CHARACTERIZATION OF LIGNINOLYTIC ENZYMES PRODUCED BY *SCHYZOPHYLLUM COMMUNE* IN SOLID STATE CULTURES FOR INDUSTRIAL APPLICATIONS

By

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DECLARATION

I hereby declare that the contents of the thesis, **"Characterization of Ligninolytic Enzymes Produced by *Schyzophyllum commune* in Solid State Cultures for Industrial Applications."** are product of my own research and no part has been copied from any published source (except the references, standard mathematical equation/formula or protocols etc). I further declare that this work has not been submitted for award of any other diploma/degree. The university may take action if the information provided is found inaccurate at any stage.

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TO

MY BELOVED LATE PARENTS

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ABSTRACT

The potential of an indigenous white rot fungus *Shyzophylum commune* IBL-06 for the production of ligninolytic enzymes in solid state fermentation of banana stalk was investigated. The production process was further improved by optimizing some physical parameters (incubation time, moisture level, pH, temperature, inoculums size) and nutritional factors (carbon and nitrogen sources, carbon: nitrogen ratio, mediators and metal ions). By optimization of different parameters the maximum activities of enzyme synthesized by *S. Commune* IBL-06 were 3745 IU/mL of MnP, 2700 IU/mL of LiP and 345 IU/mL of Laccase after 3 days incubation at pH 4.5 and 35°C temperature with inoculum size, 3mL; moisture content, 60%; C: N ratio, 20:1(glucose and ammonium nitrate as carbon and nitrogen supplements), 1mM $MnSO_4$ as mediator, 1mL and 1.25mM $MgSO_4$.$7H_2O$a, 1mL. The enzymes produced under optimum conditions were purified by $(NH_4SO_4)_2$ precipitation, dialysis and Sephadex G-100 gel filtration chromatography. The purified enzymes were run on SDS-PAGE and characterized through kinetic studies. The purified MnP was a monomeric protein with mass of 40 kDa. The optimum pH and temperature for MnP were 5 and 40°C with 0.29 mM K_M and 450mM/min V_{max} using $MnSO_4$ as substrate. The enzyme was activated by 1mM $CuSO_4$ but was inhibited by $CaCl_2$, EDTA, TEMED, β-Marcaptoethanol, $AgNO_3$ and $Pb(NO_3)_2$. The molecular weight of purified LiP was 43KDa and it displayed a single band on SDS-PAGE. LiP showed optimum pH 5.0, optimum temperature, 35°C; K_M, 0.5 mM and Vmax, 400 mM/min using varatryl alcohol as substrate. The enzyme was inhibited by $CuSO_4$, $MnSO_4$, $CaCl_2$, EDTA, TEMED, β-Marcaptoethanol, $AgNO_3$, $Pb(NO_3)_2$. Molecular mass of Laccase was 63 kDa and it had optimum pH 6.0, optimum temperature 40°C, K_M value 0.25mM and V_{max} 80mM/min using ABTS as substrate. The laccase activity was enhanced by 2mM $CuSO_4$, and was inhibited by $MnSO_4$, $CaCl_2$, EDTA, TEMED, β-Marcaptoethanol, $AgNO_3$, $Pb(NO_3)_2$. Crude ligninase extract decoulorized Novasol direct blue dye to 80%, followed by Novasol direct yellow dye to 60%, Novasol direct red to 38% and Novasol direct black to 37%. The effluent from Magna textile industry was maximally decolorized to 87% in 24 hours, followed by effluents from Crescent, Arzoo and Chenab textile industries. *S. Commune* IBL-06 produced high activities of MnP and LiP having higher catalytic activities as compared to most of the previously reported enzymes.

CHAPTER 1

INTRODUCTION

Lignin is the important aromatic polymer and cementing material of plant cell walls which together with cellulose provide physical strength to plant cell wall. Complex structure of plant lignin is made up of basic structural units of phenylpropanoids which polymerize radically and protect the plant from microbial attack. Lignin degradation, as well as its potential utilization, is very important to maintain the global carbon cycle (Bermek *et al*., 2004; Boer *et al*., 2006; Kanayama *et al*., 2002). Due to aromatic structure, water-insoluble and non-hydrolyzable bonds, lignin is more difficult to break down than cellulose or hemicellulose (Adler, 1977; Brunow, 2001). The molecular mass (MW) of lignin is high, about 100 kDa or more, which prevents its uptake inside the microbial cell (Eriksson *et al.,* 1990). Carbon content in lignin is high and, microorganisms are unable to utilize polymeric lignin as sole carbon and energy source (Kirk *et al.,* 1976). Lignin depolymerization is necessary to gain access to cellulose and hemicellulose fibers. The biological degradation of macromolecular lignin must occure through the activity of extracellular enzymes. Ligninolytic enzymes needed for the complete degradation of lignin are induced in white rot fungi (WRF) under carbon and nitrogen limitation conditions (Hatakka, 2001). During sugar utilization from polysaccharides of wood, H_2O_2 is produced by the action of glucose oxidase and glyoxyl oxidase (Asghar *et al*., 2008; Asgher *et al*., 2009; Kirk and Farrell, 1987; Hatakka, 2001) that assist the ligninolytic enzymes in their catalytic action

The microbes are being used for conversion of lignocellulosic wastes as potential energy sources for synthesis of very useful industrial products such as enzymes, hormones, organic acids, liquid fuels, single cell protein etc (Rajoka and Malik, 1994). A diverse spectrum of lignocellulolytic microorganisms, mainly basidiomycetic white rot fungi (Baldrian and Gabrial, 2003; Falcon *et al*., 1995) have been identified during the last two decades. Many microorganisms that decompose lignocellulosic material are being studied as producers of enzymes for delignification of the lignocellulosic materials present in agro-industrial residues. Although the cellulose and hemicellulose present in these materials have their value for feeding cattle, their bioavailability requires breakdown of the bonds with indigestible lignin. Pre-digestion of such materials with cellulose free ligninases, xylanases and

pectinases may transform the lignocellulosic substrate into easily hydrolysable substrates (Jordan and Mullen, 2007).

The WRF produce a wide range of lignocellulytic enzymes, also named hydrolytic enzymes, including xylanases, cellulases and ligninases (Sun *et al*., 2004). All these enzymes contribute in the degradation of the cell wall of lignocellulosic substrates (Stajic *et al*., 2006). Lignin biodegradation by WRF involves the action of ligninolytic enzymes which also have promising biotechnological applications in biopulping, denim stone washing, bioethanol production and wastewater treatment (Hofrichter *et al*., 1999). Basidiomycetes comprise of white rot, brown rot, and leaf litter fungi that may insure their nutrition in different ways. Some of them are edible or medicinal fungi; some have important biotechnological and environmental applications. WRF belonging to the basidiomycates are the most efficient and extensive lignin degraders (Akin *et al*., 1995; Gold and Alic, 1993). White-rot basidiomycetes are capable of degrading all basic wood polymers due to their capability to synthesize relevant hydrolytic (cellulases and hemicellulases) and unique oxidative ligninolytic.

WRF seem to be the unique microorganisms which show capacities of degrading and mineralizing lignin and a variety of highly toxic and recalcitrant organic pollutant compounds due to low specificity and strong oxidative abilities of lignin degradation. Furthermore, little attention has been given to the evaluation of the hydrolytic system of white rot fungi. This capacity is, at least to some extent, caused by non-specific enzymatic system produced by these fungi during the lignin degradation, including several isoenzymes of . This multienzyme system involved in the lignin degradation and mineralization is constituted of different ligninolytic enzymes including lignin peroxidase (LiP, E.C.:1.11.1.14), manganese peroxidase (MnP, E.C. 1.11.1.13) and laccase (EC 1.10.3.2) as well as H_2O_2-producing oxidases (Krause *et al*., 2003;Wesemberg *et al.,* 2003; Katia *et al.,* 2005). Ligninolytic enzymes find applications in numerous other industrial processes such as pulp bleaching, oxidation of organic polluntants, stabilization of fruit juices, biosensors development, biofuels cells, textile biofinishing, beverage processing, decreasing dough extensibility in flour, animal feed, cosmetics, clinical diagnosis enzyme immunoassays, wastewater detoxification, denim stone washing and detergent manufacturing (Boer *et al.,* 2006; Papinutti and Forchiassin, 2007; Ravankar and Lele, 2007; Asghar *et al*., 2008).

LiPs are glycosylated, heme containing enzymes that functionally require H_2O_2 for oxidation of lignin and related aromatic structures (Asghar *et al*., 2006; Papinutti and Forchiassin, 2007). LiPs are strong oxidizers of phenols, aromatic amines, aromatic ethers and polycyclic aromatic hydrocarbons (Tien and Kirk 1998; Kalmis *et al.,* 2008).The substrates of LiP include both phenolic and non-phenolic aromatic compounds. The phenolic substrates are oxidized to yield products similar to those produced by peroxidases, while oxidation of non-phenolic methoxybenzenes is unique to LiP (Kersten *et al*., 1985). The oxidation of these substrates to yield aryl cation radicals can result in either demethylation, C_α- C_β cleavage of lignin model compounds, benzylic alcohol oxidation, or hydroxylation of aromatic rings and side chains (Kirk and Farrell, 1987). The natural fungal secondary metabolites veratryl alcohol (VA) and 2-chloro-1, 4-dimethoxybenzene are redox mediators and enhance the LiP catalyzed oxidation of recalcitrant substrates (Teunissen and Field, 1998; Christian *et al.,* 2005). Kinetic analysis has revealed that cationic radical of VA converts LiP (II) and/or LiP(III) to LiP and enhances the catalytic cycle of LiP (Lan *et al*., 2006).

Manganese peroxidase (MnP) finds potential applications in biopulping and biobleaching in paper and pulp industries, as well as in bioremediation processes. MnP is an extracellular heme-containing glycoprotein produced only by ligninolytic basidiomycetes, especially during the secondary metabolism. This enzyme catalyzes the H_2O_2-dependent oxidation of Mn^{2+} to a highly reactive Mn^{3+}, the latter, stabilized by chelating dicarboxylic acids like malonate, oxalate, L-tartrate, oxaloacetate, L-malate, and methylmalonate (Makela *et al*., 2005) is a low-molecular-mass diffusible mediator, which nonspecifically oxidizes a variety of phenolic and non-phenolic substances, including lignin and toxic pollutants. MnP can also utilize peracetic acid, *m*-chloroperoxybenzoic acid, and *p*-nitroperoxybenzoic acid, as sources of oxidizing equivalents (Urek & Pazarlioglu, 2005). The aromatic structures are depolymerized via formation of phenoxy or aryl cation radicals, which finally result in the breakdown of the molecule. (Rogalski, 2006). Manganese peroxidases are secreted by most WRF studied till today. Some WRF even secrete MnP as sole lignolytic enzyme for lignin degration. (Bermek *et al*., 2004; Asghar *et al*., 2008). MnP have the capability to oxidizes a range of substrates, including phenolic organic compounds and high molecular weight lignin model compounds (Bermek *et al.,* 2004).

Laccases are dimeric or tetrameric N-glycosylated extracellular blue multicopper oxidases (Wells *et al*., 2006) with molecular masses varying from 58-90 kDa (Murugesan *et al*., 2006; Salony *et al*., 2006; Zouari-Mechichi *et al*., 2006; Quaratino *et al*., 2007) that contain four copper atoms distributed in redox sites and have advantage that they do not need H_2O_2 for substrate oxidation and have broader spectrum than peroxidases (Mishra and Kumar, 2007). Laccases oxidize mediator compounds such as N-hydroxyacetanilide (NHA), N-(4-cyanophenyl)acetohydroxamic acid (NCPA), 3-hydroxyanthranilate, syringaldehyde, ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonate), 2,6-dimethoxyphenol (DMP), violuric acid, 1-hydroxybenzotriazole (HBT), 2,2,6,6-tetramethylpipperidin-N-oxide radical, acetovanillone (AV), acetosyringone and acetohydroxamic acid (Geng *et al*., 2004; Cho *et al*., 2006; Lu *et al*., 2007; Minussi *et al.*, 2007; Quaratino *et al*., 2007) that in turn degrade dyes and a wide variety of organic and inorganic substrates, including mono-, di- and polyphenols, aminophenols, methoxyphenols, aromatic amines and organic polluntants with concomitant four electron reduction of oxygen to water (Rodriguez *et al*., 1999; Abadulla *et al*., 2000; Podgornik *et al*., 2001; D'Souza *et al*., 2006; Asghar *et al*., 2008). Laccases have gained the status of industrially relevant enzymes because of a number of diverse applications, such as delignification of lignocellulosics, cross-linking of polysaccharides, bioremediation, food technological uses, personal and medical care applications, and for biosensor and analytical applications. To utilize laccases more efficiently for these biotechnological and environmental applications and to better understand the properties of these important enzymes at a molecular and kinetic level, rather large amounts of crude and purified laccases are required (Gianfreda *et al.,* 1999; Claus, 2003; Baldrian, 2006).

The carbon sources in the medium play an important role in ligninolytic enzyme production. Mansur, (1997) showed that the use of fructose instead of glucose resulted in a 100-fold increase in the specific Lac activity of Basidiomycetes. According to Mishra and Kumar (2007), the ligninolytic enzymes are produced during the secondary metabolism under conditions of limited nitrogen. However, in *Pleurotus ostreatus* a high concentration of nitrogen in the medium (34 mm, N as glutamate) did not repress but rather slightly stimulated mineralization of lignin compared to the N-limited medium.

Lignocellulosic agroindustrial and forestry wastes and residues like wheat straw, corncobs, corn stover, sugarcane bagasse, rice straw, banana stalks, whole plants and plant

parts are efficient substrates for growing WRF for the production of industrially important lignolytic and cellulolytic enzymes (Arora and Gill, 2001; Reddy *et al*., 2003; Stajic *et al*., 2006). Among processes used for enzyme production, solid-state fermentation (SSF) using waste residues is an attractive option because it presents many advantages, especially for fungal cultivations (Pointing, 2001). SSF presents higher productivity, simpler operation, and lower cost when compared with submerged culture (SmF). In addition, SSF reproduces the conditions under which the WRF grow in nature. This type of processe has been shown to be particularly suitable for the production of industrial enzymes. Since WRF grow under solid state conditions, it can be advantageous to utilize these fungi to develop bioprocesses using lignocellulosic agricultural solid wastes as substrates in SSF. Encouraging results have been reported by growing *P. chrysosporium* and other white rot fungi in SSF (Stajic *et al*., 2006), suggesting that ligninocellulosic substrates act as inducer substrates for the production of the ligninolytic peroxidases.

WRF have the capability to degrade a wide range of xenobiotics and organic pollutant including synthetic textile dyes (Pointing, 2001; Wesenberg *et al.,* 2003) due to their extracellular non-specific ligninolytic enzyme system (Christian *et al.,* 2005). Ligninolytic fungi have been used for decolorization of a variety of textile dyes and industrial pollutants due to their capability to produce extracellualar and non-specific ligninolytic peroxidases and laccases that are very efficient oxidizers of organic pollutants and recalcitrant compounds (Cenek *et al*., 2004). The ligninolytic enzymes from white rot fungi can be directly used for bioremediation of textile industry effluents, biopolishing of cotton fabrics and as stone wash agents for biostoning of denims.

Schyzophyllum commune is probably the most widespread fungus found in every continent except Antarctica, where there is no wood to be used as a substrate. Unlike other mushroom species, the mycelium only has to produce one set of fruiting bodies per year, which can then dry out and rehydrate and keep functioning. It is an efficient wood decaying fungus that causes white rot of soft woods. Researchers have focused for the last few years on production of ligninase enzymes responsible for wood decay by the fungus through fermentation (Asghar *et al*., 2008;Travis and Anderson, 2004). The objective of this work was to quantify and characterize the extracellular peroxidase enzymes of an indigenous strain *Schyzophyllum commune* IBL-06 to determine whether the enzymatic complex produced by

the fungus could be utilized in industrial processes.

Aims and objectives:

1. Production of ligninolytic enzymes by the *Schyzophyllum commune* IBL-06 using lignocellulosic substrates
2. Optimazation of SSF process for maximum production of ligninases
3. Purification and characterization of ligninolytic enzymes produced under optimum conditions
4. Characterization of ligninolytic enzymes enzymes through kinetic studies
5. Application of ligninase extract for decolorization of textile dyes and industrial effluents

CHAPTE 2

REVIEW OF LITERATURE

2.1 Production of Ligninases

There are three major ligninolytic enzymes including LiP, MnP and laccase produced by white rot fungi (WRF) that are being evaluated for their applications in industrial and environmental biotechnology (Buchanan *et al*., 2004). Improvements of the production of these enzymes such as discovery of new fungal strains, modification of growth conditions, use of inducers and cheaper growth substrates such as agricultural and food wastes has been extensively investigated in the last two decades. In the very beginning *P. chrysosporium* was found to produce higher amounts of LiP in nutrient limited cultures to break down biopolymeric lignin (Higson, 1991) in the presence of hydrogen peroxide. The production processes for ligninase using many different strains and species of WRF have been optimized in flasks and reactors by the addition of carbon sources, nitrogen additives, mediators, surfactants and minerals to the medium. Nutritional variables including carbon, nitrogen and manganese significantly influence the production of ligninolytic enzymes by white rot fungi (Pascal *et al*., 1991).

