A Novel Approach to Delignify Lignocellulosic Materials by Using Ligninolytic Enzyme Consortium

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Lignin biodegradation is an attractive approach for producing value-added products. These valuable products are produced by the processing and refining of lignocellulosic residues. A set of ligninolytic enzymes including lignin peroxidase (LiP), manganese-dependent peroxidase (MnP), and laccase (Lac) were individually produced from *Ganoderma lucidum*, *Trametes versicolor*, and *Pleurotus ostreatus*. Solid state fermentation under pre-optimized culture conditions with varying ratios of enzymes were used for the delignification of lignocellulosic biomass residues. The fungal enzymes were purified in four steps including ammonium sulfate precipitation, dialysis, ion exchange chromatography, and gel filtration chromatography. The purified enzymes were subsequently used in varying ratios (with each containing 200 U/mL) for the delignification of wheat straw, sugarcane bagasse, and rice straw. The consortium of enzymes caused the removal of 58.5%, 46%, and 52% of the lignin from the wheat straw, sugarcane bagasse, and rice straw, respectively, at LiP: MnP: Lac ratios of 1:2:2, 1:1:2, and 2:1:2. The best delignification was observed in wheat straw (58.5%), exposing 76.54% cellulose content. The results suggested that the ligninolytic enzymes are effective catalysts for the selective partial delignification of lignocellulosic biomass residues. After delignification these lignocellulosic residues could be utilized as cost-effective substrates for the production of enzymes, biofuels, and other industrially significant products.

Keywords: Ligninolytic enzymes; Enzyme Consortium; Lignocellulosic substrates; Delignification

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INTRODUCTION

Lignocellulosic feedstock is a promising renewable material that is a natural source for modern industries; it is the backbone of bio-based economies in the developed world (Anwar *et al*. 2014). Due to their high sugar content, lignocellulosic materials such as agricultural and forestry residues have tremendous potential for the large-scale production of bioethanol and other products (Farrell *et al*. 2006; Mohanram *et al*. 2013). Approximately 1.4 billion tons of agricultural wastes are produced globally, which are primarily sugarcane bagasse, corn straw, wheat straw, and rice straw (Saini *et al*. 2015). Thus, using lignocellulosic materials reduces its negative impact on the environment by minimizing the accumulation of waste. It also has a significant positive impact on the global economy (Alonso *et al*. 2008; Moreira *et al*. 2012; Asgher *et al*. 2014).

Lignocellulosic residues are a composite material that consists of the three main

biopolymers: lignin, cellulose, and hemicellulose (Brandt *et al*. 2013). Lignin is the second most abundant natural organic polymer on earth, constituting 10 to 25% of lignocellulosic biomass (Hu *et al*. 2011; Min *et al*. 2013; Chen *et al*. 2015). Lignin is a three-dimensional macromolecule that is highly cross-linked. It is also polymerized with a large number of functional groups/linkages and is composed of three types of phenols, namely, sinapyl, coniferyl, and p-coumaryl alcohol (Lee *et al*. 2009; Brosse *et al*. 2011). Lignin is insoluble in water, and it binds cellulose and hemicellulose together (Watkin *et al*. 2015). Approximately 70% of lignocellulosic biomass is made up of cellulose (30 to 50%) and hemicellulose (15 to 35%) (Limayem and Ricke 2012). They are tightly linked to the lignin through hydrogenic and covalent bonds, making the structure resistant towards any treatment (Knauf and Moniruzzaman 2004; Limayem and Ricke 2012). The conversion of lignocellulosic materials into useful products depends on unlocking the lignin barrier to expose the cellulose and hemicellulose components (Ayeni *et al*. 2015). However, lignin degradation is one of the prominent hurdles when trying to directly utilize the biomass materials for fuel fermentation and bio-pulping in the paper and pulp industries (Liu *et al*. 2012; Poornejada *et al*. 2013). Lignin acts as a physical barrier by preventing the hydrolysis of cellulose and hemicellulose fibers through cellulases and hemicellulases (Ranjan *et al*. 2013; Sartori *et al*. 2015). Thus, the delignification of biomass is the most important step for the furthest utilization of lignocellulosic materials (Asgher *et al*. 2016 a,b). Various pretreatment methods have been employed including physical, chemical, or biological treatments alone or in combination (Ogeda and Petri 2010).

For biological treatments, various fungi show unique characteristics when the delignification of the lignocellulosic biomass produces residues. Usually, cellulose is attacked by soft and brown rot fungi. Such fungi impart minimal modifications to the lignin, which is in contrast to white rot fungi (WRF). By use of WRF, one can solely degrade the lignin component (Sun and Cheng 2002). WRF are the most efficient degraders of lignin due to their ligninolytic enzymes such as lignin peroxidase (LiP, E.C. 1.11.1.14), manganese peroxidase (MnP, E.C. 1.11.1.13), lac (E.C. 1.10.3.2), and H_2O_2-producing oxidases (Iqbal *et al*. 2011). These ligninolytic enzymes have low substrate specificity, non-sterio selectivity, and strong oxidative abilities (Iqbal *et al*. 2011; Asgher *et al*. 2014). Among WRF, *Ganoderma lucidum*, *Trametes versicolor*, and *Pleurotus ostreatus* are efficient producers of ligninolytic enzymes (Xavier *et al*. 2007; Batool *et al*. 2013).

