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Full Length Research Paper

Partial purification and characterization of ligninolytic enzymes produced by *Pleurotus ostreatus* during solid state fermentation

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The production of ligninolytic enzymes including laccase, manganese peroxidase and lignin peroxidase from *Pleurotus ostreatus* was studied under different parameters using solid state fermentation. Maximum production of enzymes was observed after 7 days in solid state fermentation (SSF) medium containing 5 g wheat straw (66% w/w moisture) in a still culture SSF. Different parameters had a significant effect on enzyme production. Maximum laccase (455.11 U/mL), manganese peroxidase (210.77 U/mL) and lignin peroxidase (54.50 U/mL) were produced when wheat straw (5 g) at 66% moisture (w/w) was used with 4 mL inoculum at pH 4.5 and 30°C in the presence of 1% (v/v) glycerol as carbon source, 0.2% w/w urea as nitrogen source, 1% (w/v) 2, 2 azinobis 3-ethylbenzthiazoline 6 sulphonate (ABTS) as an inducer for laccase and 1% (w/v) $MnSO_4$ for manganese peroxidase,1% (w/v) $CuSO_4$ as metal ion for laccase and Mn^+ for manganese peroxidase. Both enzymes laccase and manganese peroxidase produced by *Pleurotus ostreatus* were partially purified by ammonium sulphate precipitation followed by gel filtration chromatography. Additionally, the protein content of the recovered supernatants was also noted. Purification results showed an increase in purity up to 3.37 and 3.07 fold for laccase and manganese peroxidase, respectively. The Michaelis constant, K_M was 62 and 33 µM for laccase and manganese peroxidase, respectively. Lignin peroxidase was not produced during solid state fermentation of ligniocellulosic material by *P. ostreatus* because this fungus is a laccase producer. The activators ABTS and $MnSO_4$ proved good for laccase and MnP production. This shows that SSF parameters had a significant influence on catalytic activity of ligninolytic enzymes produced by *P. ostreatus* under still conditions.

Key words: Ligninolytic enzymes, *Pleurotus ostreatus*, 2,2 azinobis 3-ethylbenzthiazoline 6 sulphonate (ABTS), solid state fermentation.

INTRODUCTON

White rot fungi are the most significant lignin degraders among the wood inhabiting microorganisms. They degrade lignin by secreting extracellular redox enzymes, that is, laccase, MnP and LiP which play a key role in lignin biodegradation (Mester, 2000). *Pleurotus ostreatus* is a white rot fungus which belongs to *Basidiomycetes*. It is an important cholesterol reducing mushroom (Aguila et al., 2003). Some genera of *Basidiomycetes*, such as *Pleurotus spp.,* were found to lack lignin peroxidases (Fukushima, 1995; Galliano et al., 1988, 1991; Mansur et al., 2003) indicating that different enzymes are probably involved in lignin biodegradation and that among these enzymes, laccases play a key role. Studies on the enzymes secreted by the fungus *P. ostreatus* have shown that the concerted action of laccase and aryl alcohol oxidase produces significant reduction in the molecular mass of soluble lignosulphonates (Mansur et

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Abbreviations: EDTA, Ethylenediaminetetraacetic acid; **ABTS,** 2,2-azinobis 3-ethylbenzthiazoline 6 sulphonate; **SSF,** solid state fermentation.

al., 2003; Marzullo et al., 1995). The composition of the ligninolytic system is thus very complex and species specific (Baldrian, 2008). The Laccase, MnPs and LiPs are very closely related enzymes of white rot *Basidiomycetes* and are likely contributors to fungal ligninolysis. Many of them cleave lignin model compounds to give products consistent with those found in residual white rotted lignin and at least some depolymerised synthetic lignins (Cullen, 2008). Laccases are copper containing oxidases. They reduce molecular oxygen to water and oxidize phenolic compounds (Mester, 2000). These ligninolytic enzymes oxidize various environmental pollutants such as polycyclic aromatic hydrocarbons, Cholorophenols and aromatic dyes. The peroxidases (LiP and MnP) are heme containing enzymes having typical catalytic cycles that are characteristic of other peroxidases. LiP is able to oxidize various aromatic compounds while MnP almost exclusively Mn (II) to Mn (III), that it chelates, acting as a diffusing oxidizer (Savitha et al., 2008, Wang et al., 2009). Laccases (benzenediol: oxygen oxidoreductase, EC (1. 10. 3. 2) are multi-copper blue oxidases widely distributed in higher plants, in some insects and in a few bacteria. However, the best known laccases are of fungal origin. Laccase production occurs in various fungi (Bollag, 1984; Sadhasivam et al., 2008). Laccases catalyze the oxidation of broad range of substrates such as ortho and para diphenols, methoxy substituted phenols, aromatic amines, phenolic acids and several other compounds coupled to the reduction of molecular oxygen to water with the one electron oxidation mechanism. The substrate specificity of laccases varies from one organism to another. The spectrum of laccase oxidizable substrates can be expanded considerably in the presence of appropriate redox mediators (Johannes, 2000; Suigura et al., 2003). Due to their interesting catalytic properties, laccases have gained considerable interest in various industrial areas. The most intensively studied applications have included development of oxygen cathodes in biofuel cells, biosensors, labeling in immunoassays and organic synthesis through biocatalysis. Due to their broad substrate specificity, laccases might have great potential in varied environmental applications including pulp delignification, textile dye bleaching and xenobiotics degradation (Mazumder et al., 2009; Pickard et al., 1999; Savitha et al., 2008). These applications stimulate new fundamental research for which this enzyme can be used.

