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Studies on decolorization and xylanase production on sawdust liquid extract and studies on biobleaching activity by white rot fungi

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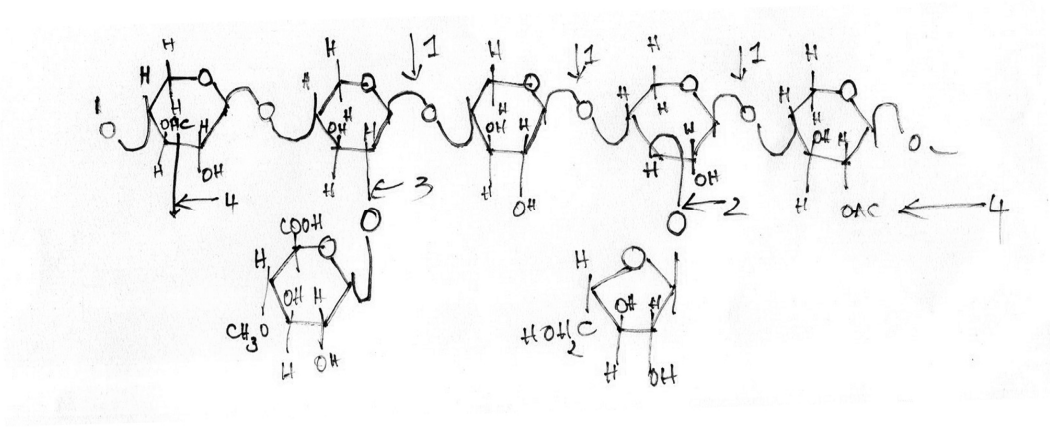
ABSTRACT

The efficiency of three white rot fungi Trametes versicolor, Lenzites betulina and Polyporus elegans in bringing about decolorization and enhancement of xylanase production was studied using sawdust liquid extract, a crude source of xylan at two different concentrations of 12% and 20%. The selected cultures were proved to be efficient in both decolorization of xylan and production of xylanases. A clear linear correlation between the enzyme production and decolorization was observed. The decolorization percentage increased with increase in enzyme production from 6th to 12th days of incubation. Trametes versicolor showed maximum decolorization and enzyme production at both the concentrations. It showed 100% of decolorization and 900ug/ml enzyme production at 12% and 95% decolorization and 750ug/ml enzyme at 20%. Lenzites betulina and Polyporus elegans showed 90% and 85% of decolorization and 700ug/ml, 650ug/ml enzyme production at 12% while 85% and 80% decolorization with 650ug/ml, 583ug/ml enzyme production at 20 % concentration of saw dust extract respectively. The study of the bleaching and delignifying (kappa number reduction) ability of wood degrading fungi on pulp were also studied. The results obtained revealed that the selected cultures were able to reduce kappa number. Trametes versicolor was able to reduce kappa number up to 10 points, Lenzites betulina and Polyporus elegans upto 6 and 2 points respectively. Xylanase enzyme was secreted during bleaching by all the three white rot fungi. Based on the results it can be interpreted that xylanases enhance the delignification process and therefore the bleaching of pulp.

Key words: Xylanases, Sawdust, Decolorization, White rot fungi, Biobleaching.

INTRODUCTION

The study of xylanases has been started in 1960's with the development and application of new technologies, such as molecular biology, structural biology and protein engineering. Using these technologies substantial progress has been achieved in research concerning structures and functions of xylanases. Xylanases [1-4- β –D xylan xylano hydrolase E.C.3.2.1.8] catalyses the random hydrolysis of 1-4 β -D- xylosidic linkages in xylan. Xylan, the second most abundant polysaccharide constitutes from 20-40% of dry weight and forms the major component of hemicellulose in plant biomass [6,31]. In wood as well as in saw dust lignin and xylan are more tightly bound together imparting brown colour [40]. Xylan structure is very complex and variable ranging from linear 1-4, β-linked polyxylose chains to highly branched "hetero" polysaccharide [Fig-1]. The prefix "hetero" denotes the presence of sugars other than D-xylose. The main chain of xylan is analogous to that of cellulose, but is composed of D-Xylose instead of glucose. Branches consist of L-arabino-furanose linked to 0-3 positions of D-xylose residues and of D-glucoronic acid (or) 4-0-methyl D-glucoronic acid linked to 0-2 positions. Both side chain sugars are linked L-glucosidically [19]. The hydrolysis of xylan is carried out by synergistic action of xylanases and associated enzymes like β-xylosidase, a-2-arabino furanosidase L-galactosidase, acetyl esterase, β-mannosidase and β-glucosidase [30]. The main products formed from hydrolysis of xylan are xylobiose, xylotriose and substituted oligomers containing 2 to 4 xylosyl residues [46, 47].