Lignin peroxidase (LiP) and manganese peroxidase (MnP) synthesis by *Schizophyllum commune, Phanerochaete chrysosporium* and *Phanerochaete sordida* is inhibited by high nitrogen and carbon contents but laccase production by most WRF is higher in high-nitrogen (24 mM) cultures as compared to those in low-nitrogen (2.4 mM) (Boyle *et al.*, 1992; Ruytimann-Johnson, 1994; Trevor, 1999). The production of MnP is enhanced by the addition of manganese in lower concentrations (0.1-1mM MnSO4) as Mn is a specific inducer of MnP production and repressor of LiP synthesis by *P. chrysosporium* (Rajan *et al* (2010). In molasses and bagasse media (Ferrara *et al*., 2002) addition of glucose, xylose, and xylan promoted cell growth and Veratryl alcohol enhanced LiP formation (28 U/L to 155 U/L) by *P. chrysosporium*. MnP and LiP were produced in the medium containing wheat straw and hemp woody core under carbon and nitrogen limiting conditions by *Phanerocheaete chrysosporium* (Kapich *et al*., 2004), suggesting that some compounds derived from these lignucellulosic substrates act as inducers for ligninolytic peroxidases. High amounts of organic nitrogen source enhanced the production of ligninolytic enzymes

where as surfactant Tween-80 did not affect the enzyme production. However, Raziye and Nurdan (2007) and Urek and Pzarlioglu, (2007) have reported that Addition of Tween 80 (0.05 %, v/v) and Mn^{2+} resulted in two times increase in MnP production (356 U/L) by *P. chrysosporium* as compared to control. Addition of low concentrations of peptone, yeast extract and Tween-80 in SSF using corncob substrate (70% moisture) also enhanced LiP production by *P. chrysosporium* (Asghar *et al*., 2006) to a maximum of 13.7 U/gds in 5 days with 20% inoculum. *P. chrysosporium* and *Phanerochaete* sp. were grown on lignocellulosic biomass from arecanut husk (Arecanut & catechu Linnaeus) in SSF carried out at different pH values (Rajan *et al* 2010). The optimum enzyme production was at pH 6.0 (52.60 IU/g) for *P. chrysosporium* and pH 5.0 (44.08 IU/g) for *Phanerochaete* sp. where as optimum temperature was 30 ± 2°C for both strains. Absence of carbon and nitrogen stimulated enzyme production in *P. chrysosporium* while Phanerochaete sp. needed additional nitrogen.

The MnP production by *G. lucidum* was 2.48 U/mg but laccase activity was very low (0.03 U/mg of protein) on oak saw dust substrate supplemented with wheat bran, millet and sucrose (Ming *et al*., 1993). Ligninase production by *G. lucidum* using pine or poplar as carbon sources has also been reported (Trevor *et al*., 1999). Cultures containing both pine and poplar together showed 5- to 10-fold-higher levels of laccase than cultures containing pine or poplar alone. LiP, MnP and laccase were produced in liquid culture by four strains of *Ganoderma spp* (Celia *et al*., 2004) in the presence of Ramazol Brilliant Blue R at 30°C with addition of wheat bran (4.5g) and propanil (2.5цg/mL). Addition of wheat bran enhanced production of ligninases, while the herbicide was found to inhibit the production of enzymes by the two best producer strains. The two *Ganoderma spp*. Inspite of having similar protein secretion patterns and biomass yields showed differences in enzymatic activities depending on the cultural conditions.

The variability in ligninases production by *Pleurotus* sp. has been reported due to genetic variations as well as variation of inoculum size, substrate to water ratio and the time of incubation. *Pleurotus sp* produced maximum MnP activities while laccase activity was about ten times lower in liquid medium and on solid straw substrate (Lang *et al*., 1996). The MnP production was optimum in SSF cultures during in 20-40 days but laccase activity was higher in LSF. The presence of complex polysaccharides containing substrates (olive mill wastewater (OMW) and wheat straw) favored the production of ligninases, celluases and

hemicellulase production by *Pleurotus sajor-caju* (Massadeh *et al*., 2010). Addition of rapidly utilized simple carbon sources enhanced biomass formation with low levels of enzymes production where as complex carbon sources enhanced both cell growth rate as well as enzymes induction in 2-14 days. The production of ligninases by *P. ostreatus* was 80-100% and it decreased to 30% by increasing incubation time and laccase activity was lower in SSF on wheat straw as compared to LSF (Baldrian and Gabriel, 2002).

Pleurotus eryngii, *P. ostreatus* and *P. Pulmonarius* were found to produce lininases in submerged fermentation (SF) and solid-state fermentation (SSF) using dry ground mandarine peels and and grapevine sawdust (Stajic *et al*., 2006). The highest levels of Lac were secreted by *P. eryngii*, under SF conditions and higher levels of LiP and MnP were produced by *P. ostreatus* and *P. pulmonarius*, under SSF conditions of grapevine sawdust. Whereas in SF, the activities of MnP and LiP from *P. ostreatus* and *P. pulmonarius* cultures were either very low or absent. In the medium with the best carbon sources (mandarine peels and grapevine sawdust, respectively), both *P. eryngii* and *P. ostreatus* produced highest Lac activity with $(NH_4)_2SO_4$ as a nitrogen source at 20-30 mM nitrogen content, respectively. *P. ostreatus* and *P. pulmonarius* produced higher activities of LiP and MnP in the presence of 0.5% peptone and NH_4NO_3 with 30 mM nitrogen content, respectively. Comparative study on laccase production and the degradation of polycyclic aromatic hydrocarbons (PAHs) by *Pseudotrametes gibbosa* and *Pleurotus ostreatus* showed that the laccase activity of *P. gibbosa* (2841.3 U/L) was 6 times higher than that of *P. ostreatus* grown under the same culture conditions. *P. gibbosa* had higher laccase production potential and was more potent for degradation of PAHs.

Pleurotus ostreatus and *P. sajor-caju* exhibited similar levels of ligninolytic and cellulolytic enzyme activities using banana wates in SSF (Reddy *et al*., 2003) and leaf biomass was more suitable substrate. *Pleurotus laciniatocrenatus* produced extracellular aryl-alcohol oxidase, laccase, MnP and manganese-independent peroxidase (MiP) activities under carbon limitation in a liquid medium supplemented with yeast extract (0.1%) and peptone (0.5%). Addition of 1mM/L vanillic acid and 150µM/L CuSO4 as mediators increased laccase production to 4 and 68.3-folds, respevtively (Saparrata and Guillen, 2005). The nature lignocellulolytic material and the method of fungi cultivation are among the major factors determining the expression as well as the ratio of individual enzymes in

ligninase complex. Comparison of *Lentis edodes* and *Pleurotus spp* showed that *Pleurotus spp* gave higher yields of CMCase (62.3U/mL), xylanase (84.1 U/mL) and SSF of tree leaves has been found to be more favorable as compared to liquid cultures for laccase and MnP secretion by most of the *L. edodes* and *Pleurotus strains* (Elisashvili *et al*., 2006). Comparative study on laccase production and the degradation of polycyclic aromatic hydrocarbons (PAHs) by *Pseudotrametes gibbosa* and *Pleurotus ostreatus* showed that the laccase activity of *P. gibbosa* (2841.3 U/L) was 6 times higher than that of *P. ostreatus* grown under the same culture conditions. *P. gibbosa* had higher laccase production potential and was more potent for degradation of PAHs.

Different strains of *Trametes spp., Trametes versicolor, T. cingulata, T. elegans* and *T. pocas* have been found to produce LiP, MnP and laccase in different ratios (Tekere *et al*., 2001). The ligninase production by *Trametes versicolor* is affected by C, N and Mn^{2+} concentration in the medium. Higher laccase activities were expressed in the media containing high carbon and low nitrogen contents but high nitrogen content of the medium favors high MnP production. Activators, such as ethanol, veratryl alcohol, and melanin enhanced laccase production by *Trametes versicolor* (Maceiras *et al*., 2001). Veratryl alcohol (VA) caused a two fold increase in laccsae production but MnP production was not affected by the addition of this alcohol. Laccse production by *F. Trogii* and *T. versicolor* was stimulated in copper-supplemented agitated and static batch cultures (Birhanli and Yesilada, 2006) to 40.29±1.97 and 12.09±0.72 U/mL, respectively. Seven times recycled pellets of *F. trogii* supplemented with copper gave higher cumulative laccase activity (223U/mL) as compared to *T. versicolor* pellets (59 U/mL), suggesting the suitability of repeated-batch method for long-term laccase production.

In *Lentinus squarrosulus* the there was a two fold increase in mycelial extension and ligninase production by the addition of 20 to 80 mM Mn^{2+} and Ca^{2+} ions to the lignocellulose waste in 6 days where as Mg^{2+} and K^{+} had non-significant effect (Wuyep *et al*., 2003). The phenomena of mycelia extension and enhanced ligninase synthesis by the addition of Mn^{2+} and Ca^{2+} is very important in biotechnological applications of *Lentinus squarrosulus* in paper and pulp, textile, tanning and oil industries. *P. radiate* secreted secreted LiP, MnP as major enzymes along with lower levels of laccase. The production of LiP was enhanced by adding veratryl alcohol and by supplementing the pulp waste effluent medium with wheat

and rye bran. *Lentinula edodes* produced maxiumum MnP in SSF medium of corn cobs in 12 days of incubation at 30°C that was substantially stimulated with the addition of with 0.5% glucose and 5mM $MnSO_4$ under optimized conditions in corncob (Boer *et al*., 2006).

An extracellular laccase with novel characteristics was produced *Termitomyces clypeatus* in submerged medium containing glucose or cellulose as the carbon source (Bose *et al*., 2007). Rogalski *et al*., (2006) obtained high yields of MnP (2304 nkat/L) in 8 days using immobilized polyurethane foam mycelium of *N. frowardii* in a low nitrogen medium using 1.36 mM nitrogen in the form of ammonium tartrate and 16 g/L glucose as carbon source with 65:1 C/N ratio and 2 mM Mn^{2+}. The enzyme productivity by using immobilized mycelia of *N. frowardii* was 1.4 times higher than with free fungus and reusable immobilized mycelium could be used in three subsequent 10 day batches with no loss of MnP activity, suggesting its potential for biotechnological applications.

High enzyme production along with the very low cost of the substrates Lignocellulosic substrates like soy and wheat bran showed the suitability of the system of *F. sclerodermeus* for industrial purposes (Papinutti and Forchiassin, 2007). Maximum MnP was produced in SSF by *F. sclerodermeus* in 15 days (14.5U g/L) and laccase peaked at 28th day of incubation (520U g/L). Recently, a newly isolated strain L-25 was found as efficient of MnP producer in potato-processing wastewater mediumn supplemented with glucose (Fujihara *et al*., 2010). The presence of glucose and initial pH affected the cell growth and MnP production. The production of MnP in potato-processing wastewater was 2.5 fold higher as compared to that in basal nutrient medium. Amino acids L-serine, L-Glutamic acid and L-aspartic acid enhanced MnP secretion, whereas L-leucine, L-phenylalanine, L-lysine and L-tyrosine caused repression of MnP induction.

2.2 Purification and Characterization of Ligninases

White rot fungi secret a variety of hydrolytic and oxidative enzymes in liquid and solid state cultures for degradation of lignocellulosic materials (Stato *et al*., 2007). In the last decade, the ligninolytic enzymes produced by different WRF have been purified and characterized by many researchers around the world for determining their suitability for applications in industrial processes. Three manganese peroxidas isozymes having almost same 45 kDa molecular mass were isolated from *P. sorbida* (Ruytimann-Johnson, 1994). The

isoelectric pH for the three isozymes were 5.3, 4.2, and 3.3 for MnPI, MnPII, and MnPIII and these were active in 3.0-6.0 pH range with optimum pHs between 4.5-5.0. The major isoenzyme, MnP2 of produced by *Lentinula edodes* in corncob SSF medium was purified to 6.76-fold with a yield of 26.6% through ultrafiltration, acetone precipitation and gel filtration (Boer *et al*., 2006). The enzyme was 44 kDa molecular mass glycoprotein with 17.8% carbohydrates moiety having optimum pH 4.5 and 40^{o}C temperature. The enzyme was stable in acidic pH range (pH 4.5–6.0) and 45^{o}C temperature. The K_m values for H_2O_2 and Mn^{2+} at pH 4.5 were 20.8 and 22.2-10.3mM, respectively and was more stable in the presence of high H_2O_2 concentrations as compared to many other MnPs.

MnP from *Aspergillus terreus* LD-1 (Kanayama *et al*., 2002) was purified to 13.1 fold and it was a monomeric protein displaying one 43 kDa band of on SDS-PAGE. The enzyme showed pH and temperature optima of 12.5 and 37 ^{o}C, respectively and it was stable in the pH range of 11.0-12.5 at 40^{o}C temperature. Using different concentrations of Mn^{2+}, 2, 6-dimethoxyphenol and H_2O_2 the Km values were 33M, 20 M and 320 M, respectively. The enzyme was completely inhibited by Hg^{+2}, Pb^{+2}, EDTA, Ag^{+2} and lactate but was activated by malate, fumarate and oxalate. A manganese peroxidase purified from culture extracts of *Trichophyton rubrum LSK-27* molecular mass of 42 kDa by SDS–PAGE (Bermek *et al*., 2004). The enzyme had very high homology to MnP and LiP from *Bjerkandera sp.* as shown by mass spectrometric analysis. *Bjerkandera sp.* MnP was more stable at high temperatures in the presence of high concentrations of H_2O_2 as compared to MnP from *Trichophyton rubrum* LSK-27 and it was able to oxidize veratryl alcohol. The MnP from *P. Chrysosporium* immobilized on polystyrene foam was purified with 23.08% yield using ethanol, methanol, glycol and ammonium sulphate and DEAE-Sepharose ion exchange chromatography, followed by gel filtration (Urek and Pzarlioglo, 2007). The purified MnP was 45 kDa molecular mass monomeric protein revealing a single band on SDS-PAGE. The temperature and pH optima for MnP were 30^{o}C and 4.5, respectively and it was stable in the pH range 4.5–6.0 at 25^{o}C and 35^{o}C at pH 4.5 for 1 h. The Km values of MnP for 2.6-dimetoxyphenol (DMP) and H_2O_2 were 28.57 and 71.4μM at pH 4.5 and the enzyme activity inhibited was noted in the presence of 2mM ascorbic acid, NaN_3, dithreitol and mercaptoethanol.

Two extracellular laccase isozymes isolated and purified from culture filtrates of *Ganoderma lucidum* showed 40 and 50kDa bands on SDS-PAGE but isoelectric focusing

revealed the presence of five major laccase isozymes with pIs of 3.0, 4.25, 4.5, 4.8, and 5.1. Another strain of *G. Lucidum* secreted three laccase isozymes (Kao *et al*., 2001) that were isolated and purified through anion exchange chromatography to 32.4 folds. The laccases were 65-68 kDa glycoproteins containing 7-10% N-linked carbohydrates. The major isozyme had optimum pH 3.5 and 20°C temperature and it was fairly stable at pH 4-10 and at temperatures lower than 40°C. The Км and V_{max} values for the isozyme were 3.7 цМ and 142 U/mL, respectively for ABTS. Two laccase isozymes with molecular masses of 55 and 86 kDa with optimum temperature 62°C were isolated and purified from another strain of G. lucidum. The major laccase was stable at 80°C and pH 4.5 and it retained almost 100% activity in 7 days and it was strongly inhibited by sodium azide and fluoride. Laccase isolated from *Sclerotium rolfsii* was a single enzyme active in the acidic pH range, showing an optimum activity at pH 2.4, using ABTS as substrate (Mansur *et al*., 2003). The ferulic acid induced laccase produced by *Marasmius quercophilus* was also a single monomeric protein that exhibited high thermal stability at 30-40°C after incubation of 24 h (Anne-Marie *et al*., 2004).The enzyme was not inhibited by EDTA, cystein or SDS and was able to polymerize 2-chlorophenol, 2, 4-dichlorophenol (DCP) and 2, 4, 6-trichlorophenol (TCP) without ABTS or HBT mediators.