The current research activity of ligninases includes screening of suitable substrates which are suitable for the optimum production of ligninases by *P. ostreatus* and optimization of different parameters including the effect of 1) moisture, 2) inoculum size, 3) C: N ratio (carbon and nitrogen ratio), 4) suitable mediators and 5) effect of macro/micro trace elements. After optimization of different parameters, characterization of ligninolytic enzymes (Laccase and MnP) from the fungus *P. ostreatus* with respect to production, partial purification and kinetic properties, is reported in this study.

MATERIALS AND METHODS

Substrate collection and preparation

Ligniocellulosic materials like wheat straw, rice straw, sugarcane bagasse, corn stover, banana stalk and corn cobs were used as substrate for the production of ligninases by *P. ostreatus*. Wheat straw and rice straw were obtained from farms of University of Agriculture, Faisalabad, Corn stover and corncobs from CPC Rafhan, Faisalabad, Sugarcane bagasse was obtained from Crescent sugar mill, Faisalabad. All the substrates were dried in an oven at 80°C to constant weight and were ground in an electric grinder to powder form and stored in airtight plastic jars to keep the substrate free of moisture.

Fermentative organism

A culture of *P. ostreatus* was available in the Industrial Biotechnology laboratory, Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad. The fungal culture was grown on potato dextrose agar slants (PDA) at pH 4.5 and 28°C and was preserved at 4°C in a refrigerator. Cultures were recultivated periodically as needed at their respective optimal growth temperature.

Inoculum preparation

Aqueous spore suspension of *P. ostreatus* was prepared by growing the fungus in an inoculum medium for 5-7 days. The inoculum medium was that of Kirk (Fukushima, 1995). This medium contained: Ammonium tartrate, (0.22 g/L), KH_2PO_4 (0.21 g/L), $MgSO_4.7H_2O$ (0.05 g/L), $CaCl_2$. H_2O 0.01 g/L, Thiamine (0.001 g/L), Tween-80 (10 mL/L), Trace element solution (10 mL/L) supplemented with 1% (w/v) glucose. The medium was adjusted to pH 4.5 and autoclaved at (121°C) for fifteen minutes. A loopful of *P. ostreatus* spores was transferred to the sterilized inoculum medium under sterile conditions and the flask was incubated at 35°C for 7 days in a shaker with continuous shaking to get the spore concentration to 1×10^6 to 1×10^8 spore/mL (Kay-Shoemake and Watwood, 1996).

Solid state fermentation protocol (screening and sample recovery)

P. ostreatus was grown on all six substrates that is, rice straw, wheat straw, corn stover, corn cobs, sugarcane bagasse and banana stalk. Experiments were performed in triplicate using 250 mL Erlenmeyer flasks in a temperature-controlled incubator. The flasks contained 5 g of the respective substrates (wheat straw, rice straw, banana stalk, corn cobs, corn stover and sugarcane bagasse) which were moistened with 10 mL of the basal medium without glucose. Each flask was sterilized at 121°C in an autoclave for 15 min and was inoculated with 5 mL of the freshly prepared fungal spore suspension. The inoculated flasks were kept at 30°C for 1-10 days in a temperature controlled incubator to determine a suitable substrate which results in high activity for the identified enzymes. After the stipulated fermentation period, the experimental and control flasks were harvested by adding 100 mL of distilled water and placed in a shaker for 30 min. The biomass was filtered using a Whatman filter paper (No.1). The filtrates were centrifuged at 10,000 rpm for 10 min at room temperature. The supernatants

obtained were stored (4°C) in sterilized glass bottles in refrigerator to prevent contamination and were used as crude enzyme extracts.

