delignification of hardwood kraft pulp (HWKP)

For biobleaching and delignification of HWKP,

whole fungal biomass and the partially purified enzymes (LiP, MnP and laccase) were used. The HWKP was obtained from Tamilnadu Newsprint and Paper Industry Limited, Karur, India).

Treatment with fungal biomass

Mycological broth amended with a glass bead (2.5cm diameter) and HWKP (0.25%w/v) was inoculated with spore suspension (10^5 spores /ml) and incubated at 25° C for 5 days on a rotary shaker (200 rpm). The resulting suspension was inoculated into HWKP suspended in sterile distilled water (2%w/v) at a concentration of 15%(v/v) and incubated at 25°C for 2 to 5 days on a rotary shaker (200 rpm) (Archibald *et al.*, 1990)

Treatment with enzymes

The HWKP with 2.5% consistency (w/v) was treated with various concentration (5,10 and 15 U/2.5g dry pulp) of mixed enzyme, LiP, MnP and laccase at 50 °C for 24 h. For mixed enzyme, LiP and MnP treatments the pulp suspension was prepared in 20mM sodium succinate buffer, pH 4.5, and for laccase treatment it was prepared in 0.1M phosphate buffer pH 7.0.

Preparation of hand sheets and paper quality

After every pulping and bleaching process, the pulps were thoroughly washed with distilled water and the pulp suspension was through a Bunchner funnel vaccum. The residue was bottled and air-dried for 24h (TAPPI, 1998). The quality of the paper made was determined in terms of kappa number and ISO brightness points. The kappa number was determined by TAPPI test methods (1998) and brightness of the paper was determined in Perkin Elmer ë 3B spectrophotometer equipped with a reflectance sphere at 457nm.

RESULTS AND DISCUSSION

Biopupling and bleaching of wood chips

Ligninolytic fungi *F.lividus* and *T.versicolor* were tested for the pretreatment of *E. grandis* wood chips to reduce the usage of chemicals in pulping and bleaching processes. The result revealed the efficiency of fungi in bleaching and delignification of wood chips. The hand sheets prepared by conventional methods of pulping and bleaching had 20 Kappa points and 14 ISO brightness points. . Incubation of wood chips with white rot fungi could decrease the refining energy requirement and could increase the paper quality (Table 1). Leatham et al. (1990) reported that incubation of aspen chips for four weeks with *Phlebia brevaspora, Ceriporiopsis subvermispora* and *Dichomictus squalens* decreased the refining energy requirement by 47-68%. Messner et al., (1992) pretreated birch wood chips with white rot fungi for four to six weeks prior to pulping; the treatment resulted in the 30 to 50% reduction in kappa number and the 0 to 4% increase in ISO brightness .In the present study it was observed that pulps obtained from wood chips and incubated with

Table 1. Effect of *F.lividus* and *T.versiocolor* pretreatment and chemical dosage on paper quality

Treatment	Kappa number (Points)		Brightness (%) (ISO Points)	
Conventional chemical Method (Control)				
W:Na_2O (1:2.8w/v)+ 0.18 Chlorine multiple	20(100)		14(100)	
Biopulping	*F.lividus*	*T.versiocolor*	*F.lividus*	*T.versiocolor*
FPW: Na_2O (1:2.8w/v)	17 (-15)	12 (-40)	14 (0)	16 (+14)
FPW: Na_2O (1:1.4w/v)	21 (+5)	17 (-15)	15 (+7)	15 (+7)
Biobleaching				
FPW: Na_2O (1:2.8w/v)+ 0.18 Chlorine multiple	10 (-50)	9 (-55)	18 (+29)	18 (+29)
FPW: Na_2O (1:2.8w/v)+ 0.15 Chlorine multiple	13 (-35)	11 (-45)	20 (+43)	18 (+29)
FPW: Na_2O (1:1.4w/v)+ 0.18 Chlorine multiple	14 (-30)	13 (-35)	18 (+29)	18 (+29)
FPW: Na_2O (1:1.4w/v)+ 0.15 Chlorine multiple	15 (-25)	14 (-30)	16 (+14)	17 (+21)

W: Wood chip, FPW: Fungal pretreated wood chip
Kappa number (%) decrease over control and brightness (%) increase over control

F. lividus and *T. versicolor* for six weeks followed by conventional cooking had 17 and 12 kappa points and 14 and 16 ISO brightness point, respectively. When pulps pretreated with *F. lividus* and *T. versicolor* was further bleached with full dosage of chemicals (chlorine multiple of 0.18), the kappa points were decreased to 10 and 9, respectively, and the ISO brightness with points were increased to 18. The pulps subjected to biopulping with *F. lividus* and *T. versicolor* followed by 50% Na_2O treatment had kappa points of 15 and 14 and ISO brightness point of 16 and 17, respectively. The quality of the biopulp in terms of increased brightness was in par with that of conventionally obtained finished sheets, revealing that pretreatment of wood chips with *F. lividus* and *T. versicolor* could reduce the chemical consumption at least by 50%.