The current study investigated a ligninolytic enzyme consortium for the selective delignification of various lignocellulosic substrates. The lignin and cellulose contents of the substrates before and after the enzymatic treatments were also determined.

EXPERIMENTAL

Lignocellulosic Materials

Wheat straw, rice straw, and sugarcane bagasse were obtained from the student farms of the University of Agriculture, Faisalabad, Pakistan. The substrates were crushed into pieces, sun dried, oven dried (60 °C), ground to fine particle size (40 mm mesh size), and stored in airtight plastic jars that were moisture free.

Fungal Strains and Inoculum Development

The pure cultures of *Pleurotus ostreatus* IBL-02, *Trametes versicolor* IBL-04, and *Ganoderma lucidum* IBL-05 were obtained from the Industrial Biotechnology Laboratory, Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan. The inoculum was prepared by growing the strains in Kirk's basal medium supplemented with 1.0% (w/v) Millipore filtered sterilized glucose solution. The composition of the medium was 0.22 g/L ammonium tartrate, 0.21 g/L KH_2PO_4, 0.05 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $CaCl_2$, and 0.001 g/L thiamine. Also, there was 10 mL of Tween 80 (10%), 10 mL of 100 mM veratryl alcohol, and 10 mL of trace metals solution. The composition of the trace minerals was 0.08 g/L $CuSO_4$, 0.05 g/L $NaMoO_4$, 0.07 g/L $MnSO_4 \cdot H_2O$, 0.043 g/L $ZnSO_4 \cdot 7H_2O$, and 0.05 g/L $FeSO_4$. Prior to sterilization, the pH of the medium was adjusted to 4.5. After 5 to 7 days of cultivation, a homogeneous spore suspension (1×10^6 to 1×10^8 spores/mL) was attained and used as inoculum (Bilal *et al*. 2015).

Ligninolytic Enzymes Production

The three ligninolytic enzymes (LiP, MnP, and laccase) were produced from *Ganoderma lucidum* IBL-05, *Trametes versicolor* IBL-04, and *Pleorotus ostreatus* IBL-02. Lignin peroxidase and manganese peroxidase were produced using wheat straw moistened with Kirk's basal medium (60% w/w moisture) containing additional 2 mL glycerol as carbon source, 2 g urea as a nitrogen source, 1 mM oxalates (1mL) as a mediator, and 1 mM $CuSO_4$ (1 mL) as metal ion activator. Laccase was produced using corn cobs as a substrate, moistened with Kirk's basal medium with yeast extract as a nitrogen source and 1 mM ABTS (1 mL) as a mediator. All the flasks were autoclaved and inoculated by adding 5 mL of freshly prepared respective inoculums under sterilized conditions. The inoculated flasks were set at pH 4.5 and allowed to ferment at 30 °C temperature without agitation. After fermentation, the extracellular enzymes were isolated by filtration and centrifugation of filtrate at 3000 × g. These individual enzymes were purified in a four step purification protocol that incorporated ammonium sulfate precipitation, dialysis, ion exchange chromatography, and gel filtration.

Purification of Enzymes

Crude enzyme extracts from *G. lucidum* IBL-05, *T. versicolor* IBL-4, and *P. ostreatus* IBL-02 were saturated separately by the gradual addition of ammonium sulfate according to their optimum ammonium sulfate saturation point, as described earlier (Iqbal and Asgher 2013). The extracts were centrifuged, and the pellets were dissolved in 50 mM tartrate buffer (pH of 4.5). The solution was dialyzed overnight against the same buffer. Enzyme activity was determined before and after dialysis. Crude enzyme solutions obtained after the dialysis were subjected to ion exchange chromatography using diethyl aminoethyl (DEAE)-cellulose. The columns were equilibrated with phosphate buffer (pH 6.5) for 24 h and eluted with a 0 to 1.0 M linear gradient of NaCl in a 50 mM malonate buffer at a flow rate of 0.5 mL/min. The active fractions of LiP, MnP, and laccase were pooled and loaded on a Sephadex-G-100 column (10 × 300 mm). A 50 mM malonate buffer was used for elution, and the flow rate was 0.3 °/min. Positive fractions collected, pooled, and stored at -20 °C. All collected samples were analyzed for enzyme activity and protein content.

Ligninolytic Enzyme Assays

Lignin peroxidase assay

Lignin peroxidase (LiP) activity was measured by the method of Tien and Kirk (1988), following the H_2O_2-dependent oxidation of veratryl alcohol to veratraldehyde at 25 °C. The reaction mixture contained 1 mL of 4 mM veratryl alcohol in 1 mL of 100 mM tartrate buffer (pH 3). The reaction was initiated by adding 0.2 mM H_2O_2, and the mixture was read at 0.5 mL. The absorbance of the reaction mixture was monitored at 310 nm (ε_{310} = 9300).