Enzyme activity assays

Laccase was assayed by monitoring 2, 2 azinobis 3-ethyl-benzthiazoline 6 sulphonate (ABTS) oxidation in sodium malonate buffer at 420 nm (Wolfenden, 1982). The activity of Manganese peroxidase (MnP) was measured by the method of Wariishi et al. (1992) at 25°C by the dependent oxidation of manganic-malonate complex at 270 nm. 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 at 310 nm.

Proteins measurement

Protein concentration was quantified using the Bradford assay (Bradford, 1976) with Bovine serum albumin as the standard.

Ligninolytic enzymes partial purification and characterization

Crude extracts obtained from *P. ostreatus* were centrifuged at 10,000 rpm for 15 min at 4°C and then concentrated further by freeze drying. Different amounts of ammonium sulphate (in powder form) were added separately to 1 mL of the crude enzyme concentrates in Eppendorf tubes to get 10-90% saturation at 0°C. These tubes were gently mixed and left over night at 4°C and centrifuged at 10,000 rpm for 15 min the next day. The supernatants were assayed for ligninolytic enzyme activity.
After optimizing the various concentrations of ammonium sulphate, the crude enzyme concentrate was placed in an ice bath and ammonium sulphate was added to attain a 40% (w/v) at 0°C. The flasks were further kept overnight at 4°C and the resultant pellet of precipitated protein was discarded. In the supernatant, more crystals of ammonium sulphate were added to attain 80% (w/v) at 0°C. It was again kept for a night at 4°C and centrifuged. This time, the pellet was recovered and the supernatant was discarded. The pellet was dissolved in a 50 mL buffer and dialyzed against distilled water for 24 h. Enzyme activity was determined before and after dialysis. Crude enzyme solutions obtained after dialysis were loaded on Sephadex G-100 columns for further partial purification to standardized homogeneity levels.100 mM phosphate buffer was used as elution buffer. The flow rate was 0.5 ml/ min. 1 mL fractions were collected and analyzed for enzyme activity and protein content.

Effect of pH/ temperature

The effect of pH on purified enzyme activity was studied in the following buffers (0.2M): Tartrate buffer, pH 3.0; sodium malonate buffer, pH 4.0; citrate phosphate, pH 5.0 and pH 6.0; sodium phosphate, pH 7.0 and pH 8.0; and carbonate buffer, pH 9.0 and pH 10.0. The effect of temperature on purified ligninolytic enzyme was determined in the range from 25 to 60°C with 5°C increments at (specify time intervals here/5 to 10 min) intervals. After 5 to 10 min of incubation, enzyme activity was measured to evaluate the effect of temperature on enzyme activity.

Effect of Substrate concentration

Michaelis constant K_M and maximum velocity V_{max} of ligninolytic enzymes (laccase and MnP) were calculated by using different concentrations (0.1-1.0 mM) of respective substrates for each enzyme that is, ABTS, $MnSO_4$ and veratryl alcohol for Laccase and MnP, respectively.

Effect of activators/inhibitors

The effect of several activators/inhibitors such as $CuSO_4$, $MnSO_4$, $FeSO_4$, ethylenediaminetetraacetic acid (EDTA), cysteine and ethanol with the concentration of 1 mM on ligninolytic enzymes (Laccase and MnP) activity was monitored under standard assaying conditions. The reaction mixtures of Laccase and MnP were incubated in their optimized buffer and respective optimum temperatures; the change in absorbance was measured spectro-photometrically at their respective wave length that is, 420 nm for laccase and 270 nm for MnP to evaluate the influence of these activators/inhibitors on enzyme activity.

RESULTS AND DISCUSSION

Initially, the maximum production of ligninase enzymes was based on the selection of a suitable substrate for *P. ostreatus*. Six different substrates; that is, wheat straw, rice straw, banana stalk, sugarcane bagasse, corn stover and corn cobs were used to determine their suitability as substrates for the optimised production of ligninase enzyme. However, among all the six substrates which we used for the production of ligninase enzymes, wheat straw proved to be a suitable substrate for the production of ligninase enzymes using *Pleurotus ostreatus*. The maximum production of Laccase enzymes (Lac 410.30 U/mL), Manganese peroxidase (MnP 197.92 U/mL) and Lignin peroxidase (LiP 52.50 U/mL) was achieved after the 7th day of solid state fermentation. *P. ostreatus* has been reported as a producer of Laccase and MnP, rather than LiP (Mazumder et al., 2009; Patel et al., 2007; Tellez et al., 2008). It was also noted that the addition of inducers such as copper, laccase production was enhanced up to 3.39 fold when compared to control cultures. Furthermore, the time required for the maximum production of laccase was reduced to 6 days. The next suitable substrate for the maximum production of ligni-nase enzymes was rice straw which produced Laccase (Lac 401.07 U/mL), manganese peroxidase (MnP 148.20 U/mL) and lignin peroxidase (51.56 U/mL) after the 7th day of solid state fermentation. Lignin peroxidase activity was found to be minimal in comparison to Lacasse and MnP produced (Patel et al., 2007), whereas manganese peroxidase showed maximum activity on the 10th day of fermentation by *P. ostreatus*. This activity was less as compared to laccase production.