Biobleaching and delignification of HWKP

Treatment with fungal biomass15 days incubated

Trametes versicolor was capable of decolorizing and delignifying the unbleached industrial kraft pulps over 2 to 5 days incubation. The brightness was increased by 28 to 45.8% and the kappa number was reduced by 9.6% (Archibald 1992; Paice *et al.*, 1993) *Ceriporiopsis subvermispora* bleached the pulp effectively after 14 days incubation. The kappa number was decreased from 6.7 to 0.8 and the brightness was increased by 47.0% (Christov *et al.*, 1995). Bjerkandara sp. strain BOS55 extensively delignified and bleached the oxygen- delignified eucalyptus Kraft pulp with brightness gain of 14 ISO units (Moreia *et al.*, 2001). In the present study (Fig 1A) *F. lividus* treatment 6 days incubation reduced the kappa number by 38.71% and increased the ISO brightness points of the paper by 29.93% *T. versicolor* treatment also reduced the kappa number (67.30%) and increased the ISO brightness points (29.8%) after five day treatment (Fig 1B).

Treatment with enzymes

The hard wood kraft pulp was treated with LiP, MnP, laccase and a mixture of these enzymes. The *F. lividus* and *T. versicolor* enzymes seemed to be efficient in bleaching and delignification of HWKP. Treatment of *F. lividus* enzyme at reduced the kappa number by 33.30% and increased the ISO brightness point by 17.3%. LiP reduced the Kappa number by 22.20% and increased the ISO brightness point by 6.50% and MnP reduced the kappa number by 25.10% and increased the ISO brightness point was increased by

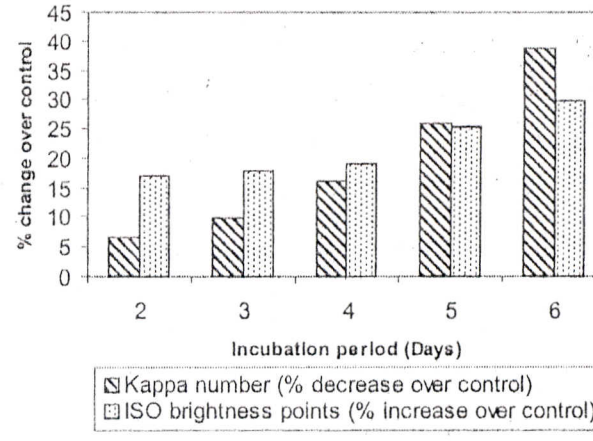

Fig: 1A

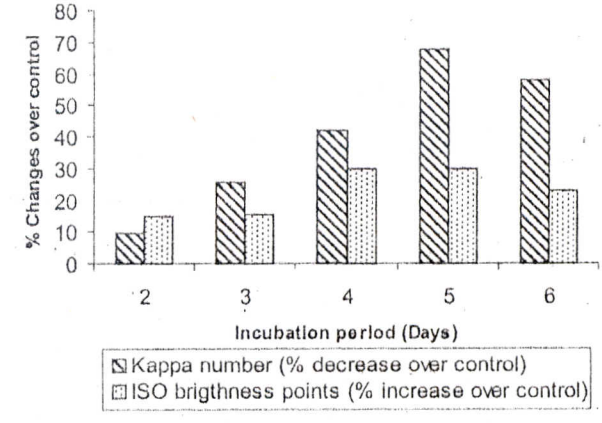

Fig: 1B

Fig.1. Bioleaching and delignification of hard wood kraft pulp (HWKP) by *Fomes lividus* and *Trametes versicolor*. HWKP suspended in sterile distilled water (2%w/v) was inoculated with *Fomes lividus* spore suspension (10^5 spores/ml; 15%v/v) (A) and *Trametes versicolor*- (B) incubated at 25°C for 2 to 5 days on a rotary shaker (200rpm). Hand sheets were prepared from the pretreated pulp and kappa number and ISO brightness point were determined. Control values-Kappa number: 20; ISO brightness point: 14.

15.1%. In laccase treatment, the kappa number was reduced by 36.1% and the ISO brightness point was increased by 19.31% (Fig 2A,B). In the case of *T. versicolor* enzymes, mixed enzyme treatment yielded the reduction of the kappa number by 36.10% at 15 U/ml concentration, but the ISO brightness point was increased to a maximum of 22.6% by 10U/ml concentration. In Lip treatment also, maxmium of 22.60% by 10 U/ml concentration. In Lip treatment also, maximum reduction in kappa number (27.8%) was observed at 15U/ml concentration, while the