Manganese peroxidase assay

MnP was assayed at 25 °C by the H_2O_2 dependent oxidation of manganic-malonate complex at 270 nm (ε_{270} = 11,570). The reaction mixture contained 1 mL of 1 mM manganese sulfate in a 1 mL sodium malonate buffer (50 mM; pH 4.5) and 100 µL of enzyme sample. The reaction was initiated by the addition of 0.5 mL of 0.1 mM H_2O_2 (Wariishi *et al*. 1992).

Laccase assay

Laccase activity was determined by monitoring the oxidation of 2, 2'-azino-bis (3-ethylbenzothiazoline- 6-sulfonic acid (ABTS) in a reaction mixture containing 1 mL of 1 mM ABTS in 1 mL of 0.1 M sodium acetate buffer with a pH of 4.5 and 50 µL of an enzyme sample (Wolfenden and Willson 1982). The oxidation was followed at 25 °C and 420 nm (ε_{420} = 36,000).

Protein content

The protein concentration was determined using a Bradford protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

Delignification of Lignocellulosic Substrates

Lignocellulosic substrates, wheat straw, sugarcane bagasse, and rice straw were subjected to enzymatic treatment for delignification. Varying ratios (v/v) of the three ligninolytic enzymes (LiP, MnP, and laccase) were used to develop a mixture of the three enzymes for delignification of the three substrates (Table 1). Varying volumes of the individual enzymes each contained 200 U/mL and were applied on the substrates at 25 °C and 40 °C.

Table 1. Ligninolytic Enzyme Based Treatments of Lignocellulosic Substrates

Lignin Peroxidase (LiP)	Manganese Peroxidase (MnP)	Laccase (Lac)
1	1	1
2	1	1
1	2	1
1	1	2
2	2	1
1	2	2
2	1	2

Each flask contained 15 g of substrates and was filled to 100 mL with 0.5 mM sodium malonate buffer (pH 4.5). The treated substrates were then analyzed to determine the lignin and cellulose contents.

Determination of Lignin Content

The lignin content was determined as described by Johnson *et al*. (1961). The sample was dissolved in 10 mL 25% (w/v) acetyl bromide in a re-distilled glacial acetic acid (≥ 99%) at 70 °C in a water bath for 30 min. The samples were stored in a special digester tube with a notched glass stopper. After 30 min, the dissolved samples were transferred into a 200 mL volumetric flask containing a 5 mL mixture of acetic acid and caustic soda in a 1:1 ratio.

Interfering substances were removed by adding 0.2 g of hydroxylamine hydrochloride. The samples were diluted to 15 mL of 99% acetic acid with an absorbency of 280 nm. The lignin content of treated and untreated samples was calculated using a lignin standard curve constructed following the same protocol.

Determination of Cellulose Content

The cellulose content of the native as well as the de-lignified biomass was determined by the method of Updegraff (1969), with minor modifications. The native and de-lignified biomass were homogenized in a Waring blender and centrifuged for 5 min at 3000 × g. The supernatant was decanted and discarded.

A total of 3.0 mL of acetic/nitric reagent was added to the pellet and mixed well with a Vortex mixer. The tubes were incubated in a boiling water bath for 30 min. Marbles were also placed on top of each tube to reduce evaporation and to create a refluxing action. The contents were centrifuged for 5 min at 5000 × g, and the supernatant was decanted. Next, 10 mL of 67% H_2SO_4 (V/V) was added to the residue, mixed well with a Vortex mixer, and incubated for 1 h.

The contents were diluted with 1 to 100 mL of distilled water and centrifuged if any precipitate or turbidity was present. A total of 1.0 mL of this diluted mixture was placed in a 150 mm × 18 mm screw cap culture tube, and 4.0 mL of distilled water was added. Tubes were placed in crushed ice, and 10 mL of cold anthrone reagent was added with a pipette in a layering effect and mixed well on a Vortex mixer. Marble was placed on top of each tube, which was placed in boiling water for 16 min and cooled in ice bath for 2 to 3 min. The tube was placed at room temperature for 5 to 10 min, and the absorbance was measured on a spectrophotometer at 620 nm against a reagent blank.

Statistical Analysis

All experiments were carried out in triplicate. The data values are presented as means ± standard error (SE). The means and standard errors were computed for each treatment, and the SE values were displayed as Y-error bars in the figures. A statistical analysis was performed by analysis of variance (ANOVA) using MINITAB 15, Windows Version (Minitab Inc., State College, PA, USA) and the probability values ($p \leq 0.05$) were considered a statistically significant difference.