Partial purification of enzymes

A mixture of crude ligninases (Laccase and MnP) was obtained from seven day old incubated cultures of *P. ostreatus* grown on wheat straw under optimum fermentation conditions. The first step towards partial

Table 1. Partial purification summary of Laccase produced during solid state fermentation of lignocellulosic wheat straw by *Pleurotus ostreatus* under optimized conditions.

Purification step	Activity/500mL	Protein content (U/mg)	Specific activity	Purification fold
Crude enzyme	211940	2184.5	97.01	1
Ammonium Sulphate	208610	1184.8	176.07	1.81
Dialysis	201805	1025.50	196.78	2.02
Sephadex G-100	191525	585.15	327.30	3.37

Table 2. Partial purification summary of MnP produced during solid state fermentation of lignocellulosic wheat straw by *Pleurotus ostreatus* under optimized conditions.

Purification steps	Activity/500mL	Protein content (U/mg)	Specific activity	Purification fold
Crude enzyme	182480	1885	96.80	1
Ammonium Sulphate	179150	1585	113.02	1.16
Dialysis	172345	1025	168.14	1.73
Sephadex G-100	162055	545	297.34	3.07

purification of crude ligninolytic enzymes was to achieve the optimum ammonium sulphate saturation point for ligninolytic enzymes (Laccase and MnP). The optimum ammonium sulphate precipitation saturation point for ligninolytic enzymes Laccase and MnP was 65 and 85%, respectively. After optimizing the various concentrations of ammonium sulphate for Laccase and MnP precipitation, the crude enzyme extracts were placed in an ice bath and crystals of solid ammonium sulphate were added to achieve an initial 35 and 50% (w/v) concentration at 4°C for Laccase and MnP, respectively. Thereafter, the pellet of precipitated protein was discarded. In the supernatant, more crystals of ammonium sulphate were added to attain 65 and 85% concentration at 4°C for laccase and MnP, respectively. The samples were again kept overnight at 4°C and centrifuged. This time the pellet was collected and supernatant was discarded. The pellet was dissolved in 50 mL of buffers sodium malonate (50 mM, pH 4.5) for laccase and MnP and dialyzed against distilled water for 24 h to remove the ammonium sulphate.

Enzyme activity was determined before and after dialysis of ammonium sulphate precipitated and finally freeze dried. All the purification steps were conducted at temperatures not exceeding 4°C. Mtui and Nakamura achieved 50 to 80% $(NH_4)_2SO_4$ saturation followed by chromatographical purification techniques for the recovery of pure ligninolytic enzymes. The crude enzyme solution obtained after dialysis was loaded on to Sephadex G- 100 columns to further purify the samples to a homogeneous level (Tables 1 and 2). 100 mM phosphate 0.15 M NaCl was used as an elution buffer. The protein fractions were eluted at a flow rate of 0.5 mL/min. 1 mL size fractions was collected and each fraction was assayed for laccase and MnP activity. Gel filtration graphs were plotted for both laccase and MnP by taking absorbance versus in each vial (Figures 1 and 2). The acceptance of gel filtration is due to its simplicity, rapidity and economy of the method. Gel filtration is an extremely gentle method which rarely causes denaturation of labile substances.