1.ENDO-1,4-β-XYLANASE [EC 3.2.1.8]
2.α-L-ARABINOFURANOSIDASE [EC 3.2.1.55]
3.α-GLUCURONIDASE [EC 3.2.1]
4.ACETYLESTERASE [EC 3.1.18]
5.Iβ·XYLOSIDASE [EC 3.2.1.37]

XYLβ 1-4XYLβ 1
↑ 5

FIG:1: STRUCTURE OF XYLAN

Xylanases have received a great deal of attention in the last 10 years. Potential applications of xylanases include bioconversion of lignocellulosic material and agro wastes to fermentative products, bio-conversion of waste materials to industrial products, bio-processing of agro

residues, biomass conversion, and bio-refinery of lignocellulosic wastes [9, 3, 2, 45]. Pulp and paper industry has given utmost preference in applying xylanases where the quantities of raw materials processed is huge as well as the use of naturally hazardous chemicals are also large [30,44,26]. Pulping is the means where by the wood is reduced to a fibrous mass for onward processing into paper and broad products [24]. Lignin component binds with cellulose fibers together with hemicelluloses. In order to produce paper, the fibers must be separated from each other. Xylan forms a barrier against effective extraction by chemicals of the residual brown colored lignin from the fibers [40]. Residual lignin is both darker and more tightly bound to the fibers together with xylan. The principal aim of pulp bleaching is to increase the brightness of pulps. Large quantities of chlorine or chlorine containing compounds are required to be used for effective increase in pulp brightness [5]. Evidence mounted throughout the 1980's that mill discharges were responsible for increasing the levels of organic chlorines detected in receiving environments and a variety of ecological effects ranging from lethal effects upon marine micro flora to deformalities [35, 36, 37, 21, 23].

Progress through intensive research and developmental activities to make technology eventually free from hazardous chemicals would be a cherished dream and this has been realized through biopulping [41,42,43,15,13,20,1]. Hydrolysis by xylanases of relocated and reprecipitated xylan on the surface of cellulose fibers formed during cooking facilitates the removal of lignin by increasing permeability to oxidizing agents [14,17,18]. Prebleaching of pulp by xylanases would decrease 20% xylan from pulp saving up to 25% of chlorine containing bleaching component [39].

Xylanases are now playing a role in clarifying juices and wines, extraction of coffee and plant-oils, in improving nutritional starch property of agricultural silage and grain feed. Xylanase based enzyme products are used in pig and poultry diets based on wheat and rice[48]. Microbial xylanases enhanced dough rheological properties manifested as an increase in loaf volume that improves its baking performance and hence has great importance in cereal industries [32,27]. The multifarious physiological roles of xylanases in fruit softening, seed germination and plant defense mechanisms [1] were well known. Several studies have shown that microbial production of enzymes can be induced by using substrates.[28,38] xylanases are co-induced in response to substrates containing xylan or hemicellulose or xylobiose or xylotriose or their residues in the medium [7]. White rot fungi come under sub division fleshy basidiomycete members. All the components of wood including lignin, cellulose and hemicellulose are used up by these fungi as their hyphae are able to penetrate wood and causes rot disease. The degraded wood appears light in color due to loss of lignin (brown colour). As the strength to the wood is because of lignin component, the tissue becomes spongy due to lignin degradation. Fungi with this ability are collectively known as White rot fungi. White rot fungi secrete high levels of plant cell wall hydrolyzing enzymes such as cellulases and xylanases into their culture media, and are employed for the hydrolysis of lignocellulosic materials[47,4,29,34].

Major impediments to exploit commercial potential of xylanases are the difficulty in obtaining yield stability and cost of xylanase production. Therefore research has been aimed to exploit commercial potential of existing and new xylanases in nature. In view of the earlier indications and the aforesaid applications, the present study is planned to improve xylanase production on natural substrates of xylan and also to optimize production of xylanases and decolorization of

saw dust extract and studying the biobleaching activity by detecting the kappa number by TAPPI method and estimating the xylanase production during bleaching by three white rot fungi *Trametes versicolor, Lenzites betulina* and *Polyporus elegans.*