RESULTS AND DISCUSSION

Production and Purification of Lignin Peroxidase (LiP)

Ganoderma lucidum IBL-05 was grown on wheat straw in solid state fermentation (SSF) for the production of LiP under pre-optimized conditions (Aslam and Asgher 2011). The crude LiP produced showed enzyme activity of 1277.9 U/mL with a specific activity of 493.3 U/mg. The LiP was completely precipitated at 80% ammonium sulfate saturation, with 1.31-fold purification. The enzyme purification was further increased to 2.6-fold after DEAE cellulose ion exchange chromatography. Specific activity was increased to 1712.2 U/mg after the sample was eluted through a Sephadex G-100 gel filtration column, as shown in Figs. 1 and 2. This resulted in a 3.47-fold purification with more than 80% of other proteins removed (Table 2). In a previous study, LiP obtained from *P. chrysosporium* was purified under optimum conditions by 60% $(NH_4)_2SO_4$ saturation (Wang *et al*. 2008). MnP produced from *Ganoderma lucidum* was purified by salting out at 65% saturation with $(NH_4)_2SO_4$ to 1.73-fold purification with a specific activity of 273.01 U/mg after ion exchange chromatography (Bilal *et al*. 2015).

Table 2. Purification Summary for Lignin Peroxidase (LiP) Produced by *G. lucidum* IBL-05 in Solid State Fermentation

Sr. No	Purification Step	Total Volume (mL)	Enzyme Activity (U/mL)	Protein Content (mg/mL)	Specific Activity (U/mg)	Fold Purification
1	Crude Enzyme	300	1277.9	2.59	493.3	1
2	Precipitation	25	1159.37	1.79	647.69	1.31
3	Dialysis	21	1024.41	1.27	806.6	1.63
4	DEAE-cellulose	19	860.7	0.65	1324.15	2.6
5	SephadexG-100	15	821.86	0.48	1712.2	3.47

Production and Purification of Manganese Peroxidase (MnP)

Trametes versicolor IBL-02 was grown on wheat straw in solid state fermentation for the production of manganese peroxidase enzymes under pre-optimized conditions (Aslam and Asgher 2011). The crude MnP created showed an enzyme activity of 1436.32 U/mL, with a specific activity of 460.21 U/mg. The manganese peroxidase was completely precipitated at 80% ammonium sulfate saturation, resulting in 1.33-fold purification. The enzyme purification was then increased to 1.72-fold after the sample was passed through a DEAE cellulose ion exchange column.

The specific activity was further increased to 801.8 U/mg after the sample was resolved through a Sephadex G-100 gel filtration column, as shown in Figs. 1 and 2. The purification was 1.74-fold with more than 80% of the other proteins removed as shown in Table 3. From a previous study, MnP from *Trametes versicolor* was purified using ammonium sulfate precipitation at a saturation point of 65%. Purification of MnP resulted in a purification of 2.4 leading to an overall yield and specific activity of 3.4% and 660U/mg, respectively.

Table 3. Purification Summary for Manganese Peroxidase (MnP) Produced by *T. versicolor* IBL-04 in Solid State Fermentation

Sr. No	Purification Step	Total Volume (mL)	Enzyme Activity (U/mL)	Protein Content (mg/mL)	Specific Activity (U/mg)	Fold Purification
1	Crude Enzyme	300	1436.32	3.121	460.21	1
2	Precipitation	22	1208.2	1.96	616.4	1.33
3	Dialysis	21	1199.1	1.61	744.7	1.618
4	DEAE-cellulose	12	809.9	1.02	794.0	1.72
5	Sephadex G-100	9	609.44	0.76	801.8	1.74

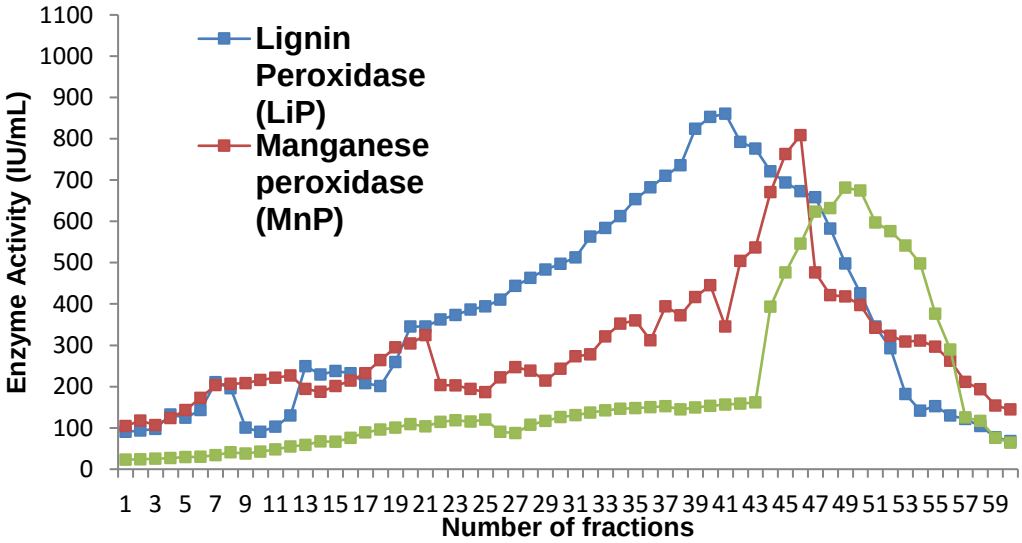

Fig. 1. Purification of lignin peroxidase, manganese peroxidase, and laccase from ion exchange chromatography