Effect of pH on enzyme activity

To check the effect of pH on enzyme activity and stability, the purified enzymes were incubated for 5 to 10 min at different pH values ranging from 3.0 to 10 using the following buffers (0.2 M): Tartrate buffer, pH 3.0; sodium malonate buffer, pH 4.0; citrate phosphate, pH 5.0 and pH 6.0; sodium phosphate, pH 7.0 and pH 8.0; and carbonate buffer, pH 9.0 and pH 10.0. Enzyme assays were performed after five to ten minutes of incubation in a temperature controlled spectrophotometer at their respective wavelength of each ligninolytic enzyme. The ligninolytic enzyme activities at different pH values for purified Laccase and MnP from *P. ostreatus* are shown in (Figure 3). Results of enzyme assay showed that the ligninolytic enzymes were completely stable in large pH range (4.0-8.0) and presented an optimum activity of 575 U/mL for laccase and 200.10 U/mL for MnP at a pH value of 4. It was observed that sodium malonate buffer of pH 4 enhanced laccase activity, and a decreasing trend towards inactivity was shown when a carbonate buffer of pH 9 and 10 (Figure 3) was used. For different substrates, pH optima and range was between 4 and 7. Similar results were achieved by Wang et al. (2009) were the optimum pH for the determination of laccase activity

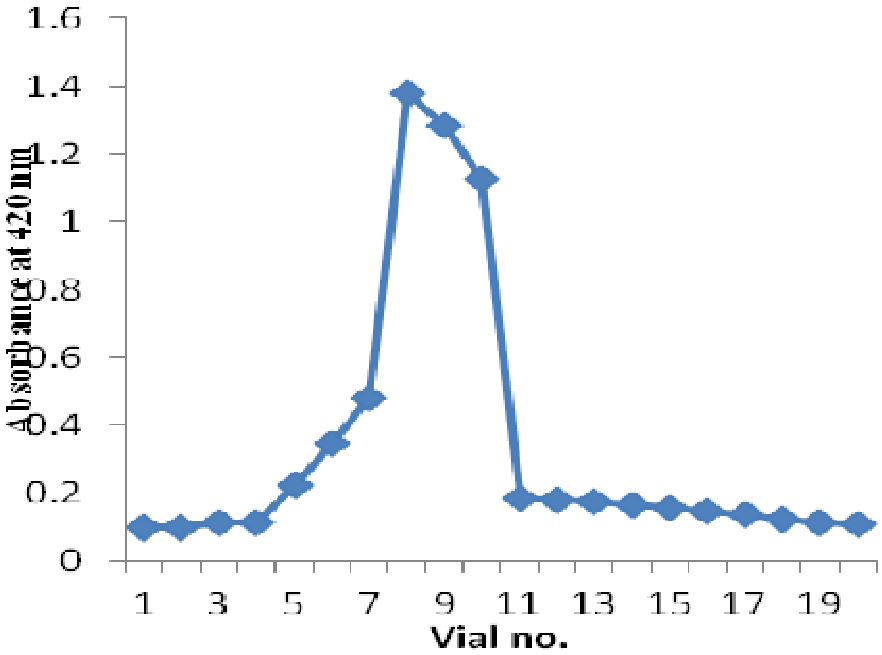

Figure 1. Gel filtration chromatography of Laccase produced during degradation of lignocellulosic wheat straw from *Pleurotus ostreatus* under optimum conditions.

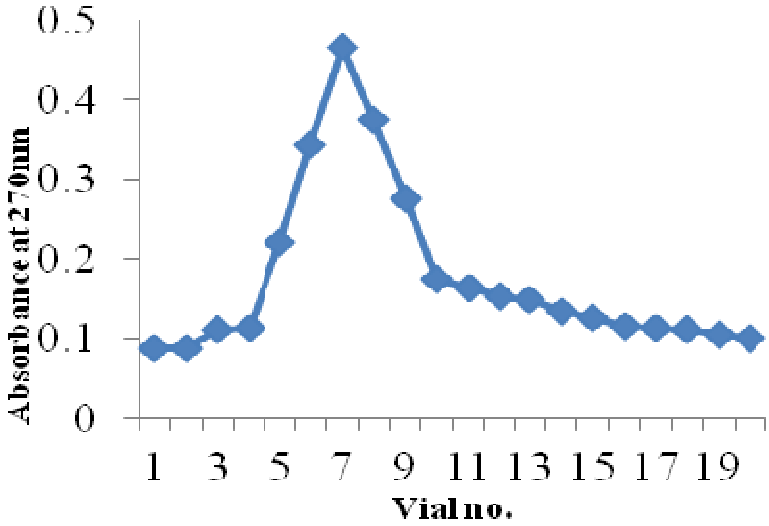

Figure 2. Gel filtration chromatography of MnP produced during degradation of lignocellulosic wheat straw from *Pleurotus ostreatus* under optimum conditions.

was 4.0.

Effect of temperature

The experiment was conducted to evaluate the effect of different temperatures on ligninolytic enzymes (Laccase and MnP) in the range of 25 to 60°C. For a variety of industrial applications, relatively high thermostability is an attractive and desirable characteristic of an enzyme. Temperature optimum for Laccase and MnP was observed at 50 and 30°C, respectively.