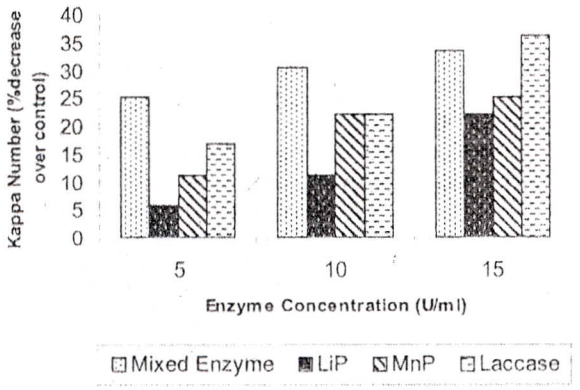

Fig :2A

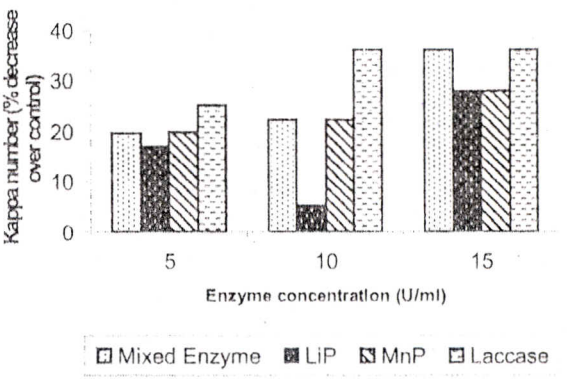

Fig :2C

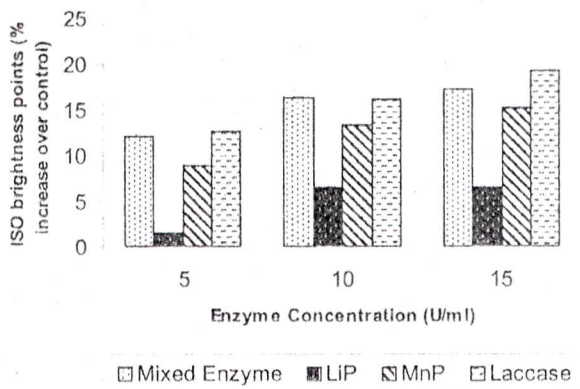

Fig :2B

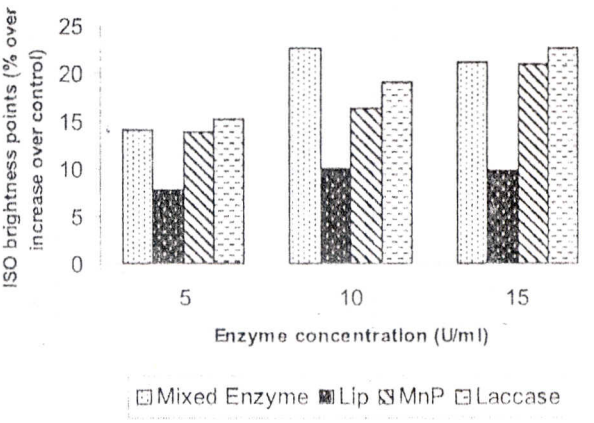

Fig :2D

Fig.2.Bioleaching and delignification of hard wood kraft pulp (HWKP) by ligninolytic enzymes of *Fomes lividus* and *Trametes versicolor*. HWKP (2.5% consistency, w/v) was treated with ligninolytic enzymes of *Fomes lividus* (2 A, B) and *T.versicolor* (2 C, D) at 5,10,15 U/2.5g dry pulp at 50°C for 24h. Hand sheets were prepared from the pretreated pulp and kappa number and ISO brightness points were determined. % Decrease in kappa number and increase in ISO brightness points were calculated. Control values-Kappa number: 20; ISO brightness points: 14.

ISO brightness point was increased to the maximum (10.0) at 10 U/ml concentration. But in MnP and laccase treatments, maximum reduction in kappa number (27.8) and 36.10% respectively) and increase in the ISO brightness points (21.0 and 22.5) was observed at 5 U/ml enzyme concentration Fig 2C,D. Arbeola *et al.* (1992) and Paice *et al* (1993) reported that ligninolytic enzymes could be employed in the pulp industry for bleaching and delignification purposes. Especially laccase and MnP have major roles in bleaching process. Call and Muckle (1995) reported that the treatment of different kinds of pulps with LIGNOZYMS laccase –Mediator system (LMS) resulted in 70% reduction in the kappa number. Sealy and Ragauskas (1998) reported that the laccase /N-hydroxybenzotriazole system yielded 52% delignification of softwood kraft pulp, whereas use of ABTS yielded 35% delignification. In the present study also, it was observed that laccase, MnP and the mixed enzyme were very efficient in bleaching and delignifying HWKP. They have decreased the kappa number by 33-36% and increased the brightness by 14-15%. This study is the first report that *F. lividus* can be used for pretreatment of wood chips and pulps effectively in the paper manufacturing process to reduce the consumption in paper industries.

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