Lignin peroxidase YK-LiP2 isolated from shaking cultures of *Phanerochaete sordida* (Hirai *et al*., 2005) was purified to homogeneity by anion-exchange, followed by gel filtration chromatography. The molecular mass of LiP was approximately 45 kDa, and its absorption spectrum was almost similar to that of *P. chrysosporium* LiP. In the steady-state kinetics Veratryl alcohol (VA) oxidation revealed an ordered bi-bi ping-pong mechanism. The enzyme was stable and more effective in the presence of high H_2O_2 concentrations (>2.5 mM) during breakdown of dimeric lignin model compounds. An other LiP produced by *Phanerochaete chrysosporium* in SSF medium of corncobs was purified by ammonium sulphate precipitation and ion-exchange FPLC (Asghar *et al*., 2006). The molecular mass of purified LiP, estimated by SDS-PAGE was 38 kDa. LiP displayed optimum activity at pH 4 and 40°C temperature and immobilization of the enzyme hydrophobioc gels of different hydrophobicity caused hyperactivation and thermostablization. Using varying concentrations of VA as substrate the Michaelis constant K_M for immobilized LiP decreased and Vmax increased for immobilized enzyme as compared to free enzyme. Lignin peroxidase produced

by *P. chrysosporium* under optimum conditions was purified by 60% $(NH_4)_2SO_4$ saturation, followed by Q FF ion exchange and Sepharyl S-300 HR gel filtration chromatography. Two fractions with LiP activity, LiP1 and LiP2 were identified using desalting column and purified by 9.6- and 7.6-fold with a yield of 22.9% and 18.6%, respectively. The isozymes gave two bands on SDS-PAGE corresponding to 38 kDa and 40 kDa molecular weight proteins (Wang *et al*., 2008).

Lignin peroxidase produced by *Pleurotus sajor caju* MTCC–141 in liquid culture growth medium with bagasse was purified (Yadav *et al*., 2009). Two isozymes of LiP with molecular masses of 38 and 40 kDa were identified. The Km values of 40kDa isozyme for veratryl alcohol, n-propanol, and H_2O_2 were found to be 57 M, 500 M, and 80 M, respectively. Optimum pH and temperature for LiP were found to be 3 and 30^oC, respectively and the isozymes were found to be inhibited by sodium azide.

Two laccase isozymes of 55 and 86 kDa molecular masses were isolated and purified from *Sclerotium rolfsii* (Ryan *et al*., 2003). The isozymes were optimally active at acidic pH 2.4 and 5.2 with ABTS as substrate. The optimum temperature for major isozyme was 62^oC. The laccase was stable at 80^oC and pH 4.5, and it retained almost 100% activity after 7 days incubation. Sodium azide and fluoride were the strong inhibitors of laccse.

2.3 Decolorization of dyes and industrial effluents

Different dyes and pigments are being in the textile, paper, plastic and other industries are not completely used in industrial processes due to inefficiencies of wet/exhaust dying processes and are ultimately released into the industrial wastewaters (Levin *et al*., 2005) and considered to be very toxic and carcinogenic persistent pollutants (Maas & Chaudhari, 2005; Salony *et al*., 2006; Revankar & Lele, 2007). There is extensive contamination of the water streams by unused dyes and dye intermediates originating from textile industries in the form of industrial effluents (Asgher *et al.,* 2009). It is therefore, imperative to develop cost effective and efficient methods for the removal of these toxic pollutants from industrial waste waters. Bioremediation is an attractive alternative option to the costly and inefficient conventional physical and chemical methods being used to remediate the industrial effluents (Watanabe, 2001).

During the last two decades, WRF have attracted the attention of researchers due to the capability of these fungi to degrade a wide range of xenobiotics and organic pollutant including synthetic dyes (Pointing, 2001; Wesenberg *et al.,* 2003) due to their extracellular non-specific LME system (Christian *et al.,* 2005). In most of the initial dye decolorization studies *Phanerochaete chrysosporium*, *Trametes versicolor* and *Pleurotus ostreatus* were used (Toh *et al*., 2003). However, in recent years the research progressed and other fungi like *Phellinus gilvus, Pycnoporus sanguineus*, *Dichomitus squalens, Irpex flavus, Daedalea flavida, Polyporus sanguineus*, *Funalia trogii* ATCC 200800, *Ischnoderma resinosum, Ganoderma* sp. WR-1 and *Ganoderma lucidum* have been found to be more efficient (Asgher *et al*., 2008). Ligninolytic fungi have been used for decolorization of a variety of textile dyes and industrial pollutants due to their capability to produce extracellualar and non-specific ligninolytic peroxidases and laccases that are very efficient oxidizers of organic pollutants and recalcitrant compounds (Cenek *et al*., 2004). Laccasses oxidize phenolic compounds creating phenoxy radicals with high oxidation reduction potential in the presence of low molecular weight mediators and non-phenolic compounds are oxidized by LiP and MnP via cation radical mechanisms (Youn *et al*., (1995). Low molecular mass redox mediators like ABTS are necessary for laccase-catalyzed decolorization of most of the dyes (Lu *et al*., 2005; Lu *et al*., 2007). However, *Pleurotus pulmonarius and Lentinula edodes* SR-1 could decolorize dyes of different structures by secreting only exracellular laccase without the addition of mediators (Nagai *et al*., 2002). LiP produced by *Trametes versicolor* could also decolorize Remazol Brilliant Blue R (RBBR) in the in the absence of its mediator VA (Christian *et al*., 2005). The decolorization ability of WRF has been found to be substantially enhanced optimizing the physical and nutritional parameters and operational conditions like initial dye concentration, inoculum age and C: N ratio (Ozsoy *et al.,* 2005; Nilsson *et al*., 2006; Sanghi *et al.,* 2006; Asgher *et al*., 2009).

The most important aspect for industrial applications of WRF is that different WRF strains produce different profiles and patterns of ligninolytic enzymes and the expression of enzymes depends on genetic make up of WRF cultures as well as chemical structure and functional groups of the dyes present in the effluent (Boer *et al*., 2004; Kariminiaae-Hamedaani *et al.,* 2007). *Phanerochaete chrysosporium, Coriolus versicolor, Ganoderma lucidum* and *Pleurotus ostreatus* isolated in Pakistan showed highly variable decolorization

efficiency on different reactive (Asgher *et al*., 2006) and Vat textile dyes (Asgher *et al.* 2008a). In case of reactive dyes, *P. chrysosporium and C. versicolor* had good decolorization efficiency for Remazol Brilliant Yellow 3GL, Procion BluePX-5R and Cibacron Blue P-3RGR. Drimarene Orange K-GL was completely decolorized by *P.chrysosporium* and *P. ostreatus* showed good efficiencies on all dyes except Remazol Brilliant Yellow where as *G. lucidum* decolorized only Remazol Brilliant Yellow. In case of Vat dyes, *C. versicolor* IBL-04 decolorized all vat dyes at varying incubation times and best decolorization was observed for Cibanon blue GFJ-MD (90.7%) in 7 days. *P. chrysosporium* also had good decolorization efficiency for all vat dyes. However, *Ganoderma lucidum* and *Pleurotus ostreatus* showed poor decolorization potential for all vat dyes.

The four acclimated mixed microbial cultures collected from basins of waste water outlets of different textile industries of Faisalabad Pakistan also diplayed variable decolorization of Drimarene Orange K-GL, Drimarene Brilliant Red K-4BL, Foron Yellow SE4G and Foron Blue RDGLN for 10 days (Asgher *et al*., (2007). The mixed culture collected from Crescent Textile industry (CRT) showed best decolorization of the four dyestuffs followed CT could completely decolorize all dyes within 3–5 days. NF culture showed 100% decolorization of Foron Yellow SE4G and Foron Blue RDGLN in 5 and 7 days, respectively. The mixeed culture from CF caused complete color removal of Foron Blue RDGLN and Drimarene Brilliant Red K-4BL in 4 and 8 days respectvely. Out of 26 WRF used for degradation of Malachite Green, Anthraquinone Blue, Congo Red and Xylidine only 10 strains decolorized all the dyes and produced laccase, LiP and MnP Levin *et al*., 2004). Six strains did not produce LiP and could not decolorize any of the dyes. *F. antarcticus* laccase activity was 0.13 U/mL, but neither LiP nor MnP were detected.

Phanerochaete chrysosporium immobilized on $ZrOCl_2$-activated pumice decolorized Direct Blue 15 dye and followed the first-order kinetics with respect to initial dye concentration. MnP catalyzed decolorization was the major mechanism and mycelial adsorption was the minor mechanism (Pazarlioglu *et al.,* 2005). MnP mediated decolorization of Direct Blue 15, Direct Green 6, and Congo red by *P. chrysosporium was enhanced* by adding Tween-80 (Urek & Pazarlioglu, 2005) or copper (Tychanowicz *et al*., 2006) to the decolorization medium. *Phanerochaete chrysosporium*, immobilized on calcium alginate biogel beads also (Zahmatkesh *et al.,* 2010) produced maximum MnP activity of 96 U/L

produced in 7 days caused 70% decolorization after 6 hour of dye addition. The immobilization of the culture and agitation increased the efficiency of *P. chrysosporium* for dye decolorization.

Crude enzyme enzyme extract produced during SSF of wheat bran by *Ganoderma lucidum* contained laccase as the dominant ligninoolytic enzyme activity (Murugesan *et al*., (2007) and the extracellular enzyme extract showed excellent decolorization capability for anthraquinone dye Remazol Brilliant Blue R and diazo dye Remazol Black-5 that could be further enhanced by the addition of HBT as mediator. RBBR ($50mgl^{-1}$) was maximally decolorized by 92% in presence HBT as mediator within 2 h. at $60^{o}C$ and pH 4.0. The laccase inhibitor sodium azide (0.5mM) completely inhibited decolorization. The laccse was highly thermostability and efficient decolorizer of dyes, suggesting that the enzyme can be effectively used to decolorize the industrial effluents. However *G. lucidum* IBL-05 produced MnP as the dominant enzyme involved along with minor activities of LiP and laccse for maximum decolourization (58.5%) of Cibacron Turquoise P-GR dye in 7 days (Hafiz *et al,* 2008) that was found to be substantially improved by optimizing medium composition, pH, and temperature. The type of carbon and nitrogen sources played a significant role in MnP synthesis by the fungus and dye removal and 85% decolorization was observed after 3 days when the medium was supplemented with molasses (0.1%) as a carbon source. MnP was also the major enzyme (256U/mL) secreted by *G. lucidum* IBL-05 during decolorization of Solar golden yellow R (Bibi *et al.,* (2009). Addition of starch (1%) enhanced fungal growth, MnP production and dye decolourisation to 96% whereas, addition of nitrogen sources inhibited dye decolourisation and enzyme formation. In another recent study MnP (1295 U/mL) was the main enzyme activity synthesized by *G. lucidum* IBL-05 for bioremediation practical textile industry effluents (Asgher *et al.,* 2010). The decolorization (49.5 %) of Arzoo textile industry (ART) effluent was improve to 95% with maxiumum MnP activity (1295 U/mL) using 1% starch as carbon supplement to the Kirk's medium at pH 3 and 35°C. MnP formation and effluent decolorization was inhibited by additional nitrogen.

The newly isolated *Schizophyllum commune* IBL-06 and *Ganoderma lucidum* IBL-05 showed different decolorization efficiencies for Solar golden yellow R direct textile dye. (Asgher *et al,* 2008b). Addition of glucose (1%) caused a dramatic increase in decolorization by *S. commune* IBL-06 and complete (100%) dye color was removed in 2 days. The

additional nitrogen sources showed an inhibitory effect MnP synthesis that was found major enzyme (764 U/ml) secreted by *S. commune* IBL-06 for Solar golden yellow R degradation. The major peroxidase involved in decolorization of Cibacron Red FN-2BL by *Schizophyllum commune* IBL-6 in modified Kik's medium MII was also MnP along with lower LiP and laccase activities (Bhatti *et al.,* 2008). 0.1% Cibacron Red FN-2BL at 0.1% concentration was enhanced with 1% glucose addition after 3 days of incubation at pH 4.5 and 30°C, whereas most of the nitrogen additives had inhibitory effects on enzyme synthesis and dye removal.

The process parameters like pH, temperature, dye concentration and additional carbon and nitrogen sources were found to have significant influence on ligninase production and decolorization of Cibanon blue GFJ-MD by *Coriolus versicolor* IBL-04 (Asgher *et al* (2008a). Maximum (98.5%) decolorization Cibanon blue GFJ-MD (0.01%) was achieved in 3 days at pH 5 and 30 ^{o}C temperature in the Kik's medium receiving 1% starch as carbon source. The supplementary nitrogen inhibited laccase activity as well as dye decolorization. Adsorption of the dye on fungal mycelia was negligible, suggesting that and laccase catalyzed oxidation was the major decolorization mechanism. *Coriolus versicolor* IBL-04 showed best decolorization results (36.3%) for Arzoo Textile Industry (ART) effluent in 6 days out of the four effluents from different textile units of Faisalabad, Pakistan (Asgher *et al.,* 2009). MnP (486U/mL) was the main enzyme present in the culture filtrates and LiP and laccse were undetectable. The synthesis of MnP and ART effluent decolorization could be enhanced by using 1% starch as carbon source. The presence of copper in the medium was also found to influence the production and activities of laccase, MnP and glyoxal oxidase produced by *Trametes trogii* for decolorization of Ponceau 2R, malachite green and anthraquinone blue (Levin *et al*., 2005). The laccase activity was more important in xylidine degradation and MnP activity was more important factor in malachite green decolorization.

Pleurotus ostreatus IBL-02 produced lacasse (460 U/mL) as major enzyme, followed by MnP (443 U/mL) and LiP (157 U/mL) to decolorize Drimarene Blue K2RL (0.01%) in r 5 days. The decolorization was enhanced to 97.86% in 3 days (Kanwal *et al.,* (2010) by optimizing pH and temperature and with the addition of glucose (1%) as carbon source and ammonium sulphate (1%) as nitrogen source. The purification of laccase from *P. eryngii* revealed two peaks where as three peaks was observed for both *P. ostreatus* and *P.*

pulmonarius laccses. The phenol red oxidation by purified laccses was inhibited by Mn^{2+} ions (Stajic *et al*., 2006).

Agaricus bitorqus A66 was found to decolorize Novasol direct dyes Direct Black, Direct Blue GL, Direct Red 2BL and Direct Flavin to different extents (Bibi *et al*., 2010) but showed best performance (77.42% color removal) on Direct Black. By optimizing physical and nutrional parameters the maxiumum dye decolorization increased from 77 in 6 days to 99.68% in only 5 that was strongly correlated with high LiP activity. Varatryl alcohol substantially enhanced LiP synthesis and dye decolorization where as $MnSO_4$, ABTS and Guiacol had non-significant influence on the process.

Stationary cultures of a highly degradative strain *Irpex lacteus* exhibited 380-fold and 2-fold increase in production of MnP and laccase as compared to submerged cultures. Surfactant Tween 80 increased MnP levels to 260-fold that were correlated to efficient decolorization of Reactive Orange azo dye. The decolorization of anthraquinone dye Remazol Brilliant Blue R was not correlated to MnP activity. (Novotny *et al*., 2004). Decolorization of Reactive Blue 19 (RBBR) and Reactive Black 5 (RB5) by *Datronia sp. KAPI0039* was influenced by dye concentration, fungal inoculum size and pH (Vaithanomsat *et al*., 2010). Optimal decolorization of 1,000mg $L^{-}1$ RBBR and RB5 was achieved using 2% (w/v) inoculum at pH5 and it was correlated to high laccase activity (759.81UL−1) whereas the activity of MnP and LiP were not detected.

2.4 Applications of WRF and ligninases in Industries

Ligninases find wide range of commercial applications in oxidation of dyes, bioremediation of industrial effluents, degradation of lignin and lignosulphonates, drug analysis, ethanol production from lignocellulosic biomass, clarification of musts and wines, to improve the whiteness in a conventional bleaching of cotton (Mayer and Staples 2002; Rodriguez Couto *et al*., 2002; Lorenzo *et al*., 2002; Sigoillot *et al*., 2004) and biostoning of denims (Pazarlioglu *et al*., 2005). Agricultural wastes possess a high economic potential for paper and pulp industries. One of the major limitations to its use is the presence of lipophilic low molar-mass constituents in wood chips that result in low permeability and tear index of pulp, pitch deposition and effluent toxicity (Roncero *et al*., 2003; Van-beek *et al*., 2007). New biotechnological solutions such as fungal pre-treatment of wood chips can reduce pitch and

paper quality problems. WRF are not only capable of producing lignin-degrading enzymes, but are also able to penetrate the substrate to transport these enzymes into materials such as wood chips by hyphal extension (Messner and Srebotnik, 1994). One of the most important industrial uses of WRF and their oxidative enzymes is in biobleaching and biopulping in the pulp and paper industry to replace eco-unfriendly toxic chlorinated chemicals to save on mechanical pulping energy costs (Hakala *et al*., 2005). Additional benefits are obtained by removal of wood extractives with less pitch problems and less effluent toxicity.