Results in Figure 4 showed that at temperatures higher than 55°C enzymes loss their activity rapidly. Zheng et al. (2009) reported optimum temperature for its activity as 50°C when fermentation optimization and characteriza-

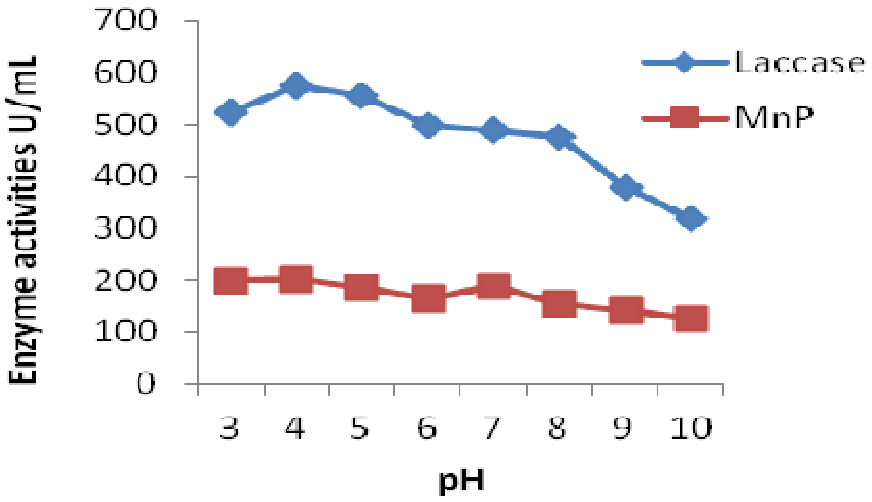

Figure 3. Effect of different pH values on partially purified ligninolytic enzymes from *Pleurotus ostreatus*.

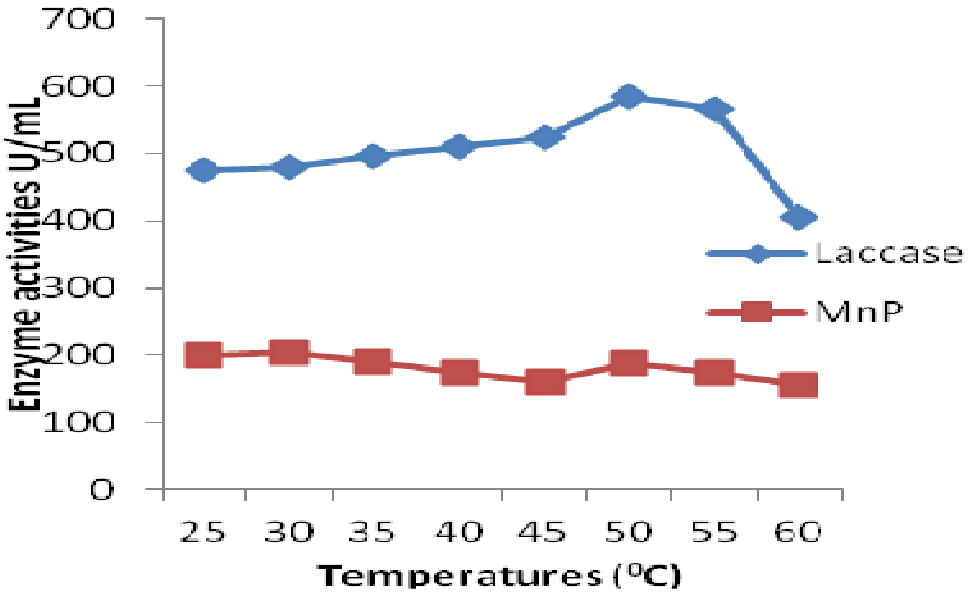

Figure 4. Effect of different temperatures on partially purified ligninolytic enzymes from *Pleurotus ostreatus*.

tion of the laccase from *P. ostreatus* strain 10969 was carried out.

Effect of substrate concentration on Michaelis saturation constant K_M and the maximum substrate conversion rate V_{Max}

The substrate specificity of the partially purified ligninolytic enzymes laccase and MnP were examined with different substrate concentrations (0.1- 1.0 mM) that is, ABTS and $MnSO_4$, respectively. Enzyme activity was measured under standard assaying conditions. K_M and V_{max} for ligninolytic enzymes Laccase and MnP were determined by incubating each enzyme with varying concentrations of their assaying substrates; the results were plotted as an illustration of enzyme activity (U/mL) against concentration of substrate (S), which yield a hyperbolic curve, as shown in the Figures 5 and 6 for laccase and MnP respectively with the values K_M and V_{max}.

