ISSN: 0379-5462
IPPTA: Quarterly Journal of Indian Pulp and Paper Technical Association
Vol. 29, E1, 2017, pp.74-77

PRODUCTION OF FUNGAL XYLANASE AND LACCASE ENZYMES FOR ENZYMATIC PRE-BLEACHING APPLICATION


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Abstract :

The need for adoption of greener technologies has led to a growing interest in the use of enzymes in the production of paper. Of the various enzymatic options available to the paper industry, enzymatic prebleaching of the pulp has been the most significant one. The use of the either hydrolytic or oxidative enzymes like xylanase and laccase has been widely applied for the pretreatment of pulp before bleaching. More recently the use of mixed enzyme systems consisting of a combination of hydrolytic and oxidative enzyme has been explored.

Both xylanase and laccase enzymes are commercially available. The application of commercial enzymes for enzymatic prebleaching application has been demonstrated by CPPRI before. In the present study CPPRI made an effort to produce both xylanase and laccase enzymes from isolated fungal strain. The produced enzymes were studied individually as well as in combination for their effect on improving the bleachability of the mixed hardwood pulp. The effect of different enzyme on the strength properties of pulp was also studied. The study indicated that application of a mixed enzyme system results in higher brightness gain and improved strength properties compared to individual enzyme application.

INTRODUCTION :

The world's total production of paper and paperboard reached approximately 400 million tons in 2012, and the global demand for paper and paperboard for packaging purposes has increased by 75% in the last five years (Sakhawy et.al.2015). Considerable interest has been focused on the use of hydrolytic enzymes like xylanases that degrade xylan components in plant cell walls into simple sugars. Commercial applications of xylanases include pulp bleaching, food and animal feed industries, fuel, textile industries and in water management. They are required in bulk amounts and have significant application in paper and pulp industries as hydrolysis of xylan releases lignin from paper pulp and reduce usage of chemical bleaching agents. Throughout world pulp and paper mill industrial effluents contain toxic and harmful organic compounds as byproducts of pulping and bleaching processes. These effluents contain toxic heavy metals, lignin and its derivatives in addition to colour-imparting phenol and resinous compounds (Valls and Roncero 2009). Dark colour of unbleached pulp is due to the deposition of lignin and to remove colour one or more bleaching sequences like chlorine bleaching, oxidizing or reducing chemicals and alkaline extractions are needed (Khandeparkar and Bhosle 2007; Ziaie-shirkolaee et al. 2008). Due to use of these strong oxidants, chlorinated lignins and phenols are discharged into wastewaters. To substitute chlorine and to implement environmentally sound bleaching sequences by chlorine dioxide, hydrogen peroxide, oxygen, or ozone, among others can be used to get "totally chlorine-free" (TCF) bleaching. The drawback to introduce these environmentally sound technologies in pulp and paper industry is because it is difficult to attain high brightness degree as residual lignin and lignin-derived compounds that are

more recalcitrant to degradation in TCF bleaching. To overcome these difficulties, enzymatic biobleaching using xylanases and laccases is an efficient alternative in many industrial applications. The application of enzymes like xylanases, cellulases or laccases in paper pulp bleaching is important as they reduce release of pollutants during bleaching and can also enhance the bleaching effect of chemical reagents by affording substantial savings (Valls and Roncero 2009). Simultaneous action of xylanase and laccase may prove to be a promising strategy for achieving higher degree of pulp bleaching. Due to action of xylanase, lignin is exposed which will be degraded and removed simultaneously due to presence of laccases, therefore, leading into improved level of delignification (Zhang, D. et al. 2016)

In the present work, mixed enzyme preparation consisting of indigenously produced fungal xylanase and fungal laccase was evaluated for its ability to bleach mixed wood pulp.

Material and Method

Raw materials (Pulp)

The mixed wood pulp (Eucalyptus/Poplar, 60:40) corresponding to kappa number 16.1, obtained through Kraft pulping from STAR Paper Mill, Saharanpur, (Uttar Pradesh), India. Pulp was washed, air dried, and stored in air tight polythene bags for further studies.

Chemicals

All assay reagents were purchased from Sigma–Aldrich while all media components were purchased from Hi Media Laboratories Pvt. Ltd. The chemicals used were purchased from Fischer Scientific (Waltham, USA). Wheat bran was obtained locally.

Enzyme Production

Xylanase

The Xylanase enzyme used in the study was a crude enzyme produced from an isolated fungus in the laboratory (Xylanase enzyme activity, 150 U/ml).

Microorganism

Fungus was isolated from soil collected from pulp and paper mill. The fungus was purified, grown, and maintained on Potato dextrose agar.

Production

Xylanase production was carried out in flask level with 100 ml malt extract broth inoculated with 10% of 5 day old fungal discs and incubated at optimum temperature (30°C) and optimum pH (6.0). Crude xylanase enzyme was extracted after 4 days used in present study.