An elution curve was plotted between the fraction no. *vs.* enzyme activity (IU/mL). In ion exchange chromatography, a total of 60 fractions were collected in case of each enzyme. The highest enzyme activity for lignin peroxidase was 860.7 IU/mL in the range of 39 to 45 fraction numbers. MnP gave best optimal enzyme activity of 809.9 IU/mL in the range of 45 to 48 fraction numbers, whereas the highest enzyme activity for laccase was 698.87 IU/mL in the range of 49 to 53 fraction numbers, as shown in Fig. 1.

Gel filtration is an important technique for the purification of proteins. Sephadex G-100 column (16 cm x 1 cm) was used for the purification of LiP. The samples were applied to a column having pH 7. However, a total of 30 fractions each containing 1.5 mL of enzyme were collected. An elution curve was plotted between the fraction no. *vs.* enzyme activity (IU/mL). The highest enzyme activity was 821.86 IU/mL for Lignin peroxidase in the range of 21 to 25 fraction numbers. Manganese peroxidase gave the highest enzyme activity of 609. 44 IU/mL in the range of 21 to 28 fraction numbers whereas the highest enzyme activity was 673.33 IU/mL for laccase in the range of 21 to 23 fraction numbers, as presented in Fig. 2.

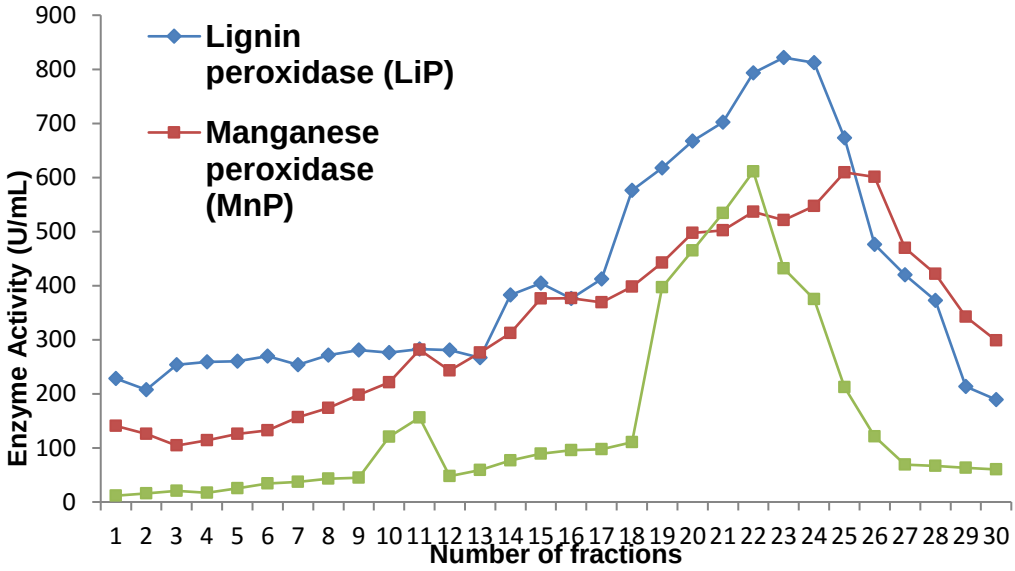

Fig. 2. Purification of lignin peroxidase, manganese peroxidase, and laccase from gel filtration chromatography

Production and Purification of Laccase (Lac)

Lac was produced from *Pleurotus ostreatus* IBL-02 under previously optimized SSF conditions (Asgher *et al*. 2012). The crude laccase showed the activity of 911.11 U/mL, with a specific activity of 219 U/mg. Lac was completely precipitated at a saturation of 80% ammonium sulfate, giving a purification of 2.29-fold. The enzyme purification was increased to 3.09-fold after the sample was passed through a DEAE cellulose ion exchange column. Specific activity was further increased to 833 U/mg after the sample was eluted from the Sephadex G-100 gel filtration column. This resulted in a 4.03-fold purification, as shown in Figs. 1 and 2, with more than 80% of the other proteins removed (Table 4). Mtui and Nakarmura (2008) demonstrated that ammonium sulfate saturation in the range of 50 to 80% is suitable for the recovery of ligninolytic enzymes. Freixo *et al*. (2008) and Aslam and Asgher (2011) also reported an 80% saturation point for laccase, leading to 3.37-fold purification. Khammuang and Sarnthima (2009) salted out the laccase enzyme from the *Ganoderma* species at 70% saturation with a 1.34-fold purification.