The potential benefits of biochemical pulping by WRF include decreased lignin content of pulp, reduction of pulping time, reduced consumption of bleaching chemicals and improved tensile or bursting strength properties of pulps and paper strength properties of pulp (Bajpai *et al*., 2001; Selvam *et al*., 2006; Franco *et al.,* 2006; Mardones *et al*., 2006). Biopulping of wood chips by WRF prior to mechanical pulping also reduces the electrical energy requirements during refining and potentially increases mill throughput, (Kirk *et al*. 1994; Zhao *et al*., 2006; Maijala *et al*., 2008). An economic analysis (Zhao *et al*., 2006) of a 600 tons/day thermomechanical pulp (TMP) mill indicated that, based on energy savings alone, the process is economically feasible and results in an overall savings of about US$ 10 per ton. Increasing the mill throughput by 20% achieves addional savings of more than US$ 40 per ton of pulp. Replacement of TMP for kraft pulp results in additional savings indicating that biopulping is feasible from both on engineering and economic standpoint. The specific energy consumption in the refining of Scots pine and Norway spruce wood chips treated with *Physisporinus rivulosus* and MnP decreased about 11 and 6%, respectively with pulps refined to low freeness values (CSF 85-130 ml) with improved strength, light scattering and opacity properties (Maijala *et al*., 2008).

Ganoderma australe treated biopulps presented a selective pattern of biodelignification with lower kappa numbers, same strength and fibrillation degree and approximately the same screened pulp yield as compared to control (Franco *et al.,* 2006; Mendonc *et al.,* 2008). Biotreatment of wood chips from *Pinus radiata* and *Acacia dealbata* by *C. subvermispora* and *G. australe* gave pulp yield of 45-49% and 31-51%, respectively (Munoz *et al*., 2007) with higher glucan (93-95 %) and lower lignin content (4-6%) than control pulps (82% glucan and 13% lignin). *Eucalyptus nitens* wood chips pretreated with *C. subvermispora* for 15 days caused 13.3% lignin and 2% glucan degradation and the pulping

of biotreated samples required lower active alkali charge to reach the target kappa number, exhibited better pulping selectivity, enhanced pulp yield by 3% and 1.5% for the pulps of 22 and 16 kappa numbers, respectively and improved strength properties (Mardones *et al*., 2006). Where as biodegradation *of Eucalyptus grandis* wood pulps by *C. subvermispora* revealed 9% lignin loss (Asgher *et al.,* 2010; Ghodake *et al*., 2009). *C. subvermispora* (Pilát) also performed better than *Phanerochate chrysosporium* Burds, and *Trametes versicolor* (L.) on chips and pulps of *Acacia mangium* and *Eucalyptus camaldulensis* causing adecrease in Kappa number (28% and 25% for Acacia and Eucalyptus, respectively) without serious viscosity loss and the final pulp had improved brightness and strength properties (Asgher *et al*., 2008). Bleaching hardwood kraft pulp by *Phlebia* sp. MG-60 (Li *et al*., 2003) resulted in pulp brighteness 11 points and decreased kappa number by 6 points. *Pycnoporus sanguineus* was able to reduce lignin content of *Pinus taeda* wood chips to 11% in 14 days of treatment, and wood suffered notable structural changes of lignin and hemicelluloses with an increase of 15% in porosity of decayed wood confirming the physical changes due to fungal attack (Levin *et al*., 2007). Treatment of wheat straw pulp by *Streptomyces cyaneus* improves pulp brightness (Berrocal *et al*., 2000), had a positive effect on both the burst and tear indices of the pulps but a negative impact on tensile index (Berrocal *et al.,* 2004). Biobleaching and delignification of hard wood and kraft pulp (HWKP) by pretreatment with *F. lividus* and *T. versicolor* in shaking culture reduced the kappa number by 38.7 and 67.7% and increased pulp brightness by 29.8 and 29.9%, respectively (Selvam *et al*., 2006). Recently, the co-culturing of *C. subvermispora* and *P. ostreatus* (Chi *et al*., 2007) could significantly stimulate wood decay, and laccase and MnP activities when compared to monocultures.

A range of extracellular enzyme activities and low molecular mass mediators is involved in biopulping by different WRF. *C. subvermispora* lacks lignin peroxidase (LiP) activity but produces laccase and MnP isoenzymes (Souza-Cruz *et al*. 2004; Mendonc *et al*., 2008). Where as LiP appears to be the key lignin-degrading enzyme in *P. chrysosporium* (Zabell and Morrell, 1992). *P. chrysosporium* is widely used in biopulping, on the basis of high growth rate, rapid metabolism of lignin, high optimum temperature and, low phenol oxidase activity (Istek *et al*., 2005). *Physisporinus rivulosus* T24 produces high MnP (24 nkat/g dry woods), laccase and oxalic acid when grown on spruce (*Picea abies*) wood chips (Hakala *et al*., 2005), degrades lignin selectively and is promising for use in biopulping of

softwood. *Phlebia* sp. MG-60, secretes LiP, MnP, and Lac to bleach unbleached hardwood kraft pulp when only 0.5mM H_2O_2 is added continuously (Li *et al*., 2003). *C. subvermispora* extretes MnP as the main oxidative enzyme with oxalic acid being the major organic acid during biopulping of *Pinus taeda* wood chips. Strong Fe^{3+}-reducing activity and high catechol concentrations observed in wood extracts indicates that Fe^{2+} would be available in solution during the wood decay process involving H_2O_2 mediated degradation of oxalate by MnP in lignin degradation mechanism (Aguiar *et al*., 2006). Biopulping of *Eucalyptus grandis* by *C. subvermispora* in SS- cultures supplemented with corn steep liquor (CSL), glucose and Mn^{2+} revealed enhanced MnPs and xylanase activities with minor effect on oxalate production suggesting that oxalate was not a limiting agent during biopulping (Asgher *et al.,* 2010; Ghodake *et al*., 2009; Sigoillot *et al*., 2004; Garcia *et al*., 2004). The shortcomings of lignin degradation by WRF enzymes can be overcome by the application of laccase/xylanase system that can completely replace the complex and expensive laccase/mediator system to be used for biopulping and biobleaching (You *et al*., 2008).

The traditional technology of producing a stone-washed look in denim fabrics or jeans involves washing of the fabrics in the presence of pumice. Cellulases can partially replace pumice stones to create an abrasive effect at the fiber surface producing the stone-washed look. Lac produced by WRF has the capability to bleach dyed denim fabrics to lighter shades (Campos *et al*., 2001). Abadulla *et al.,* (2000) reported the degradation of indigo on fabrics, with two Lac preparations from *Polyporus sp*, and *Sclerotium rolfsii*. In another study Campos *et al.,* (2001) observed degradation of indigo both in and on fabrics using purified Lac from *Trametes hirsute* and *S. rolfsii* in combination with redox-mediators and reported that bleaching of fabrics by the laccases (based on K/S values) is correlated with the release of indigo degradation products. In a recent study, Pazarlioglu *et al*., (2005) found that a Lac from *T. versicolor* used for denim washing was more effective with out a mediator as compared to commercial Lac with a mediator. Laccase can also be used to assay the total antioxidant concentration (TAC) blood plasma without the interference encountered with the hydrogen peroxide and metmyoglobin mediated assay method. An accurate and reliable laccase-based assay method has been developed for detection and determination of TAC in human plasma (Mazumder *et al.,* 2008).

Each industrial application of enzymes requires specific enzymes with specific kinetic

and thermostability characteristics. Keeping in view the many industrial applications of ligninolytic enzymes there is a dire need to explore more and more strains for the production of enzymes with suitable characteristics for different applications. *Schyzophyllum commune* an efficient wood decaying fungus that causes white rot of soft woods. This study was therefore, focused on hyperproduction of ligninolytic enzymes by an indigenously isolated strain of *Schyzophyllum commune* **and to** characterize them for determining their suitability as industrial catalysts.

CHAPTER 3

MATERIALS AND METHODS

3.1 Lignocellulosic substrate

The lignocellulosic banana stalks were collected from fruit and vegetable market of Ghulam Muhammad Abad, Faisalabad, Pakistan. The stalks were chopped into pieces and sun dried. The sun dried chips were dried in oven at 60°C, and ground to 40 mm mesh size and stored in airtight plastic jars.

3.2 White rot fungus

The indigenous white rot fungus *Schyzophyllum commune* IBL-06 available in the Industrial Biotechnology Laboratory, Department of Chemistry and Biochemistry University of Agriculture Faisalabad was used for ligninase production. During the study, the fungal culture was periodically multiplied on potato dextrose agar (PDA) slants sporulation medium (Table 3.1) at pH 4.5 and temperature 35°C and stored at 4°C in refrigerator.

Table3.1 Composition of sporulation medium for *Schyzophyllum commune* IBL-06

Sr. No.	Ingredients	Quantity(g/L)
01	Potato extract	250
02	Glucose	20
03	Agar agar	15
04	Ammonium tartarate	0.22
05	$MgSO_4.7H_2O$	0.05
06	$CaCl_2.2H_2O$	0.01
07	KH_2PO_4	0.21
08	Thiamine	0.001
09	10% Tween-80	10 mL/L
10	100mM veratryl alcohol	10 mL/L
11	Chloramphenicol	1cc/L

3.3 Inoculum development

For preparation of inoculum, the *S. commune* IBL-06 was grown in a liquid medium (pH 4.5) containing (g/L): glucose 2; $MgSO_4 \cdot 7H_2O$, 0.05; $CaCl_2 \cdot 2H_2O$, 0.1; NH_4Cl, 0.12 and thiamine, 0.001 (Asghar *et al.*, 2006). The inoculum flask was autoclaved at 121^oC (15 lb pressure) for 15 minutes and inoculated with loopful culture of the fungus transferred aseptically from the PDA slant in laminar air flow (Dalton, Japan). After inoculation, the flask was incubated at 35°C in orbital shaker (150 rpm /min) for 5 days to get homogeneous spore suspension containing 1×10^6-10^8 spores/mL. The spore counting was performed using hemocytometer by the method of Kolmer (1959).

3.4 Solid state fermentation of banana stalk

Triplicate flasks contained 5g of lignocellulosic substrate banana stalk. The substrate was moistened to 60% moisture (w/w) by adding 7.5 mL of Krik's basal medium (Table 3.2) of pH 4.5 (Tien and Kirk, 1988). The flasks were sterilized in a laboratory scale autoclave (Sanyo, Japan), allowed to cool at room temperature and inoculated with 2 mL of homogenous inoculum of *S. commune* IBL-06. The inoculated flasks were allowed to ferment at 35°C for stipulated time period in a still culture incubator (Sanyo, Japan).

Table3.2 Composition of Krik's basal medium

Sr. No.	Ingredients	Quantity(g/L)
01	Glucose	10
02	Ammonium tararate	0.22
03	KH_2PO_4	0.21
04	$MgSO_4.7H_2O$	0.05
05	$CaCl_2$	0.01
06	Thiamine	0.001
07	Tween 80 (10%)	10mL
08	100 *m*M veratryl alcohol	10mL
09	Trace elements solution	10mL
10	Chloramphenicol	1cc

3.5 Fermented biomass harvesting

The triplicate flasks were harvested after every 48 hours. To the fermented solid biomass, 100 mL of 50 mM sodium malonate buffer (pH 4.5) was added and the flasks were shaken (120 rpm) for half an hour. The contents were filtered through Whatman No.1 filter paper (125mm) and residues were discarded. The filtrates were centrifuged (3,000×g, 10 min, 4°C) to remove the fungal pellets and carefully collected clear supernatants were used as enzyme extracts for determining the activities of LiP, MnP and laccase

3.6 Optimization of SSF parameters

The SSF process for the production of ligninolytic enzymes using banana stalk as substrate was optimized by varying different physical and nutritional parameters. The Classical Strategy of optimization was adopted; varying one variable at a time in triplicate and maintaining the previously optimized at optimum level.

3.6.1 Optimization of incubation period

Sets of triplicate flasks containing banana stalk (5g) as substrate was moistened (60% w/w moisture). The pH was adjusted to 4.5 and the flasks were sterilized, inoculated (2 mL inoculum) with *S. commune* IBL-06 and incubated at 35°C for 2-12 days under still culture conditions. Triplicate flasks were harvested after every 48 hours.

3.6.2 Selection of basal nutrient medium

To find out a simple and economical nutrient medium for moistening the substrate for SSF, Kirk's basal nutrient medium and three modified simpler media were used.

Medium-I: It was a relatively costly Krik's basal salts medium (Tein and Kirk, 1998) composed of (g/L):ammonium tartarate, 0.22; potassium dihydrogen phosphate, 0.2; magnesium sulfate, 0.05 and calcium chloride, 0.01 along with thiamine,1 mg/L; 10% Tween-80 solution, 10mL/L, 100mM varatryl alcohol,1 mL/L and trace elements solution, 10mL/L. The trace elements solution contained (g/L): CuSO4, 0.08; Na2MoO4, 0.05; MnSO4. H2O, 0.07; ZnSO4.H2O, 0.043 and Fe2 (SO4)3, 0.05

Medium-II: It contained all the ingredients of Kirk's medium except two costly chemicals varatryl alcohol and Tween 80

Medium-III: It was the simplest and most economical medium having the following composition (g/L): Urea, 0.03; KH_2PO_4, 0.02; $MgSO_4.7H_2O$, 0.1 and $CaCl_2.2H_2O$, 0.02.

Medium-IV: It was modified form of M-III medium with the addition of K_2HPO_4 and was composed of (g/L): Urea, 0.04; KH_2PO_4, 0.1; K_2HPO_4, 0.2; $MgSO_4$ $7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.05.

3.6.3 Optimization of initial pH of the medium

The substrate banana stalk was moistened with the selected nutrient medium (M-II) of varying pH and initial pH of the solid substrate medium was adjusted to varying pH levels (Table 3.3) before sterilization in autoclave. Media of varying pH were uniformly inoculated and incubated at 35^{o}C for optimum fermentation time observed in the time course study. pH of the medium that showed best ligninase yields was selected as optimum initial pH.

Table 3.3 Composition of growth media of banana stalk for ligninase production at varying pH

Parameters	**Treatments**						
	T1	T2	T3	T4	T5	T6	T7
Banana stalk (g)	5	5	5	5	5	5	5
Moisture (%)	60%	60%	60%	60%	60%	60%	60%
pH	3	3.5	4	4.5	5	5.5	6

3.6.4 Optimization of incubation temperature

Banana stalk (5g) was moistened to 60% moisture (w/w) and adjusted to optimum pH 4.5. The flasks were autoclaved, inoculated and triplicate sets of flasks were incubated at varying temperatures (Table 3.4) for optimum time period in the still culture incubator. The

temperature supporting maximum fungal growth and ligninolytic enzymes formation was selected as optimum temperature for *S. commune* IBL-06.

Table 3.4 Composition of growth media for ligninase production at varying incubation temperatures

Parameters	**Treatments**				
	T1	T2	T3	T4	T5
Banana stalk(g)	5	5	5	5	5
Moisture content (%)	60	60	60	60	60
Initial pH	4.5	4.5	4.5	4.5	4.5
Temperature (°C)	25	30	35	40	45

3.6.5 Optimization of moisture level

Banana stalk was moistened with different volumes of M-I medium to adjust varying initial moisture contents (Table 3.5). Triplicate flasks were inoculated with 2mL inoculum and were subjected to SSF under optimum conditions. The liginase activities determined in case of each treatment to check the optimum production of enzyme.

Table 3.5 Composition of growth media for ligninase production with varying moisture contents

Parameters	Treatments						
	T1	T2	T3	T4	T5	T6	T7
Banana stalk (g)	5	5	5	5	5	5	5
pH	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Temperature (°C)	35	35	35	35	35	35	35
M-II medium (mL)	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Moisture (%)	40	45	50	55	60	65	70

3.6.6 Optimization of inoculum size

The effect of inoculum size in the range of 1-7mL per 5 g substrate on production of ligninolytic enzymes synthesis by *S. commune* IBL-06 was investigated under optimum conditions. Triplicate flasks of banana stalk medium of optimum moisture and pH were autoclaved and inoculated with varying volumes of inoculum (Table 3.6) before incubation at optimum temperature for optimum time period. According to the volume of inoculum, the initial moisture content was adjusted accordingly by using lesser volumes of the M-I medium for moistening the substrate.