Laccase

The Laccase enzyme used in the study was a crude enzyme produced from an isolated fungus in the laboratory (Laccase enzyme activity, 1200 U/ml).

Microorganism

Fungus was isolated from rotted wood by removing upper surface with sterilized forceps. The fungus was purified, grown and maintained on malt extract agar.

Screening

Selected fungal strains for Ligninolytic activity was done in MEA plates supplemented with Gallic acid, Phenol red and ABTS. These supplemented MEA plates are inoculated with Fungal mycelial disc and incubated at 350C for three days in dark as described in Experimental. After three days plates were observed for the appearance of Brown, Yellow and Green colures respectively.

Production

Laccase enzyme production was carried out in Erlenmeyer flasks of 250 ml capacity, each containing 5g of wheat bran and 15ml of mineral salt media (MSM) solution at pH 7.0 of Mandel and Reese (1957) was autoclaved and used for Laccase production by solid state fermentation. Temperature, pH and incubation time were optimized for better production of enzyme i.e. temperature 32°C, pH 5.0, days 3rd. Fungal colony grown on PDA plates at 32°C for 3 days was used as inoculums. Culture medium was inoculated with 5 discs of fungal growth cut with the help of cork borer of 8mm diameter. The flasks were gently tapped intermittently to mix the contents and incubated at for 3 days. After fermentation, 50 ml, 0.05 M citrate phosphate buffer (pH5.0) was added to the fermented matter and the contents were mixed for 30 min at 500 rpm on a magnetic stirrer and filtered through a muslin cloth by squeezing. The extract was centrifuged at 10,000 rpm for 10 min and the enzyme extract was used for estimation of Laccase activity.

Enzyme assay

Enzyme activity of both the indigenous enzyme was determined by following methods.

Xylanase

Xylanase activity was determined according to the Bailey method (Bailey et al. 1992). The substrate solution contained 1% birchwood xylan (Sigma) dissolved in 0.05 M sodium phosphate buffer (pH 7.0). The reaction mixture consisted of 1.8 ml substrate solution and 0.2 ml of appropriately diluted enzyme. After 30 min incubation at 50°C, the liberated reducing sugars (xylose equivalent) were estimated by the DNS reagent method.

Laccase

Laccase enzyme activity was analysed following the procedure described in Arora and Sindhu (1985). Enzyme activity is assayed at 30°C by using 10 mM guaiacol with 100 mM sodium citrate buffer. The change in absorbance of the reaction mixture containing guaiacol was monitored at 470 nm for 30 minutes of incubation. One unit of activity was defined as the change in absorbance at 470 nm of 0.001 per minute per mL of enzyme.

Xylanase and Laccase enzyme treatment of the pulp

The mixed hard wood pulp was treated with xylanase and laccase individually (X or L) as well as in combination (X

+ L) using the optimized enzyme dose for optimized time period. Xylanase and laccase enzyme dose were optimized on pulp based on the kappa number reduction, and brightness improvement of the pulp after enzyme treatment. Optimized dose of both the enzyme were added to the pulp after sufficient dilution and mixed properly by kneading mechanism. Control pulps were prepared identically to the enzyme treated pulp with enzyme replaced with water. After enzyme treatment, pulps were washed and subjected to characterization.

Table 1. Enzymatic Pretreatment Conditions

S.no	Particular	Control	Enzyme treated pulp			
			X.	L.	X. +L. (Full Dose)	X.+L. (Half Dose)
1	Enzyme dose (IU/gm)	-	15	25	15+25	7.5+12.5
2	Mediator (%)	-	-	0.2	0.2	0.1
3	Temperature (^oC)	50	50	50	50	50
4	Treatment time (min)	180	180	180	180	180
5	Consistency (%)	10	10	10	10	10
6	ph	7.4	7.6	7.3	7.2	7.8

After enzymatic treatment, pulps were subjected to ECF bleaching following [D0E(p)D1] sequences and the final pulp brightness was measured.

Characterization of pulp after enzymatic treatment

After enzyme treatment, enzyme treated and untreated pulps were analyzed for kappa number and brightness.

Characterization of enzymatic pulp after ECF bleaching

After bleaching, both enzyme-treated and untreated pulps were analyzed for brightness. Handsheets were prepared at neutral pH following TAPPI Test method 205 and analysed for strength and optical properties such as burst index, tensile and tear index values.