It was noted that values of laccase catalytic constant was slightly higher as compared to MnP. The Michaelis Menten constant K_M for laccase was 62 µm according to

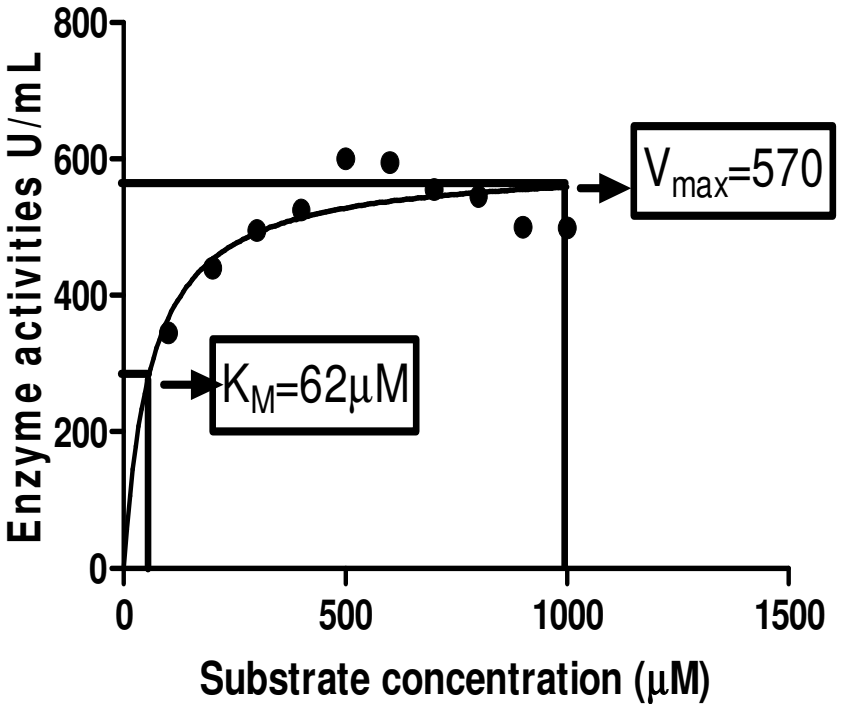

Figure 5. Determination of K_M and Vmax for partially purified Laccase produced from *Pleurotus ostreatus* through Michaelis–Menten equation.

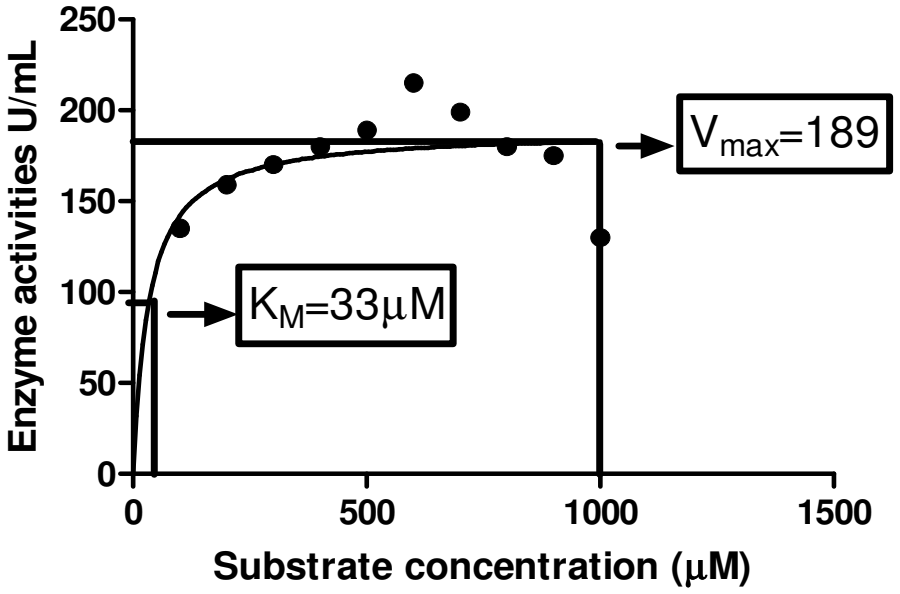

Figure 6. Determination of K_M and Vmax for partially purified MnP produced from *Pleurotus ostreatus* through Michaelis –Menten equation.