Table 4. Purification Summary for Laccase (Lac) Produced by *P. ostreatus* IBL-02 in a Solid State Fermentation

Sr. No	Purification Step	Total Volume (mL)	Enzyme Activity (U/mL)	Protein Content (mg/mL)	Specific Activity (U/mg)	Fold Purification
1	Crude Enzyme	600	921.11	4.156	222	1.00
2	Precipitation	50	836.44	1.45	577	2.59
3	Dialysis	50	826.66	1.09	758	3.41
4	DEAE-Cellulose	15	698.87	1.01	692	3.11
5	Sephadex G-100	12	673.33	0.67	1005	4.52

Delignification of Lignocellulosic Residues

The partially purified ligninolytic enzyme extracts (LiP, MnP, and laccase) produced under optimized conditions from *Ganoderma lucidum* IBL-05, *Trametes versicolor* IBL-04, and *Pleurotus ostreatus* IBL-2 were used for the enzymatic delignification of sugarcane bagasse (SCB), wheat straw (WS), and rice straw (RS) at both 25 °C and 40 °C.

Enzyme-Assisted Delignification of Wheat Straw

The enzymatic treatment of wheat straw was carried out using a mixture of ligninolytic enzymes using different ratios (Tables 5 and 6). However, the best delignification of 58.5% was observed from the mixture of three enzyme consortiums LiP, MnP, and laccase in a ratio of 1:2:2 produced by three different fungi. The cellulose content was about 76.54%.

There are different advantages of the microbial and enzymatic processes that include high delignification, low sugar losses, positive environmental impacts, and the development of consolidated processes (Placido and Capareda 2015). Treatment of lignocellulosic residues (*i.e.*, wheat straw with ligninolytic enzyme extracts, obtained from the WRF *T. versicolor*, *Bjerkandera adusta*, and *Fomes fomentarius*) *versus* the non-treated straw control showed an increase in the digestibility of the treated straw. Supplementation of different doses of a mixture of three exogenous ligninolytic enzymes, Lac, LiP, and MnP, failed to elicit any changes in either the cell wall components or the *in vitro* digestibility of straw. However, treating the straw with the same enzymes for 24 h at a ratio of 2:5 (v/ w) showed a marked reduction in these cell wall components as well as in the *in vitro* digestibility (Mahesh and Mohini 2013). Asgher *et al*. (2013) found a significant loss in the lignin content (39.6%) of wheat straw after the treatment with ligninolytic enzyme extracts at the dose level of 25 mL, respectively

Table 5. Percent of Lignin and Cellulose Content after the Enzymatic Treatment of Wheat Straw (WS) with a Mixture of Ligninolytic Enzyme Consortium at 25 °C

Enzyme combination	Ratio	Lignin (%)		Delignification (%)	Cellulose (%)	
		Before treatment	After treatment		Before treatment	After treatment
LiP:MnP:Lacc	1:1:1	24.60 ± 0.13	11.06 ± 0.42	52.80	24.00 ± 0.36	43.20 ± 0.27
LiP:MnP:Lacc	2:1:1	24.60 ± 0.13	13.04 ± 0.72	47.00	24.00 ± 0.36	52.43 ± 0.41
LiP:MnP:Lacc	1:2:1	24.60 ± 0.13	13.80 ± 0.31	43.90	24.00 ± 0.36	63.89 ± .72
LiP:MnP:Lacc	1:1:2	24.60 ± 0.13	10.47 ± 0.37	57.00	24.00 ± 0.36	56.00 ± 0.51
LiP:MnP:Lacc	2:2:1	24.60 ± 0.13	14.27 ± 0.42	41.00	24.00 ± 0.36	49.23 ± 0.42
LiP:MnP:Lacc	1:2:2	24.60 ± 0.13	10.20 ± 0.41	58.50	24.00 ± 0.36	76.54 ± 0.27
LiP:MnP:Lacc	2:1:2	24.60 ± 0.13	11.90 ± 0.51	51.60	24.00 ± 0.36	54.67 ± 0.13

Knezevic *et al*. (2013) reported a reduction of 34.1% in the lignin content of wheat straw after incubating it for 14 days with *Dichomitus squalens*. Compared with the data, *T. versicolor* BEOFB 320 showed a higher delignification rate of 20.9% for 14 days with wheat straw fermentation, while levels of the analyzed ligninolytic enzyme activities were similar and even lower (Knezevic *et al*. 2013). The wheat straw showed a lignin loss of 18.5%, while in wheat straw from *P. radiata* and *T. versicolor* it was 12.5% (Arora *et al*. 2002).