Result and Discussion

Characterization of the unbleached pulp after enzyme treatment

Unbleached pulp samples of both enzyme-treated and untreated were characterized for kappa number, brightness (% ISO), and other parameters of interest. Results are shown in Table 2. Enzyme-treated pulps showed slight reduction in kappa number and improvement in brightness when compared with the control. When both the enzyme used in combination the kappa no is more lower (12.60) in comparison to xylanase (13.32) and laccase (12.92) individually in respect to control (14.00). Brightness order was achieved higher in combination (30.60) in comparison to xylanase (29.90) and laccase (29.15) individually in respect to control (28.90). but after reducing the enzyme dose just half in combination there was increasing result in terms of brightness gain and kappa no. reduction when compared to use of X and L individually in normal enzyme dose.

Several reports described the reduction in kappa number by different xylanases.

Table 2: Characterization of enzyme treated and untreated unbleached pulp

S.no	Particular	Control	Enzyme treated pulp			
			X.	L.	X. +L. (Full Dose)	X. +L. (Half Dose)
1	Kappa no	14.00	13.32	12.92	12.60	12.75
2	Brightness %ISO	28.90	29.90	29.15	30.60	29.60
3.	Yellowness % ISO	37.05	35.77	36.23	35.15	36.28
4.	Whiteness % ISO	0.00	0.00	0.00	0.00	0.00

Characterization of enzyme treated and untreated bleached pulp

Bleached pulp samples of both enzyme-treated and untreated were characterized for brightness (% ISO), and other parameters of interest. Results are shown in Table 3. Brightness order was achieved higher in combination (85.35) in comparison to xylanase (84.65) and laccase (84.45) individually in respect to control (83.05). Brightness improvement was achieved 2.3 unit in case of combination of both X and L enzyme, whether when both enzyme used individually 1.4 and 1.6 unit improvement achieved respectively but after reducing the enzyme dose just half in combination there was increasing result in terms of brightness improvement i.e. 1.85 which is more in case when we used X and L individually in normal enzyme dose.

Table 3. D0E(p)D1 Bleaching of Enzyme treated and untreated pulps

Parameter	Control	Enzyme treated Pulp	
Brightness improvement unit	-		**1.85**
Yellowness %ISO	7.01		5.70

Table 3 :$D_0E(p)D_1$ Bleaching of Enzyme treated and untreated pulps

Parameter	Control	Enzyme treated Pulp			
		X	L	X+L	$X_{1/2}+L_{1/2}$
Brightness % ISO	83.05	84.45	84.65	85.35	84.90
Brightness improvement unit	-	**1.40**	**1.60**	**2.30**	**1.85**
Yellowness %ISO	7.01	6.90	6.25	5.30	5.70
Whiteness% ISO	71.20	71.85	72.40	75.80	75.20

Effect of enzyme treatment on Strength properties of the pulp

Enzyme-treated pulps and untreated pulps were refined in a PFI mill up to 250 to 350 mL CSF. Hand sheets of the final pulp were made and evaluated for desired strength properties. Results are shown in Table 4

Table 4: Strength properties of enzyme treated and untreated bleached pulps

S.No.	Particular	Control	Xylanase treated	Laccase treated	X+L treated	$X_{1/2}+L_{1/2}$ treated
1	Burst index Pa.m2/g	2.84	2.89	2.78	3.93	3.73
2	Tensile index, Nm/g	32.50	40.40	44.91	47.43	47.43
3	Tear index, Nm.m2/g	7.19	6.99	6.21	8.08	6.96
4	Double Fold	21	12	23	22	22

The pulp quality in terms of burst, tensile, tears indexes and double fold of the hardwood after enzymatic treatment were increased. The treatment of pulp with xylanase only decreases the tear index, which may be explained by action of xylanases that reduce the intrinsic fibrillar resistance due to removal of superficial hemicelluloses (Batalha et al. 2011). The increase in xylanase dose decreased the strength properties and bulk of wheat straw soda-AQ pulp. The decreased strength may have been due to the removal of hemicellulose, therefore decreasing the fiber bonding capacity (Ates et al. 2009). Xylanase treatment of eucalyptus kraft pulp improved the pulp properties such as tensile strength and burst factor by up to 63% and 8%, respectively (Beg et al. 2000).

Conclusion

Indigenously produced xylanase and laccase enzymes by isolated fungal strains at optimized conditions showed good enzyme activity i.e. 150 IU/ml and 1200 IU/ml respectively. Both enzymes for pulp bleaching were found better when used simultaneously compared to application of individual xylanase and laccase enzyme. At a dosage of 15+25 IU/gm (X+L) brightness gain of 2.3 units was achieved compared to increase of 1.4 & 1.6 units with xylanase & laccase dosage of 15 & 25 IU/gm respectively. Even after reducing the dose of simultaneous xylanase and laccase enzyme to 7.5+12.5 IU/gm brightness gain was still higher at 1.85 units when compared to brightness of control pulp. Strength properties of enzyme treated pulp were found improved when compared to control pulp. Moreover, cost-effective production of xylanase and laccase enzyme process is of commercial significance and thus paving way for adoption of enzyme based technologies in pulp bleaching by paper mills.

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