EXPERIMENTAL SECTION

About twenty wild species of white rot fungi were collected from forests as well as from common timber depots and identified according to the characteristics of their basidiocarps. Among the collected white rot fungal species, three basidiomycetes fungi selected were *Trametes versicolor, Polyporus elegans, Lenzites betulina* for the present study. The selected white rot fungi were characterized by the following features. *Trametes versicolor (*turkey tail fungus) was characterized by the distinctive brightly colored banding pattern on the fruiting body resembling the tail of strutting turkey. *Lenzites betulina (*multi color gill polypore) was characterized by their white to creamy pore surface covered with coarse hairs, concentrically zoned with colors. *Polyporus elegans* was characterized easily by its fairly small size with whitish pore surface and half-black central stem. Pore surface is white when young, becoming brown on age. A piece of basidiocarp was cut and first dipped in sterile water containing antibiotic then in spirit and exposed to little flame for surface sterilization. After surface sterilization, the inner piece of it was placed on malt agar plates and incubated. After incubation period the cultures were sub-cultured on to malt agar slants.

1. Estimation of xylanase production in the presence of liquid and crude source of xylan (sawdust) by the selected cultures

Five percent of sawdust was taken, boiled and filtered. The filtrate obtained is used as liquid source of xylan. To estimate the xylanase production, the sawdust filtrate obtained was added to the malt extract medium in two concentrations of 12% and 20%. Initial pH and optical density at 445nm of the medium was noted. To the above medium, with saw dust filtrate, the selected cultures were seeded and incubated for 12 days. At the end of 6, 8, 10, 12 days of incubation culture filtrate was taken and the following aspects were studied.

1.1. Determination of decolorization Percentage of 12% and 20% sawdust extract

Decolorization percentage was determined by change in the initial and final optical density of the filtrate at 445 nm. Optical density at 445nm of the culture filtrate was observed and results are given in Tables-1 and 3

1.2. Estimation of xylanase production at 12% and 20% sawdust extract

At the end of 6, 8, 10, 12 days of incubation, culture filtrate was taken and estimated for xylanase activity in terms of xylose production and results are recorded in Tables- 2 and 4

2. Study of bio-bleaching activity of selected cultures

The selected cultures were cultured on 20gms of sterilized pulp and incubated for 35days. At the end of 7, 14, 21, 28, 35 days pulp samples were tested for the following aspects.

2.1. Detection of Kappa number using TAPPI (Technical Associations of the pulp and paper industry) method

At the end of 7, 14, 21, 28, 35 days of incubation, pulp samples were taken and kappa number was detected by using Standard TAPPI method (TAPPI Test Method T 236 om-99). Kappa number was calclulated using the following formula and results are set out in Table-5

$$K = \frac{p \times f}{w}$$

and

$$p = \frac{(b-a)\,N}{0.1}$$

Where
K= kappa number.
f= factor for correction to a 50% permanganate consumption, dependent on the value of p (see table).
w= weight of moisture- free pulp in the specimen, grams.
p= amount of 0.1 N permanganate actually consumed by the test specimen, ml.
b= amount of the thiosulphate consumed in the blank determination, ml.
a= amount of the thiosulphate consumed by the test specimen, ml.
N= normality of the thiosulphate.

Factors in table are based on the equation:
Log k= log p/w+0.00093(p-50).

Table: factors f to correct for different percentages of permanganate used

P+	0	1	2	3	4	5	6	7	8	9
30	0.958	0.960	0.962	0.964	0.966	0.968	0.70	0.973	0.957	0.977
40	0.979	0.981	0.983	0.985	0.987	0.989	0.991	0.994	0.996	0.998
50	1.000	1.002	1.004	1.006	1.009	1.011	1.013	1.015	1.017	1.019
60	1.022	1.024	1.026	1.028	1.030	1.033	1.035	1.037	1.039	1.042
70	1.044									

2.2. Estimation of production of Xylanases

At the end of 6, 8, 10, 12th days of incubation, pulp samples were taken and estimated for xylanase activity in terms of xylose production and results are presented in Table-6

RESULTS AND DISCUSSION

1. Estimation of xylanase production in the presence of liquid and crude source of xylan (sawdust) by the selected cultures

For the present study, saw dust was selected as a crude source of xylan. The crude extract of saw dust was added to the medium at 12 % and 20% concentration and cultures were seeded and incubated for 12 days. At the end of 6, 8, 10 and 12 days of incubation decolorization activity (%) and xylanase production were estimated and the results were recorded.