Table 6. Percent of Lignin and Cellulose Content after the Enzymatic Treatment of Wheat Straw (WS) with a Mixture of Ligninolytic Enzyme Consortium at 40 °C

Enzyme combination	Ratio	Lignin (%)		Delignification (%)	Cellulose (%)	
		Before treatment	After treatment		Before treatment	After treatment
LiP:MnP:Lacc	1:1:1	24.60 ± 0.13	12.21 ± 0.32	50	24.00 ± 0.36	42 ± 0.92
LiP:MnP:Lacc	2:1:1	24.60 ± 0.13	10.34 ± 0.72	58	24.00 ± 0.36	54 ± 0.51
LiP:MnP:Lacc	1:2:1	24.60 ± 0.13	13.13 ± 0.51	47	24.00 ± 0.36	65 ± 0.62
LiP:MnP:Lacc	1:1:2	24.60 ± 0.13	11.23 ± 0.41	42	24.00 ± 0.36	53 ± 0.73
LiP:MnP:Lacc	2:2:1	24.60 ± 0.13	12.72 ± 0.24	48	24.00 ± 0.36	52 ± 0.14
LiP:MnP:Lacc	1:2:2	24.60 ± 0.13	10.76 ± 0.63	56	24.00 ± 0.36	72 ± 0.53
LiP:MnP:Lacc	2:1:2	24.60 ± 0.13	13.21 ± 0.72	46	24.00 ± 0.36	51 ± 0.29

Enzyme-Assisted Delignification of Sugarcane Bagasse (SCB)

The enzymatic treatment of sugarcane bagasse was carried out using a consortium of ligninolytic enzymes according to different ratios, as shown in Tables 7 and 8. The best delignification of 46% was achieved using a mixture of an enzymatic consortium of LiP, MnP, and laccase in a ratio of 1:1:2. This exposes the cellulose content to approximately 67% at 40 ºC when an enzyme consortium of 1:1:2 was used.

Table 7. Percent of Lignin and Cellulose Content after the Enzymatic Treatment of Sugarcane Bagasse (SCB) with a Mixture of Ligninolytic Enzyme Consortium at 25 °C

Enzyme combination	Ratio	Lignin (%)		Delignification (%)	Cellulose (%)	
		Before treatment	After treatment		Before treatment	After treatment
LiP:MnP:Lacc	1:1:1	22.90 ± 0.67	16.70 ± 0.62	18.00	21.00 ± 1.12	39 ± 0.42
LiP:MnP:Lacc	2:1:1	22.90 ± 0.67	13.73 ± 0.23	40.00	21.00 ± 1.12	42 ± 0.13
LiP:MnP:Lacc	1:2:1	22.90 ± 0.67	15.34 ± 0.87	33.00	21.00 ± 1.12	52 ± 0.42
LiP:MnP:Lacc	1:1:2	22.90 ± 0.67	13.81 ± 0.18	39.00	21.00 ± 1.12	62 ± 0.15
LiP:MnP:Lacc	2:2:1	22.90 ± 0.67	15.28 ± 0.24	33.00	21.00 ± 1.12	57 ± 0.62
LiP:MnP:Lacc	1:2:2	22.90 ± 0.67	16.85 ± 0.23	26.00	21.00 ± 1.12	69 ± 0.32
LiP:MnP:Lacc	2:1:2	22.90 ± 0.67	14.16 ± 0.15	38.00	21.00 ± 1.12	56 ± 0.52

Table 8. Percent Lignin and Cellulose Content after the Enzymatic Treatment of Sugarcane Bagasse (SCB) with a Mixture of Ligninolytic Enzyme Consortium at 40 °C

Enzyme combination	Ratio	Lignin (%)		Delignification (%)	Cellulose (%)	
		Before treatment	After treatment		Before treatment	After treatment
LiP:MnP:Lacc	1:1:1	22.90 ± 0.67	17.87 ± 0.23	22	21.00 ± 1.12	38 ± 0.42
LiP:MnP:Lacc	2:1:1	22.90 ± 0.67	13.16 ± 0.12	42	21.00 ± 1.12	39 ± 0.52
LiP:MnP:Lacc	1:2:1	22.90 ± 0.67	14.43 ± 0.13	37	21.00 ± 1.12	59 ± 0.62
LiP:MnP:Lacc	1:1:2	22.90 ± 0.67	12.35 ± 0.92	46	21.00 ± 1.12	67 ± 0.21
LiP:MnP:Lacc	2:2:1	22.90 ± 0.67	14.65 ± 0.45	36	21.00 ± 1.12	59 ± 0.25
LiP:MnP:Lacc	1:2:2	22.90 ± 0.67	13.32 ± 0.42	41	21.00 ± 1.12	65 ± 0.42
LiP:MnP:Lacc	2:1:2	22.90 ± 0.67	13.87 ± 0.16	39	21.00 ± 1.12	59 ± 0.52

Previously, various researchers accomplished considerable lignin disruption by employing ligninolytic enzymes. Delignification of sugarcane bagasse by *Ceriporiopsis subvermispora* over 30 days resulted in a pulp yield of 46 to 54% (Costa *et al*. 2005). Xu *et al*. (2009) produced the ligninolytic and cellulolytic enzymes from *Irpex lacteus* by using corn stover as a substrate under the solid state of fermentation. After 120 days of incubation, 80% of lignin, 75% of cellulose and 63% of hemicellulose were degraded. *Phanerochaete chrysosporium* had a remarkable ability to degrade the lignin selectively and to enhance the *in vitro* digestibility (Sharma and Arora 2010). Also, various enzyme extracts from *T. versicolor, B. adusta,* and *F. fomentarius* have shown the ability to degrade the cell wall components by improving the enzymatic digestibility and nutritional values (Rodrigues *et al*. 2008).