Table 3.6 Composition of growth media for ligninase production with varying inoculum size

Parameters	Treatments						
	T1	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**
Banana stalk (g)	**5**	**5**	**5**	**5**	**5**	**5**	**5**
Moisture (%)	**60**	**60**	**60**	**60**	**60**	**60**	**60**
pH	**4.5**	**4.5**	**4.5**	**4.5**	**4.5**	**4.5**	**4.5**
Temperature (°C)	**35**	**35**	**35**	**35**	**35**	**35**	**35**
Inoculum (mL)	**1**	**2**	**3**	**4**	**5**	**6**	**7**

3.6.7 Selection of additional carbon source

Microorganisms require easily metabolizable carbon and energy source for their growth and metabolic activities. Different carbon sources (Table 3.7) were added to the optimum medium of banana stalk and the carbon source giving maximum enhancement in ligninase production was selected for further studies.

Table 3.7 Composition of growth media for ligninase production with different carbon sources

Parameters	Treatment						
	T1	T2	T3	T4	T5	T6	T7
Banana stalk (g)	**5**	**5**	**5**	**5**	**5**	**5**	**5**
pH	**4.5**	**4.5**	**4.5**	**4.5**	**4.5**	**4.5**	**4.5**
Temperature (°C)	**35**	**35**	**35**	**35**	**35**	**35**	**35**
Moisture (%)	**60**	**60**	**60**	**60**	**60**	**60**	**60**
Inoculum(mL)	**3**	**3**	**3**	**3**	**3**	**3**	**3**
Carbon Source (1g)	**Glucose**	**Galactose**	**Fructose**	**Lactose**	**Maltose**	**Sucrose**	**Molasses**

3. 6.8 Selection of additional nitrogen source

Nitrogen source and its concentration is very important factor for microbial growth kinetics. Different nitrogen additives were used to investigate their stimulating/inhibitory influence on ligninolytic enzymes production by *S. commune* IBL-06 under optimum conditions (Table 3.10). The additive causing maximum enhancement in ligninase production was selected as best nitrogen source was better nitrogen source for enzyme production.

Table 3.8 Composition of growth media for ligninase production with different nitrogen sources

Parameters	Treatments				
	T1	T2	T3	T4	T5
Banana stalk (g)	**5**	**5**	**5**	**5**	**5**
pH	**4.5**	**4.5**	**4.5**	**4.5**	**4.5**
Temperature (°C)	**35**	**35**	**35**	**35**	**35**
Moisture (%)	**60**	**60**	**60**	**60**	**60**
Inoculum (mL)	**3**	**3**	**3**	**3**	**3**
Carbon source (glucose, g)	**1**	**1**	**1**	**1**	**1**
Nitrrogen Source (0.2g)	**$(NH_4)_2H_2PO_4$**	**$(NH_4)_2SO_4$**	**$(NH_4)_2NO_3$**	**Peptone**	**urea**

3.6.9 Optimization of Carbon: Nitrogen ratio

After selection of best carbon and nitrogen source, effect of varying C: N ratios on ligninase production by *S. commune* IBL-06 in SSF of banana stalk were investigated. Taking into consideration the inherent nitrogen and carbon contents of banana stalk, and the selected carbon (glucose) and nitrogen (ammonium nitrate) additives, the triplicate flasks were adjusted to varying C: N ratios (Table 3.9) after making necessary calculations

Table 3.9 Composition of growth media for ligninase production with varying carbon: nitrogen ratios

Parameters	**Treatments**				
	T1	T2	T3	T4	T5
Banana stalk (g)	5	5	5	5	5
pH	4.5	4.5	4.5	4.5	4.5
Temperature (°C)	35	35	35	35	35
Moisture (%)	60	60	60	60	60
Inoculum (mL)	3	3	3	3	3
C:N ratio	5:1	10:1	15:1	20:1	25:1

3.6.10 Effect of Mediators

Different fungal metabolites and organic and inorganic compounds act as mediators in the catalytic cycles of different ligninolytic enzymes. Different mediators were added to the banana stalk medium and the flasks were processed under optimum conditions (Table 3.10). $MnSO_4$ showed maximum enhancing effect of ligninase synthesis by the fungus.

Table 3.10 Composition of growth media for ligninase production with different mediators

Parameters	Treatments				
	T1	T2	T3	T4	T5
Banana stalks (g)	5	5	5	5	5
pH	4.5	4.5	4.5	4.5	4.5
Temperature (°C)	35	35	35	35	35
Moisture (%)	60	60	60	60	60
Inoculum (mL)	3	3	3	3	3
C:N ratio	20:1	20:1	20:1	20:1	20:1
Mediators (1 mM, 1mL)	Veratryl alcohol	$MnSO_4$	ABTS	oxalate	H_2O_2

3.6.11 Effect of metal ions

To enhance the growth and production of ligninases, different metal ions were used in the form of their salts (Table 3.11). The triplicate flasks were processed under optimum conditions.

Table 3.11 Composition of growth media for ligninase production with varying metal ions

Parameters	Treatments					
	T1	T2	T3	T4	T5	T6
Banana stalk (g)	**5**	**5**	**5**	**5**	**5**	**5**
pH	**4.5**	**4.5**	**4.5**	**4.5**	**4.5**	**4.5**
Temperature (°C)	**35**	**35**	**35**	**35**	**35**	**35**
Moisture (%)	**60**	**60**	**60**	**60**	**60**	**60**
Inoculum (mL)	**3**	**3**	**3**	**3**	**3**	**3**
C:N ratio	**20:1**	**20:1**	**20:1**	**20:1**	**20:1**	**20:1**
Mediator ($MnSO_4$ (1 mM, mL)	**1**	**1**	**1**	**1**	**1**	**1**
Metal ions (1 mM, 1mL)	**$MgSO_4$**	**$CaCl_2$**	**$FeSO_4$**	**$ZnSO_4$**	**KCl**	**$CuSO_4$**

3.7 Analytical

3.7.1 Ligninolytic enzymes assays

3.7.1.1 MnP assay

Manganese peroxidase activity was determined by the method of Wariishi *et al.* (1992). $MnSO_4$ was added to the enzyme extract in sodium malonate buffer in the presence of H_2O_2. Manganic ions (Mn^{+3}) form a complex with melonate which absorbs at 270 nm. (ε_{270} 11570 $M\,cm^{-1}$)

Chemicals and reagents

1 mM $MnSO_4$, 0.1 mM H_2O_2, 50 mM Sodium Malonate Buffer

Assay procedure

Manganese peroxidized assay mixture (2.6 mL) contained 1 mL of $MnSO_4$, 1 mL of 50mM sodium malonate buffer of pH 4.5, 0.5mL of H_2O_2 and 0.1 mL of enzyme extract. Blank cuvette contained 0.1 mL of distilled water instead of enzyme solution. Absorbance of each sample was taken after 10 min interval at 270 nm.

3.7.1.2 LiP assay

The LiP was assayed by the method of Tien and Kirk (1988). The rate of oxidation of veratryl alcohol to veratraldehyde was monitored in 100mM tartarate buffer of pH 3 in the presence of H_2O_2.

Chemicals and reagents

100mM Tartarate buffer (pH 3), 4 mM veratryl alcohol, 0.2mM hydrogen peroxide

Assay procedure

The assay was performed in a 2.6 mL of reaction mixture containing 1 mL of 100mM tartarate buffer of pH 3, 1 mL veratryl alcohol, 500 µL H_2O_2 and 100 µL of enzyme extract. The blanks contained 100µL of distilled water instead of enzyme aliquote. The absorbances of samples were read at 10 min intervals at 310 nm (ε_{310} 9300 $M\,cm^{-1}$). One unit of enzyme activity was defined as uM of varatraldehyde formed per min.

3.7.1.3 Laccase assay

Laccase activity was determined by monitoring the rate of 2, 2-azinobis (3-ethylbenzthiazoline)-6 sulphonate (ABTS) oxidation by the culture supernatants (Shin and

Lee 2000) at pH 4.5 and 35 ^{0}C temperature in 50mM malonate buffer of pH 4.5. The oxidation was followed at 420nm (ε_{420} 36000 M cm^{-1}).

Chemicals and reagents

0.3mM ABTS, 50 mM Sodium Malonate Buffer

Assay procedure

Laccase activity was performed in 2.1 mL reaction mixture containing 1 mL of 50 mM malonate buffer (pH 4.5), 1 mL of 0,3mM ABTS and 0.1 mL of enzyme solution. The absorbance of each sample was measured at 420nm at 10 min. interval. Blanks contained 0.1 mL distilled water instead of enzyme extract.

3.7.2 Determination of protein contents

During enzyme purification, the protein contents of the samples were estimated by the method of Bradford (1976) using Bovine serum albumin (BSA) as standard.

Bradford reagent

Dissolved 100mg of Coomassie brilliant blue G-250 in 50 mL of 95 percent ethanol and. added 100 mL of 85% concentrate phosphoric acid and the volume was made to 1 liter. It was filtered through Whatman No.1filter paper and stored at 4°C in refrigerator.

Procedure

Twenty µL of diluted enzyme extract (enzyme: water: 1:4 V/V) and 5 mL of Bradford reagent were added in a test tube. The absorbance was taken at 595nm with spectrophotometer.

Standard curve of BSA for protein estimation

In numbered test tubes, different volumes of standard BSA solution (1mg/mL) were taken and diluted to a final volume of 100 µL (Table 3.12). Then 5 mL Bradford reagent was added to each test tube and absorbances were read 595 nm. Graph was plotted between different concentration of BSA and absorbances as shown in Fig 3.1.

Table 3.12 Concentrations of BSA solutions used for constructing standard curve

Sr. No	BSA solution (µL)	Distilled water (µL)	Total volume (µL)	Absorbance at 550 nm
1	20	80	100	0.14
2	40	60	100	0.28
3	60	40	100	0.39
4	80	20	100	0.5
5	100	0	100	0.62

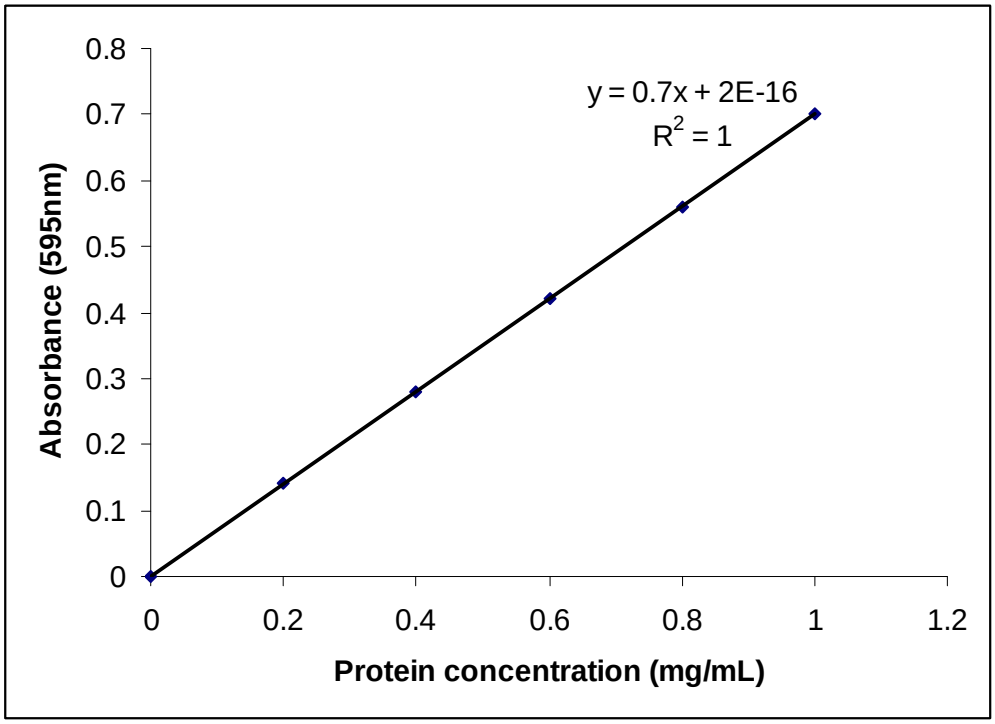


Fig. 3.1 Standard curve for protein estimation

Protein estimation

Quantity of protein was determined using standard curve made from different known concentrations of BSA:

Protein in mg/mL = Standard factor × absorbance of sample

Where as

Standard factor= Slope × dilution × volume of sample

3.8 Purification of ligninolytic enzymes

Ligninase crude extract produced by *S. commune* IBL-06 under optimum conditions was filtered and the filtrated was centrifuged at 3,000×g for 15min at 4°C. The supernatant was collected and concentrated by freeze drying. The crude extract was used for isolation, purification and characterization of LiP, MnP and laccase.

3.8.1 Fractional precipitation with ammonium sulphate

Different amounts of solid ammonium sulphate were added separately to 1mL of the crude liginases concentrates in appendorff tubes to get 10-90% saturation at 0°C and gently mixed. These tubes were left over night at 4°C and centrifuged at 3,000×g rpm for 15min. The supernatants were assayed for ligninases activity. In each step, the crude enzyme concentrate was placed in ice bath and crystals of ammonium sulphate were added to attain the particular levels of saturation (%) for the three enzymes. The tubes were kept overnight at 4°C. The contents were centrifuged at 3,000×g for 30min at 4°C and the pellet of precipitated protein was discarded. In the supernatant, more crystals of ammonium sulfate were added to attain next level of saturation and the whole procedure was repeated. After 55 % saturation, the pellets were collected for MnP assay. The pellet was dissolved in minimum quantity of malonate buffer and dialyzed against distilled water for 72 hours with changes of water at different intervals to remove ammonium sulfate. After 75 % saturation, the supernatant, the pellets were dissolved in tartrate buffer and dialyzed for LiP study. For laccase the ammonium sulphate was added to the remaining supernatant till 90% saturation. After centrifugation, the pellet was dissolved in buffer and dialyzed to remove salt. Total proteins and liginase activities were determined before and after ammonium sulfate precipitation and dialysis. The purified enzymes were separately freeze dried.

3.8.2 Gel filtration

After dialysis, the partially purified enzymes were individually loaded (300µL/run) on separate Sephadex G-100 columns (length x width - 16×2.0) for further purification. For LiP 50mM tartrate buffer pH 3.5 was used with a flow rate 1mL/min (Asghar *et al*., 2006) and 1mL size fractions were collected. For gel filtration study of MnP using Sephadex G-100 column, 50mM malonate buffer pH 4.5 was used (Bermek *et al*., 2004) at a flow rate of

1mL/min and positive fractions were collected and monitored at 280nm. The fractions containing laccase were collected using 20mM phosphate buffer pH 5.7 as eluent with flow rate of 1mL/min (Anne-Marie et al., 2004). The eluted fractions were monitored at 280 nm and proteins were estimated. After elution, the column was washed with buffer. The purified enzyme fractions in each case were pooled and lyophilized to reduce total volume.

3.9 Molecular Weight Determination by Native and SDS-PAGE

The purified LiP, MnP and laccase were run on Native polyacryl amide gel electrophoresis (PAGE) and sodium dodecyl sulphate-PAGE (SDS-PAGE) for estimation of their molecular weights and molecular organizations, following the method of Laemmli, (1970). The lyophilized samples of individual enzymes were dissolved in minimum amounts of distilled water and subjected to Native and SDS- PAGE on 10% polyacrylamide gels. The approximate molecular masses of the ligninases was determined by calibration against broad range (21-116 kDa) molecular weight markers (β-Galactosidase, 116kDa; Phosphorylase B, 97kDa; albumin, 66kDa; ovalbumin, 45kDa; carbonic anhydrase, 30kDa and trypsin inhibitor, 21kDa).

3.9.1 Reagents and Buffers

Resolving gel Buffer: 3.0 M Tris-HCl (pH 8.8)

Tris (36.3 g) was dissolved in distilled water and 24 mL of 2M HCl was added. Final volume was made up to 100mL mark with distilled water.

Stacking gel Buffer: 0.5 M Tris-HCl (6.8)

Tris (6g) was dissolved in distilled water and titrated with 2M HCl to final pH 6.8. The final volume was made up to 100mL mark with distilledwater.

Stock Bis acrylamide Solution (30%):

Acrylamide (29.2g) and Bisacrylamide (0.8g) were dissolved in distilled water. The volume was made up to 100mL mark and the solution was stored in dark colored bottle at 4°C.