1.1. Determination of decolorization Percentage of 12% saw dust

It was evident from the results that the organisms selected were efficient in decolorization of saw dust extract. Three white rot fungi achieved the effective color removal of saw dust by 100, 95and 85% respectively in 12 days of incubation. It is revealed that *Trametes versicolor* exhibited 25-100% decolourization, *Lenzites betulina* exhibited 15-90% decolourization activity and *Polyporus elegans* exhibited 10- 85% decolourization. Of the cultures selected, *Trametes versicolor* exhibited maximum decolorization 0f 100% next *Lenzites betulina* up to 90% and *Polyporus elegans* up to 85 %.

Table-1 Determination of decolorization Percentage of 12% saw dust

	DECOLORIZATION PERCENTAGE (%)			
ORGANISM	6TH DAY	8THDAY	10THDAY	12TH DAY
Trametes versicolor	25 (±0.57)	50(±0.50)	75((±0.47)	100(±0.40)
Lenzites betulina	15(±0.50)	30(±0.50)	65(±0.50)	90(±0.47)
Polyporus elegans	10(±0.57)	25(±0.50)	60(±0.50)	85(±0.50)

All ± values are significant at 5% level.

1.2. Estimation of xylanase production at 12% saw dust extract

The selected cultures were able produce xylanases on crude source of xylan and there was gradual increase in the xylanase production from 6th day to 12th day of incubation by the three cultures. From the above results, it can be concluded that the decolorization activity increased along with an increase in xylanase production by the selected cultures. Rakisudan *et al*. (2006) [33] tested different xylan-containing agricultural bye-products for substrate specificity and observed rice husk was to be the best substrate in the case of *Aspergillus niveus RS2.* Judith Liliana *et al.* (2002) [11] performed xylanase production by *Aspergillus awamori* on sugar cane baggage.

Table-2 Estimation of xylanase production at 12% saw dust extract

	PRODUCTION OF XYLANASE (ug/ml)			
ORGANISM	6TH DAY	8THDAY	10THDAY	12TH DAY
Trametes versicolor	283.35(±0.33)	550.00(±0.47)	700.00(±0.47)	900.00(±0.47)
Lenzites betulina	150.00(±0.33)	350.00(±0.50)	533.34(±0.50)	700.00(±0.50)
Polyporus elegans	116.67(±0.33)	316.67(±0.50)	483.35(±0.50)	650.00(±0.50)

All ± values are significant at 5% level.

Table-3 Determination of decolorization Percentage of 20% sawdust

	DECOLORIZATION PERCENTAGE (%)			
ORGANISM	6TH DAY	8THDAY	10THDAY	12TH DAY
Trametes versicolor	20(±0.33)	40(±0.33)	65(±0.47)	95(±0.47)
Lenzites betulina	15(±0.50)	30(±0.50)	50(±0.50)	85(±0.50)
Polyporus elegans	10(±0.50)	25(±0.50)	45(±0.50)	80(±0.50)

All ± values are significant at 5% level.

1.3. Determination of decolorization Percentage of 20% saw dust

It can be concluded from the results that the organisms selected were efficient in decolorization of saw dust extract. *Trametes versicolor* exhibited 20- 95% decolourization *Lenzites betulina* exhibited 15-85% decolourization activity and *Polyporus elegans* exhibited 10- 80%

decolourization. Of the cultures selected *Trametes versicolor* exhibited maximum decolorization 0f 95% followed by *Lenzites betulina* bringing 85% and *Polyporus elegans* up to 80 %.

1.4. Estimation of xylanase production at 20% saw dust extract

The selected cultures were able produce xylanases on crude source of xylan and there was gradual increase in the xylanase production from 6th day to 12th day of incubation by the three cultures. Xylan degrading system of *Phanerochaete chrysporium* was well studied and reported to be producing about 3.86(U) mg-1 when grown on oat spelt arabino xylan and birch wood xylan [22,8]. It is evident from the results that as the xylanase production increases there was increase in decolorization activity by the selected cultures.

From the results obtained it was interesting to note that the decolorization activity increased along with an increase in xylanase production by the selected cultures. There was a clear linear correlation between xylanase production and decolorization of sawdust extract. The brown color of wood is because of xylan, the hemicellulose component of wood. The pulp and paper industry striving hard to decolorize and bleach the wood pulp from brown to white by using higher amounts of chlorine. From the results obtained it can be suggested that biobleaching can become an efficient method in pulp and paper industry which in turn may reduce the amount of chlorine usage.