Enzyme-Assisted Delignification of Rice Straw (RS)

The purified ligninolytic enzyme extracts (LiP, MnP, and laccase) were used to degrade the lignin in rice straw. Different consortia of the enzymes caused a marked reduction in the lignin content of the substrate (Tables 9 and 10). Among different ratios of the enzyme extract, the maximum delignification was 52%, which was attained with the LiP, MnP, and laccase ratio of 2:1:2. When the temperature was increased from 25 °C to 40 °C, there was a reduction in the lignin loss to approximately 45.6%.

There is a prominent decrease in the total lignin content of rice straw *via* enzymatic treatments. Mohamram *et al*. (2013) used *T. hirsuta* and *M. roridum* for the pretreatment of rice straw leading to the selective delignification of rice straw at 19% and 70% enrichment at in the holocellulosic contents. The alkali extract analyzed by liquid showed prominent differences in the degradation of the cell wall by *T. hirsuta* and *M. roridum*. *T. hirsuta* removed 15% more phenolic compounds and 38% more glucan than *M. roridum*, while *M. roridum* removed 77% more xylan than *T. hirsuta*. Chang *et al*. (2012) screened different fungi for the selective delignification of rice straw and found

that traditional white-rot fungus *Phanerochaete chrysosporium* has a lignin degradation of 28.3% and a holocellulose degradation of 28.4%.

Table 9. Percent of Lignin and Cellulose Content after the Enzymatic Treatment of Rice Straw (RS) with a Mixture of Ligninolytic Enzyme Consortium at 25 °C

Enzyme combination	Ratio	Lignin (%)		Delignification (%)	Cellulose (%)	
		Before treatment	After treatment		Before treatment	After treatment
LiP:MnP:Lacc	1:1:1	23.60 ± 0.73	16.14 ± 0.14	31.60	40.00 ± 0.39	56 ± 0.15
LiP:MnP:Lacc	2:1:1	23.60 ± 0.73	19.5 ± 0.91	17.00	40.00 ± 0.39	49 ± 0.42
LiP:MnP:Lacc	1:2:1	23.60 ± 0.73	16.71 ± 0.32	29.00	40.00 ± 0.39	51 ± 0.14
LiP:MnP:Lacc	1:1:2	23.60 ± 0.73	16.31 ± 0.14	30.00	40.00 ± 0.39	61 ± 0.42
LiP:MnP:Lacc	2:2:1	23.60 ± 0.73	13.32 ± 0.61	44.00	40.00 ± 0.39	47 ± 0.12
LiP:MnP:Lacc	1:2:2	23.60 ± 0.73	16.76 ± 0.52	28.00	40.00 ± 0.39	71 ± 0.32
LiP:MnP:Lacc	2:1:2	23.60 ± 0.73	11.32 ± 0.14	52.00	40.00 ± 0.39	73 ± 0.12

Table 10. Percent of Lignin and Cellulose Content after the Enzymatic Treatment of Rice Straw (RS) with a Mixture of Ligninolytic Enzyme Consortium at 40 °C

Enzyme combination	Ratio	Lignin (%)		Delignification (%)	Cellulose (%)	
		Before treatment	After treatment		Before treatment	After treatment
LiP:MnP:Lacc	1:1:1	23.60 ± 0.73	17.11 ± 1.82	23	40.00 ± 0.39	54 ± 0.34
LiP:MnP:Lacc	2:1:1	23.60 ± 0.73	18.36 ± 0.98	22	40.00 ± 0.39	48 ± 0.27
LiP:MnP:Lacc	1:2:1	23.60 ± 0.73	15.12 ± 0.59	36	40.00 ± 0.39	50 ± 0.15
LiP:MnP:Lacc	1:1:2	23.60 ± 00.73	14.98 ± 0.61	37	40.00 ± 0.39	63 ± 0.15
LiP:MnP:Lacc	2:2:1	23.60 ± 0.73	13.09 ± 0.26	46	40.00 ± 0.39	49 ± 0.42
LiP:MnP:Lacc	1:2:2	23.60 ± 0.73	13.92 ± 0.52	41	40.00 ± 0.39	70 ± 0.33
LiP:MnP:Lacc	2:1:2	23.60 ± 0.73	12.76 ± 0.32	47	40.00 ± 0.39	57 ± 0.28

CONCLUSIONS

1. Ligninolytic enzymes produced by white rot fungi were effective biocatalysts for the selective delignification of various substrates.
2. Out of the three substrates, the best delignification of 58.5% observed under wheat straw with a LiP:MnP:Laccase enzyme ratio of 1:2:2. This increased the exposed

cellulose content to about 76.54%.

3. However, the other two substrates, sugarcane bagasse and rice straw, were maximally delignified to 55% and 52% with 1:2:2 and 2:1:2 ratios of the ligninolytic enzymes.

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