Michealis menten calculations, and in the line weaver burk plot it was 71 µm. For MnP, the K_M was 33 µm. Sadhasivam worked on production, purification and characterization of mid-redox potential laccase from a newly isolated *Trichoderma harzianum* WL1 (Sadhasivam et al., 2008) and reported that purified laccase showed K_M values of 180 and 60 µM, respectively for the substrates ABTS and guaiacol, results which have a similar order of magnitude to the ones obtained in this study.

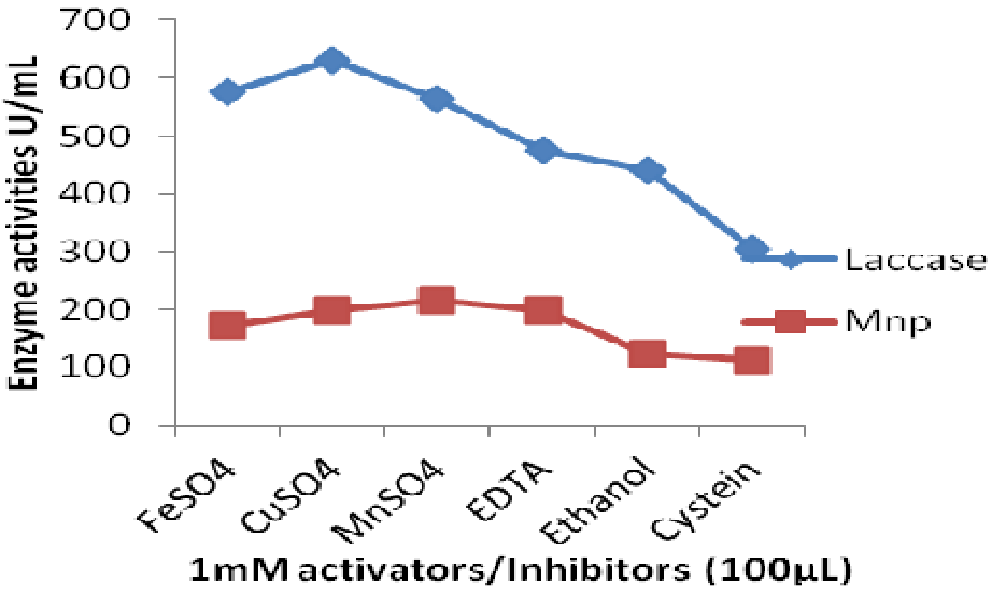

Figure 7. Effect of different Activators/Inhibitors (mM) on production of ligninolytic enzymes by *Pleurotus ostreatus.*

Effect of activators/ Inhibitors

Several activators/inhibitors such as $CuSO_4$, $MnSO_4$, $FeSO_4$, EDTA, Cysteine and ethanol with a concentration of up to 1 mM were evaluated for the effect on ligninolytic enzyme activity. Activators including $CuSO_4$ and $MnSO_4$ had an enhancing effect on the production of each of the ligninolytic enzymes whereas inhibitors EDTA and Cysteine caused inhibition as shown in Figure 7. $CuSO_4$ among $MnSO_4$ and FeSO4 was an effective activator for laccase while $MnSO_4$ enhanced MnP production. Zhu et al. (2003) reported an increase in laccase production due to the addition of $CuSO_4$ and found that Cu^{2+} (1 mM) also had a positive effect on laccase production, thus enhancing activity to 360 U/ml. It has also been reported that, the activation of the laccase by Cu^{2+} may be due to the filling of type 2 copper binding sites with copper ions. It was also determined that the activation or inhibition of proteolytic enzymes by trace metals can influence extracellular enzymes production by changing their turnover rate (Sadhasivam et al., 2008).

Conclusion

Results of optimized experiments indicated that the SSF parameters like additional carbon and different inexpensive nitrogen supplements, low molecular mass mediators and various metal ions had a significant influence on the production of ligninolytic enzymes. The major enzymes produced by *P. ostreatus* were partially purified by ammonium sulphate precipitation and gel filtration chromatography. After gel filtration chromatography it was come to know that 3.37 and 3.07 fold purification; pH 4; Temperature 50 and 30°C were obtained for laccase and MnP in comparison to crude enzyme samples. The Michaelis constant K_M of 62 and 33 µM for laccase and MnP were determined using ABTS and $MnSO_4$. Among several activators such as $CuSO_4$ and $MnSO_4$ had an enhancing effect on ligninolytic enzyme production whereas inhibitors like EDTA, Cysteine, and ethanol indicated to detrimental to be detrimental to the activity of both MnP and Lacasse.

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