Table-4 Estimation of xylanase production at 20 % saw dust extract

	PRODUCTION OF XYLANASE (ug/ml)			
ORGANISM	**6TH DAY**	**8THDAY**	**10THDAY**	**12TH DAY**
Trametes versicolor	250.00(±0.50)	500.00(±0.50)	650.00(±0.50)	750.00(±0.50)
Lenzites betulina	116.67(±0.50)	316.67(±0.50)	466.68(±0.50)	650.00(±0.50)
Polyporus elegans	83.35(±0.50)	300.00(±0.50)	433.34(±0.50)	583.35(±0.50)

All ± values are significant at 5% level.

2. Study of bio-bleaching activity of selected cultures

2.1. Detection of Kappa number using TAPPI (Technical Association of the pulp and paper industry) method:

Pulping is the means where by the wood is reduced to a fibrous mass for onward processing into paper and other products. The principal aim of pulp bleaching is to increase the brightness of pulps. Enzymatic solubilization of the hemicelluloses settled on the pulp fibers would be an "environmentally compatible" technology to improve the accessibility of the brown lignin to chemical bleaching together with substantially reduced quantities of bleaching chemicals required to achieve the same degree of bleaching and brightness.

It is evident from the results that the selected white rot fungi exhibited affective bio bleaching activity. Of the selected cultures *Trametes versicolor* showed maximum activity and was able to reduce kappa number up to 10 points. *Lenzites betulina* reduced kappa number up to 6 points and *Polyporus elegans* upto 2 points. The present results are in accordance with the earlier investigations on biobleaching carried out by other fungal cultures as given. Ryuichiro Kondo *et al.* (1994)[16], Harazino *et al.* (1996)[9] and Hirofurni *et al.* (2005)[10] visualized that white rot fungi *Phanerochaete sordida YK-624* brightened pulp up to 21.4 points to 54.0% brightness

higher than *Phanerochaete chrysosporium* [13.4%] and *Coriolus versicolor*. Kalagiri *et al.* (1995)[12] studied *Phanerochaete chrysosporium* and *Trametes versicolor* in the solid state fermentation with low nitrogen and high carbon medium and reported that pulp brightness increased by 15-30 points thereby decreasing Kappa number. It was reported by Moreira *et al.* (1997)[25] that *Bjerkandera sp. Strain Bo555* increased 80% of brightness on eucalyptus kraft pulp hand sheets.

Table-5 Detection of Kappa number using TAPPI method

ORGANISM	REDUCTION IN KAPPA NUMER
Trametes versicolor	10(±0.50)
Lenzites betulina	6(±0.50)
Polyporus elegans	2(±0.50)

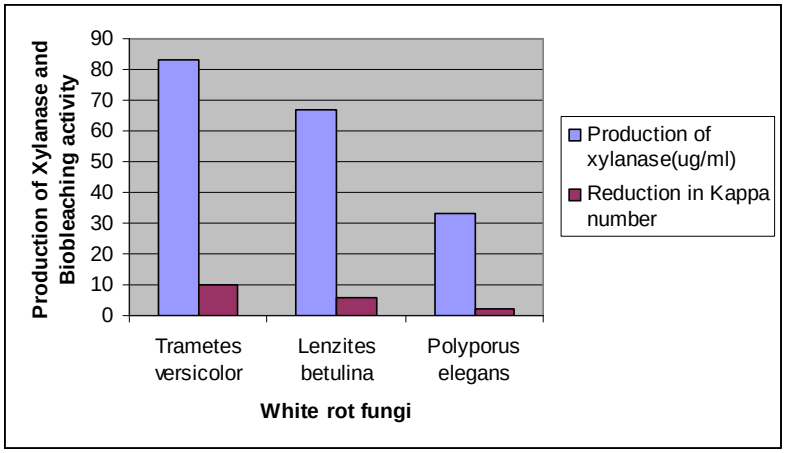
All ± values are significant at 5% level.

Table-6 Estimation of xylanase production

ORGANISM	PRODUCTION OF XYLANASE(ug/ml)
Trametes versicolor	83.35(±0.50)
Lenzites betulina	66.68(±0.50)
Polyporus elegans	33.34(±0.50)

All ± values are significant at 5% level.

Figure-2 Linear correlation between enzyme production and Biobleaching activity



2.2. Estimation of production of Xylanases

It was clear from the results that there was xylanase production by all the selected cultures. *Trametes versicolor* produced maximum enzyme, moderate production by *Lenzites betulina* and least production by *Polyporus elegans.* It was also noticed that increased enzyme production lead to increased biobleaching activity there by reducing the kappa number with the resultant brightness of the pulp [Fig-2].

The significance of the present work lies in the fact that hazardous chemical usage can be replaced by fungal cultures for biobleaching activity.

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