3.9.2 Preparation of resolving gel (Running gel 12.5%)

The following reagents were mixed together in a 100 mL beaker for preparation of resolving gel for native PAGE and SDS-PAGE*

1. Distilled Water 4mL

2. Tris Buffer (pH 8.8) 3mL
3. 30% Acrylamide 4mL
4. TEMED 10µL
5. APS (5%) 125µL

*Additionally 125µl SDS was added for SDS-PAGE denaturing gel

The resolving gels were prepared by pouring the above mentioned mixture into the gel apparatus that was assembled by sandwiching 2 spacers between two glass plates. 1mL butanol was layered on the top of the gel to get even surface after polymerization.

3.9.3 Preparation of stacking gel

The following reagents were mixed together in a 100mL beaker.

1. Distilled Water 3mL
2. Tris Buffer (pH 6.8) 0.8mL
3. 30% Acrylamide 0.6mL
4. TEMED 7µL
5. APS 50µL

The stacking gel mixture was then poured on the top of polymerized resolving gel. The comb (well marker) was immediately inserted and the stacking gel was allowed to polymerize. For SDS-PAGE 50 µL freshly prepared 1% SDS was also added.

3.9.4 Running buffer (pH 8.3)

Tris base (3.02g) and glycine (18.8g) was mixed to prepare stock solution for native gel in distilled water just before use. pH was adjusted to 8.3 with HCl and final volume was made to 1L mark with distilled water. Running buffer for SDS-PAGE also required 0.1% SDS (w/v).

3.9.5 Preparation of enzyme samples

Individual enzyme samples (50 µL) were mixed with equal volume of dye (running and sample dye) (Fermentos Kit). The samples were kept in boiling water for 15 min and loaded on the gel. For SDS-PAGE 50 µL of 1% SDS was also added.

3.9.6 Preparation of proteins markers ladder

Standard protein markers ladder (Fermentos, UK) consisted of 7 proteins ranging from 14.4 to 116.0 kDa molecular weights. The protein ladder was supplied in gel loading buffer (50%

glycerol, 2% 30mM NaCl, ImM NaN_3, 62.5 mM, Tris/HCl pH7, 0.01% bromophenol blue and 50mm DTT) and applied directly to polyacrylamide gel.

3.9.7 Running of PAGE

Polyacrylamide gel was run at a constant voltage of 100 volts. The PAGE was stopped when tracking dye front reached at bottom of the gel.

3.9.8 Preparation of Staining Solution

Dissolevd 40% methanol, 10% glacial acetic acid, 0.25% Coomassie blue G-250, 1.25g in distilled water up to 500mL mark.

3.9.9 Destaining solution

Methanol (40%) and glacial acetic acid (10%) were dissolved in distilled water; For making the volume up to 5L, 2L methanol, 2.5 L dH_2O and 500 mL glacial acetic acid were added.

3.9.10 Protein staining

A cut was applied on the lower side of the gel to illustrate the direction of electrophoresis. The gel was treated with 20% (v/v) isopropyl alcohol in 50 mM sodium acetate buffer (pH 5) and washed thrice. Then the gel was immersed in the same buffer to remove isopropyl alcohol. The gels were finally stained with Coomassie blue G-250 stain (Merril (1990). Gel was soaked in staining solution for over night (8-10 h) and protein bands were stained in order to improve the intensity of bands. Gels were left in the stain for hours with no increase in the background (Coughlan, 1988).

3.9.11 Destaining the gels

To get clear looking bands, the gel was destained for 3-4 hours. The respective gels of individual enzymes were then taken to the gel documentation system for taking pictures of standard and sample protein bands and estimation of molecular weights of LiP, MnP and laccase.

3.10 Characterization of Ligninolytic Enzymes

The purified lignolytic enzymes were subjected to characterization through kinetic studies by studying the following:

3.10.1 Effect of pH on enzyme activity

Ligninases were assayed at different pH ranging from 2-9. MnP were assayed at 40°C at pH 3-3.5 (20 mM succinate buffer, 50 mM sodium tartrate buffer), pH 4-5 (50mM malonate

buffer, 20 mM succinate buffer), pH 6-7 (100mM citrate buffer, 100mM phosphate buffer), pH 8-9(100mM phosphate buffer, 50 mM carbonate buffer). Similarly LiP and Laccase were studied at optimum temperature 35°C with above mentioned buffers.

3.10.2 Effect of temperature on enzyme activity

Ligninases were assayed at different temperature ranging from 25 – 70°C at pH 4.5. The methodology was adopted (Asgher et al., 2006 for LiP, Anne-Marie et al., 2004 for laccase, Bermek et al., 2004 for MnP).The assay methodology was same as described earlier.

3.10.3 Effect of activators / inhibitors

Organic compounds such as TEMED, Marcaptoethanol, EDTA and metal ions $Pb(NO_3)_2$, $CaCl_2$, $MgSO_4$, $CuSO_4$ were studied ranging 1-5 mM. All the organic compounds studied above and heavy metals having inhibitory effects reduce the activity of liginases. $CuSO_4$ and $CaCl_2$ having mediation effect on liginase to some extent.

3.10.4 Effect of substrate concentration; determination of K_M and V_{max}

The Michalis-Menten kinetic constants (K_m, V_{max}) were determined by using different concentration of MnSO4, Vertryl alcohol ranging from 1mM-5mM (w/v) as described by Bermek et al., 2004 for MnP, effect of LiP digestion of Vertryl alcohal by Asgher *et al*., 2006. For study of Laccase different concentration of ABTS were prepared ranging from 0.1-0.5 mM method by described Anne-Marie *et al*., 2004. Ligninases activity were dertermined in different concentration of substrates keeping the enzymes concentration constant.

3.11 Application for Ligninases for decolorization of dyes and Industrial effluents

3.11.1 Decolorization of dyes

Partially purified enzymes were used in the activity ratios present in crude extract harvested under optimum conditions for decolorization of four textile dyes including Novasol direct black (Saba Pvt.) λmax-499nm, Novasol direct yellow (Saba Pvt.) λmax-415nm, Novasol direct blue (Saba Pvt.) λmax-578nm and Novasol direct red (Saba Pvt.) λmax-357nm. Ten mL of the enzyme mixture was transferred to 100mL of 0.01 % individual dye solutions and the flaks were incubated at pH 4 and 35^{o}C for 24 hours in rotary shaker (120rpm).

3.11.2 Decolorization of textile industry effluents

Different dye containing textile industry effluents of different colours were collected from Sitara textile (SIT), Nishat textile (NIT) K&N textile (KNT) and Crescent textile (CRT) units of Faisalabad. These textile effluents had different pH, colour and wavelength of maximum absorbance (λmax) given in table 3.13. All the industries were using mixtures of different dyes but none of them disclosed the composition of dye mixtures being used by them. Ten mL of the enzyme mixture (In the ratio present in original crude extract) was transferred into the triplicate flasks containing 100 mL of the individual effluents and the flasks were incubated (120rpm) at pH 4 and 35oC for 24 hours.

Table 3.13 Characteristics of textile industry waste waters used for decolorization studies using purified ligninolytic enzymes mixture

Wastewater source industry	Color	λmax (nm)	Initial pH
Sitara Textile (SIT)	Navy blue	590	9.2
Nishat Textile (NIT)	Black	615	6.8
K&N Textile (KNT)	Greenish blue	667	6.0
Crescent Textile (CRT)	Red	515	8.7

3.12 Statistical Analysis

All the data on optimization of LiP, MnP and laccase production, purification and characterization were subjected to statistical analysis using Analysis of Variance (ANOVA), as described by Steel *et al*., (1997). The data values have been presented as Mean + S.E. of the triplicate samples analyzed in triplicate and the S.E. values have been displayed as Y-error bar s in figures.

CHAPTER 4

RESULTS AND DISCUSSION

The indigenous white rot fungus *Schyzophyllum commune* IBL-06 was used for the production of ligninases in solid state fermentation of a lignocellulosic substrates banana stalk. Different experiments were conducted for optimization of different parameters such as time period medium composition, pH, temperature, inoculum size, moisture content, different carbon and nitrogen sources, carbon: nitrogen ratio, surfactants and mediators to maximize ligninases production by *Schyzophyllum commune* using banana stalk as substrate and Kik's medium in solid state cultures. It has already been reported that secretion of MnP, LiP and laccase by different white rot fungi is strongly dependent on growth conditions (Rogalski *et al*., 2006). The enzymes produced by *Schyzophyllum commune* IBL-06 was partially purified and characterized by studying the effects of temperature, pH and respective substrate concentrations. The results have been discussed in the following headings:

4.1 Optimization of parameters for enhanced production of ligninases

4.2 Partial purification of the ligninases

4.3 Characterization of the ligninases

4.1 Optimization of parameters for enhanced production of ligninases

The nutritional and physical parameters for the production of ligninolytic enzymes using banana stalk as substrate were optimized. The Classical Strategy of optimization was adopted under; varying one variable at a time in triplicate and maintaining the previously optimized at optimum level. The carbon: nitrogen ratio was optimized in one experiment after the selection of most suitable carbon and nitrogen supplements.

4.1.1 Effect of incubation time

After every 48 hrs the triplicate SSF flasks were harvested and culture supernatants were analyzed for ligninolytic enzymes and dry weight of biomass (residue) was also recorded. The results of time course study showed that maximum production of MnP (1017 IU/mL), LiP (743.7 IU/mL) and laccase (75 IU/mL) was achieved in 8 days of SSF of banana stalk by

S. commune IBL-06 (Table 4.1a). As the incubation time increased the biomass weight also increased. It was noted that ligninases production steadily increased with an increasing fermentation time and further increase in fermentation time showed a decrease in ligninases activities (Fig. 4.1). It was also important to note that *S. commune* IBL-06 produced MnP (1017 IU/mL) as the major enzyme activity, followed by LiP (733 IU/mL) and laccase (35 IU/mL). Analysis of Variance (ANOVA) (Table 4.1 b) revealed a significant ($P \leq 0.05$) effect of varying time period for ligninases production. Comparison of means revealed significant differences among all treatment means.

Different white rot fungi have been reported to produce maximum ligninolytic enzymes after different time periods due to genetic variation among the strains as well as nature and composition of the substrates used (Heinzkill *et al*., 1998; Giardina *et al*., 2000; Patel *et al.,* 2009). *Termitomyces clypeatus* a white rot fungus was found to produce maximum MnP activity after 6 days of incubation using lignocellulosic substrates (Bose *et al*., 2007). MnP enzyme produced by *P. chrysosporium* peaked on the 7th day of cultivation (Zahamatkesh *et al*., 2010) and White-Rot Fungus *Datronia sp*. KAPI0039 produced maximum laccase and MnP after 4 and 8 days of cultivation, respectively (Vaithanomsat *et al*., 2010). Previously, the optimum incubation time range for most white rot fungi has been reported between 4-10 days (Giardina *et al*., 2000; Asgher *et al*., 2006; Bose *et al*., 2007).

Table 4.1 (a) Activities of ligninase and biomass weight produced by *S. commune* IBL-06 with varying time periods*

Incubation time (Days)	Ligninase activities (U/mL)			Biomass weight (g)
	MnP	LiP	Laccase	
2	523±10.1F	381±12.0F	9±5.2F	5.0±0.5D
4	651±11.3E	408±13.1E	27±7.1E	5.2±0.4C
6	927±10.2C	699±10.1B	45±8.2D	5.2±0.6C
8	1017±15.1A	733±8.3A	75±9.1A	7.0±0.8A
10	970±16.2B	691±7.1C	55±4.5B	5.7±0.7B
12	845±13.5D	541±5.3D	46±5.1C	5.0±0.3D

*Moisture, 60%; pH, 4.5; temperature, 25^{o}C; Medium, M-I

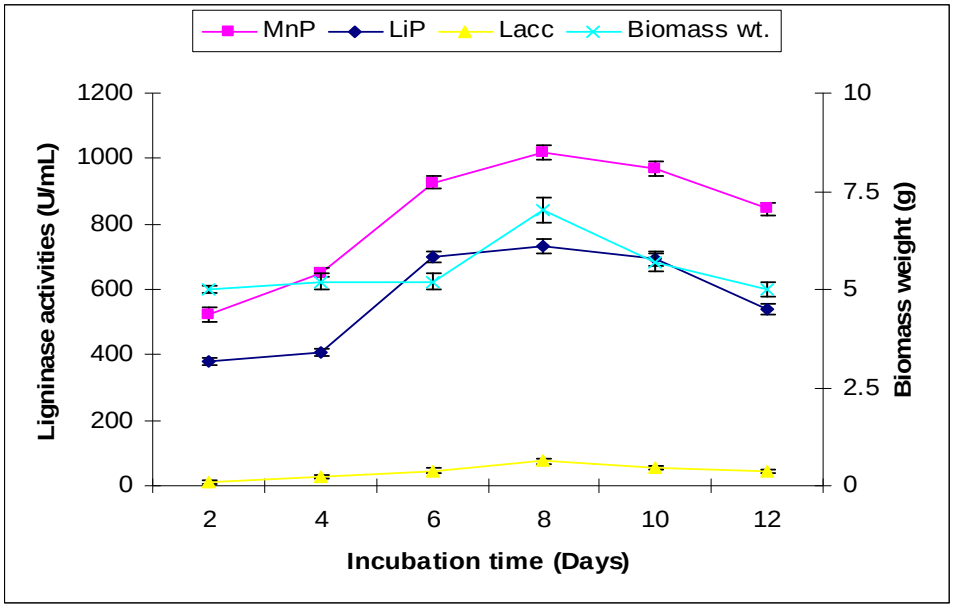


Fig. 4.1: Effect of incubation time on production of ligninases and biomass by *S. commune* IBL-06

Table 4.1(b): Analysis of variance of the data on ligninases production with varying time periods

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatments	3	2918332	2918332	972777	102.40*	0.000
Block	5	170691	170691	34138	3.59	0.025
Error	15	142499	142499	9500		
Total	23	323152				

* = Significant (P≤0.05)

4.1.2 Effect of medium composition

Four basal nutrient media were used to moist banana stalk for maximum production of ligninases. Medium M-II supported maximum fungal growth and ligninase production in 8 days (Table 4.2), followed by M-IV, M-I and M-III (Figure 4.2). Statistical analysis of the data by ANOVA (Table 4.2 b) revealed significant (P≤0.05) effect of medium composition on ligninases production. The difference between mean activities in

M-I and M-II media was non-significant. All other treatment means varied significantly. M-II was the modified Kirk's medium containing all ingredients except the costly chemicals like varatryl alcohol and tween-80. It was very significant achievement in terms of finding a cheaper medium because most of the researchers had been using Kirk's medium for growing WRF in liquid as well as solid cultures.

Table 4.2 (a) Activities of ligninases and biomass weight produced by *S. commune* IBL-06 with varying media compositions*

Media	Ligninase activities (U/mL)			Biomass weight (g)
	MnP	LiP	Laccase	
M-I	1015±17.5B	688±5.1B	85±5.1D	6.6±1.3B
M-II	1048±11.7A	750±10.2A	115±7.1A	6.9±1.1A
M-III	899±10.1D	591±17.5C	101±5.1B	5.6±0.6C
M-IV	921±16.0C	451±16.8D	95±4.0C	6.6±0.8B

*Moisture, 60%; pH, 4.5; temperature, 25°C; incubation time, 8 days

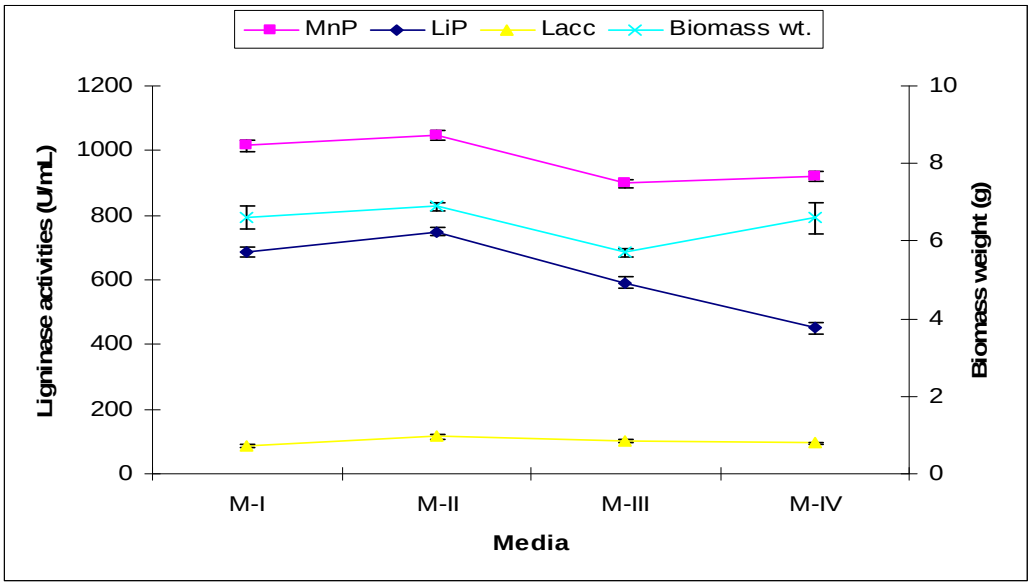


Fig. 4.2: Effect of medium composition on production of ligninase and biomass by *S. commune* IBL-06

Table 4.2 (b): Analysis of variance of the data on ligninases production on selection of suitable medium

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	2386673	2386673	795558	149.58*	0.000
Block	3	23034	23034	7678	1.44	0.294
Error	9	47869	47869	5319		
Total	15	2457576				

* = Significant (P≤0.05)

4.1.3 Optimization of initial pH of the medium

The initial time course experiment was run using pH 5 medium of banana stalk. To optimize pH for maximum enzyme production, media adjusted to varying pH were used. The maximum MnP (1125 IU/mL), LiP (783 IU/mL) and laccase (197 IU/mL) activities were observed in the medium of pH 4.5 (Table 4.3). Consistent with the enzyme activities the biomass dry weight also increased by increasing the medium pH and reached its maximum (8.99 g) at pH 4.5. It was noted that ligninases production steadily increased with an increase in initial medium pH from 3.0-4.5 and further pH increase showed a decrease in ligninase production (Fig. 4.3). The analysis of variance (Table 4.3 b) of the data showed that pH had highly significant (P<0.05) effect on the ligininase production. Maximum ligininase production was shown at pH 4.5 and differences among all treatment means were also found significant (P <0.05).

Different microbial strains show maxiumum cel growth and metabolic activities under different pH conditions. Optimal pH was found between 4.5-5.0 for production of ligninolytic enzymes by *Phanerochaete sordida* grown in milled wood lignins extracted in solid state medium (Ruttimann *et al.,* 2008). The lignolytic enzymes production (LiP and MnP) by *Pleurotus ostreatus* in the medium containing liquid culture aflatoxin as substrate was also maximum at pH 4 (Motomura *et al.,* 2003). WRF in most of the cases have shown optimum mycelial growth and to produce higher activities of ligninolytic enzymes at pH 3-6 (Xu, 1997; Radha *et al*., 2005; Shin and Lee, 2000; Motomura *et al*., 2003; Yamanaka *et al*., 2008). Maximum LiP and MnP activities were produced by *Pleurotus ostreatus* in the pH range 4.0-5.0 at 25°C by using cellulosic substrates in liquid culture fermentation (Snajdr and Baldrian, 2007).

Table 4.3 (a): Activities of ligninases and biomass weight produced by *S. commune* IBL-06 with varying initial pH*

pH	Ligninase activities (U/mL)			Biomass weight (g)
	MnP	LiP	Laccase	
3.0	395±18.9G	337±13.0G	45±1.3G	5.0±0.9F
3.5	785±15.7E	561±11.0D	75±3.4E	5.0±0.6F
4.0	901±10.3BC	689±11.0B	89±5.0CD	7.7±1.0C
4.5	1051±13.4A	753±12.0A	117±7.5A	8.9±0.9A
5.0	989±18.8B	684±10.0C	101±1.4B	8.8±1.2B
5.5	807±17.1D	553±14.1E	88±2.4C	5.8±0.6E
6.0	493±10.1F	348±10.1F	70±1.0F	5.9±0.8D

*Moisture, 60%; temperature, 25°C; Medium, M-II; incubation time, 8 days

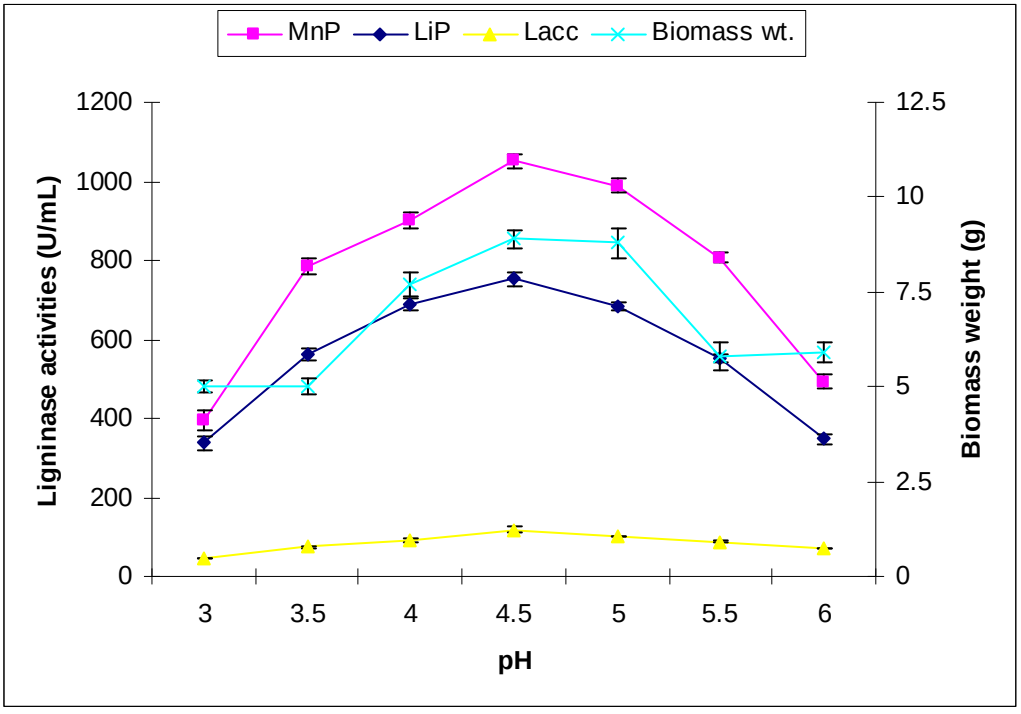


Fig. 4.3: Effect of pH on ligninase production and biomass weight produced by *S. commune* IBL-06

Table 4.3 (b): Analysis of variance of the data on ligninases production with varying pH values

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatments	3	4641202	4641202	1547067	140.02*	0.000
Block	6	300044	300044	50007	4.53	0.006
Error	18	198885	198885	11049		
Total	27	5140131				

* = Significant (P≤0.05)

4.1.4 Optimization of incubation temperature

To optimize the temperature for growth and ligninase production by *S. commune* IBL-06, the triplicate flasks of optimum pH were incubated at varying temperatures. The maximum activities of MnP(1445 IU/mL), LiP(987 IU/mL) and laccase (128 IU/mL) were produced in the flasks incubated at 35°C (Table 4.4a). The dry weight of biomass also increased with the temperature and peaked at 35˚C. A further increase in temperature caused a decreased in biomass weight and enzymes synthesis by the fungus (Figure 4.4). The analysis of variance (Table 4.4b) showed a significant (P≤0.05) effect of temperature variation on ligninase production by *S. commune* IBL-06. The differences among all treatment means were also found significant (P≤0.05).

A variation in incubation temperature has a significant influence on synthesis of ligninolytic enzymes and their activities. The temperatures ranging from 25-37°C have been found optimum for ligninase production by different WRF (Zadrazil *et al*., 1999; Arora and Gill 2000; Tekere *et al*., 2001; Tripath *et al.,* 2008). Higher temperatures denature the metabolic enzymes of microorganisms leading to inhibition of growth and enzyme formation. The change in temperature may also affect fatty acid synthesis and membrane fluidity (Vyas *et al.,* 1994; Tripath *et al.,* 2008). Shin *et al*., (1997) observed rapid enzyme inactivation when *Pleurotus ostreatus* was grown at above 35°C temperatures.

Table 4.4 (a) Activities of ligninases and biomass weight produced by *S. commune* IBL-06 under optimum conditions*

Temperature (°C)	Ligninase activities (U/mL)			Biomass weight (g)
	MnP	LiP	Laccase	
25	1069±7.0D	767±9.1D	113±1.0C	5.6±0.7D
30	1247±6.0B	832±7.2C	115±1.1B	7.4±0.4B
35	1445±7.6A	987±4.5A	128±2.3A	10.1±0.7A
40	1215±9.0C	871±9.4B	109±2.4D	6.9±0.4C
45	945±7.0E	680±5.3E	73±2.0E	6.5±0.3E
50	401±6.0F	270±4.5F	32±1.0F	5.2±0.8F

*Moisture, 60%; pH, 4.5; Medium, M-II; incubation time, 8 days

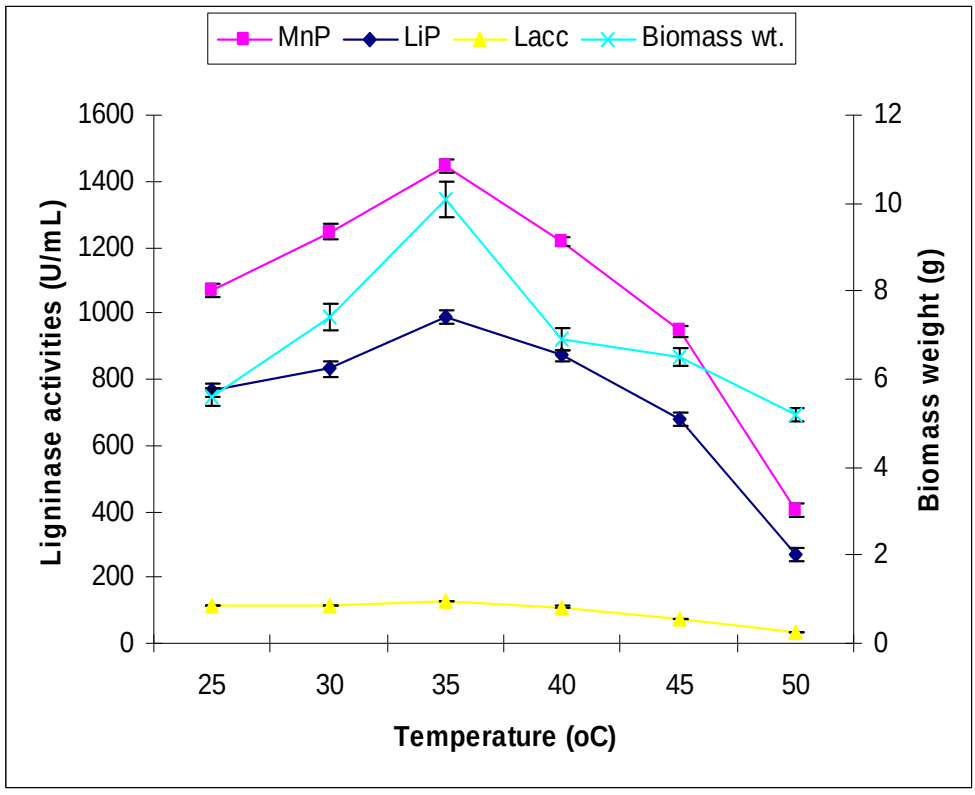


Fig. 4.4: Effect of varying temperature on ligninase production and biomass weight produced by *S. commune* IBL-06

Table 4.4 (b): Analysis of variance of the data on ligninases production with varying temperatures

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	5249969	5249969	1749990	155.83	0.000
Block	5	269424	269424	53885	4.80	0.008
Error	15	168457	168457	11230		
Total	23	5687850				

* = Significant (P≤0.05)

3.1.5 Effect of inoculum size

Inoculum is very important consideration in media optimization in microbial fermentations. For the optimization of inoculum size, the fermentation flasks were inoculated with varying volumes of homogenous spore suspension of *S. commune* IBL-06 and processed under optimum conditions of pH and temperature for 8 days. The fungal growth and ligninolytic enzymes production gradually increased with increasing the inoculum size and peaked in the flasks receiving 3mL inoculum (Table 4.5). Higher inoculum volumes resulted in lower enzyme formation (Fig.4.5). Statistical analysis (Table 4.5 b) of the data revealed a highly significant (P≤0.05) effect of inoculum size on ligninases production by *S. commune* IBL-06.

Optimum spore density is the most important consideration in microbial fermentations. SSF cultures require higher inculum density as compared to LSF submerged cultures because in SSF, there is no mass transfer and the microoraganisms have to penetrate the solid substrate matrixi to get nutrients. Lower inculum volumes need longer lag phase before starting growth and enzymes synthesis where as higher spore density in increasing inoculum volumes may causes faster depletion of nutrients, resulting poorer ligninase formation in secondary growth. Higher inoculum volumes also increase the water content, thus creating problems of aeration in the solid substrate cultures (Galhaup *et al*., 2002; Asgher *et al*., 2006; Patel *et al*., 2009). Maximum liginase activity such as MnP, 1590 IU/mL and laccase, 252 IU/mL was observed at optimum condition moisture, 75% and inoculum

size, 6 ml by *Ganoderma lucidum* IBL-06 using rice straw as the lignocellulosic substrates in solid state fermentation after 10days at 35 ^{o}C (Asgher *et al*., 2008).

Table 4.5 (a) Activities of ligninases and biomass weight produced by *S. commune* IBL-06 with varying inoculum size*

Inoculum size (mL)	Ligninase activities (U/mL)			Biomass weight (g)
	MnP	LiP	Laccase	
1	670±7.4F	658±7.6F	66±0.7E	6.9±0.8E
2	1235±5.1D	1165±5.0A	102±1.5C	7.3±0.6C
3	1471±5.7A	1079±5.0B	125±1.7A	9.3±0.9A
4	1401±9.8B	1036±5.0C	109±1.2B	7.9±0.1B
5	1277±5.7C	980±9.0D	77±0.1D	7.0±0.1D
6	1101±5.5E	825±4.0E	57±1.1F	6.0±0.9F

*Moisture, 60%; Medium, M-II; pH, 4.5; temperature, 25^{o}C; incubation time, 8 days

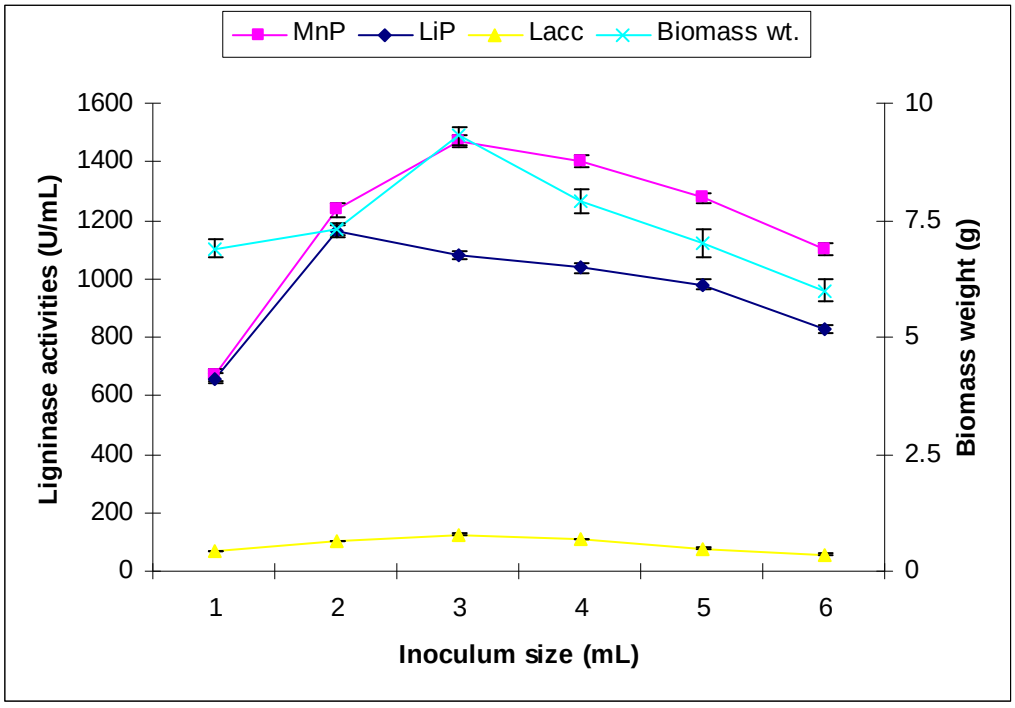


Fig. 4.5: Effect of inoculum size on ligninase production and biomass produced by *S. commune* IBL-06

Table 4.5 (b): Analysis of variance of the data on activities of ligninases with varying inoculum size

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	7285262	7285262	2428421	111.18*	0.000
Block	5	416439	416439	83288	3.81	0.020
Error	15	327628	327628	21842		
Total	23	8029329				

* = Significant (P≤0.05)

4.1.6 Optimization of moisture level

Substrate was moistened with different volume of selected M-II medium to varying moisture levels before inoculation. With an increase in the moisture content up to 60 % (w/w), the fungal growth and ligninase production by *S. commune* IBL-06 increased. With 60% moisture optimum activities of MnP (1531 IU/mL), LiP (1109 IU/mL) and laccase (176 IU/mL) were produced (Table 4.6 a). A further increase in the moisture content of banana stalk repressed fungal growth and enzyme production (Fig.4.6). Statistically analysis of the data (Table 4.6b) revealed a significant difference in the production of ligninases by varying moisture levels.

In SSF the microbial growth occurs on or near the surface of the solid and optimum moisture level in SSF is governed by the water holding capacity of the substrate and water requirements of the fungus (Kim *et al.,* 1985; Asgher *et al.*, 2006). Higher and lower water contents adversely affect the primary metabolic activities of microbes leading lower ligninase production in secondary growth (Rodriguez *et al*., 1998; Raghavarao *et al.,* 2003; Regina *et al*., 2008). Low moisture contents may also result in reduced solubility of nutrients, lower substrate swelling and higher water tension (Lonsane *et al.,* 1992). In a recent study the maximum laccase yield was obtained by *Ganoderma sp*. using wheat bran as substrate with 70% initial moisture content (Revankar *et al.,* 2007).

Table 4.6 (a) Activities of ligninases and biomass weight produced by *S. commune* IBL-06 with varying moisture contents*

Moisture content (%)	Ligninase activities (U/mL)			Biomass weight (g)
	MnP	LiP	Laccase	
40	619±5.1G	378±9.3G	108±4.0F	5.7±0.4G
45	951±6.2F	698±7.1F	116±1.0E	6.9±0.3F
50	1273±5.2D	856±8.3E	129±1.1D	8.0±0.1D
55	1389±8.3C	915±4.2C	158±5.0C	8.6±0.3B
60	1531±7.5A	1109±5.1A	177±3.1A	9.2±0.8A
65	1467±7.7B	1101±5.5B	176±3.5B	8.4±0.2C
70	1017±8.1E	912±7.3D	90±2.7G	7.5±0.3E

* Medium, M-II; pH, 4.5; temperature, 25°C; incubation time, 8 days

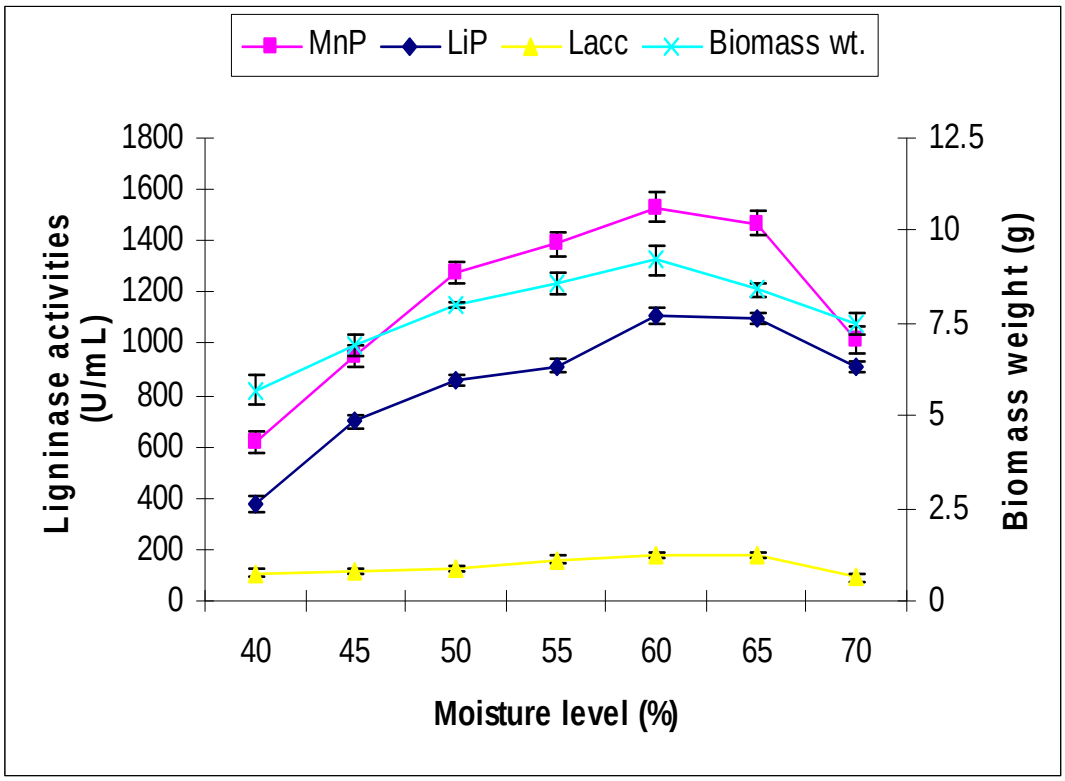


Fig. 4.6: Effect of varying moisture level on ligninases and biomass production by *S. commune* IBL-06.

Table 4.6 (b): Analysis of variance of the data on activities of ligninases with varying moisture levels

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatments	3	12490363	12490363	4163454	80.96	0.000
Block	6	1076503	1076503	179417	3.49	0.018
Error	18	925686	925686	51427		
Total	27	14492551				

* = Significant (P≤0.05)

4.1.7 Selection of additional carbon source

Different carbon sources were used as additional carbon supplements in banana stalk SSF medium under pre-optimized conditions to enhance fungal growth and production of lignolytic enzymes. All carbon sources enhanced fungal growth and ligninase synthesis by *S. commune* IBL-06. Glucose was the most enhancing carbon supplement and it produced maximum biomass (10.5g) and MnP (2211 IU/mL), LiP (1607 IU/mL) and laccase (298 IU/mL) as shown in table 4.7. The results depicted in fig. 4.7 showed that maximum ligninase production was achieved in glucose containing medium but maximum biomass weight was harvested from the flasks receiving molasses as carbon sources. It was noted that with the addition of glucose there was a significant shortening of lag phase and ligninase secretion was maximum only in 5 days. Analysis of the variance (Table 4.7 b) of the data revealed a significant difference in production of ligninase enzymes by different carbon sources.

Glucose and corn-steep liquor enriched liquid medium was found to promote cell growth and ligninase production by *Phanerochaete chrysosporium* (Ferrara *et al.,* 2002). Addition of glucose as carbon source also enhanced growth of *P. sajor-caju* and production of laccase, LiP and MnP (Massadeh *et al.,* 2010). However, use of fructose instead of glucose resulted in the 100 fold increase in laccase production by *Basidiomycete* CECT 20197 (Mansur *et al*., 1997). LiP and MnP have different sensitivities to carbon and nitrogen content of the medium; supplementing nitrogen during cultivation represses MnP but can stimulate LiP production (Bonnarme *et al*., 1990).

Additional carbon sources have also been reported to enhance ligninase formation by WRF for achieving maximum yield of LiP (Robinson *et al.,* 2001; Selvam *et al.,* 2006; Bonnarme *et al.,* 1991; Istvan *et al.,* 2000). Increase in lignin peroxidase (LiP) activity was observed upon supplementation of medium with veratryl alcohol by *Phanerochaete chaysosporium* (Collins and Dobson, 1995; Linko and Haapala, 2004).

Table 4.7 (a) Activities of ligninases and biomass weight produced by *S. commune* IBL-06 with varying carbon sources*

Carbon sources (1g)	Ligninase activities (U/mL)			Biomass weight (g)
	MnP	LiP	Laccase	
Control	1471±8.1D	1079±5.1E	125±3.5G	9.3±0.5B
Glucose	2211±6.5A	1607±4.2A	298±7.1A	10.5±0.4A
Galactose	1547±8.6B	1558±9.4B	201±3.1C	7.6±0.5D
Fructose	1478±9.1C	1209±4.5C	158±3.2F	7.4±0.7E
Lactose	1309±7.2E	1101±6.7D	161±2.1E	6.9±0.4G
Sucrose	1281±8.2F	989±7.3F	161±2.3E	6.8±0.3H
Maltose	1141±7.5G	979±3.3G	179±3.2D	7.1±0.4F
Molasses	1119±9.2H	1589±6.4H	285±4.1B	8.6±0.5C

* Medium, M-II; Moisture, 60%; pH, 4.5; temperature, 25^{o}C; inoculum size, 3 mL; incubation time, 5 days

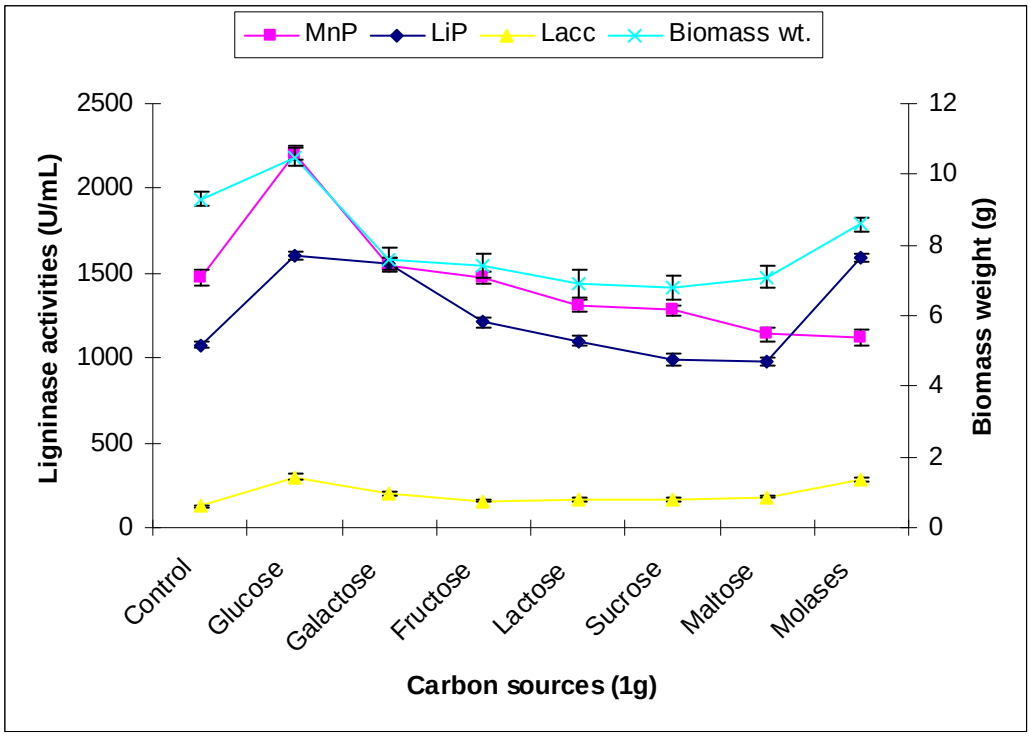


Fig. 4.7: Effect of different carbon sources on ligininase and biomass production by *S. commune* IBL-06

Table: 4.7 (b) Analysis of variance of the data on activities of ligninases with varying carbon sources

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatments	3	25339600	25339600	8446533	519.41*	0.000
Block	6	148174	148174	24696	1.52	0.228
Error	18	292711	292711	16262		
Total	27	25780484				

* = Significant (P≤0.05)

4.1.8 Selection of nitrogen source

To investigate the effect of nitrogen addition on ligninase production by *S. commune* IBL-06 in banana stalk medium different nitrogen additives were used under pre-optimized conditions. It was noted that all nitrogen sources, except ammonium tartrate enhanced ligninase production. As in case of carbon sources, addition of nitrogen also shortened the lag phase and optimum enzyme activities of were produced in only 3 days (Table 4.8a). The maximum biomass weight (7.0g), and activities of MnP (2718 IU/mL), LiP (2050 IU/mL) and laccase were harvested in the medium containing NH_4NO_3 as nitrogen supplement, followed by $(NH_4)_2SO_4$ and $(NH_4)_2H_2PO_4$ (Fig 4.8). Ammonium tartrate showed growth inhibition probably because it was already a component of the basal nutrient medium and further addition of this salt increased its concentration to inhibitory levels. Statistical analysis through ANOVA ($P \leq 0.05$) showed significant difference in ligninase production with different nitrogen sources (Table 4.8b). Comparison of means showed that there was non-significant difference between enzyme activities produced with $(NH_4)_2H_2PO_4$, $(NH_4)_2SO_4$ and peptone.

The source and concentration of nitrogen are very significant factors in regulating the synthesis of lignolytic enzymes by WRF (Mikiashvili *et al*., 2005; Songulashvili *et al*., 2007). However, different WRF strains behave differently in nitrogen enriched media. LiP and MnP genes also have different sensitivities to nitrogen source and content of the medium. The best nitrogen sources for peroxidases production by *Pleurotus species* were peptone in a concentration of 0.5% and NH_4NO_3 with a nitrogen concentration of 30 mM, respectively (Stajic *et al*., 2006). In line with our findings, the best nitrogen source was NH_4NO_3 for production of MnP and Laccase by *Ganoderma leucidum* (Stajic *et al*., 2010). Maximum LiP was produced in solid state cultures of *P. chrysosporium* with the addition of low concentration of peptone, yeast extract and Tween-80 (Asghar *et al*., 2006), where as yeast extract was the best nitrogen source for enhancing fungal biomass and ligninase yield by 41-69% by *Phanerochaete chrysosporium* as compared to a control medium(Raziye and Nurdan, 2007).

Table 4.8 (a) Activities of ligninases and biomass weight produced by *S. commune* IBL-06 with varying nitrogen sources*

Nitrogen sources (1g)	Ligninase activities (U/mL)			Biomass weight (g)
	MnP	LiP	Laccase	
Control	2213±15.5F	1607±8.2G	288±5.1F	7.0±0.4B
Urea	2299±13.4E	1786±9.5E	299±6.2E	7.1±0.5A
$(NH_4)_2H_2PO_4$	2480±11.6D	1896±6.1D	315±4.2C	6.3±0.7E
$(NH_4)_2SO_4$	2519±12.8C	2043±5.1B	301±8.3D	6.9±0.4C
$(NH_4)_2NO_3$	2718±8.5A	2050±5.2A	327±4.5A	7.0±0.3B
Peptone	2619±9.9B	1789±9.3F	318±5.5B	7.0±0.4B
Amm. tartarate	1858±6.3G	1954±6.3C	245±7.6G	6.4±0.5D

* Medium, M-II; Moisture, 60%; pH, 4.5; temperature, 25°C; inoculum size, 3 mL; glucose, 1 g; incubation time, 3 days

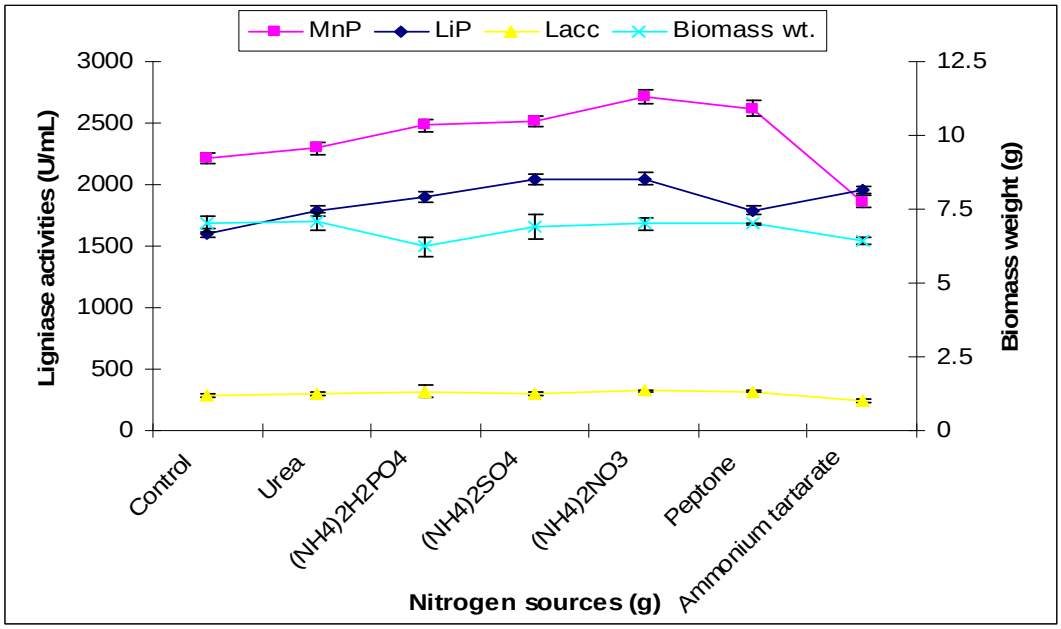


Fig. 4.8: Effect of different nitrogen sources on ligninases and biomass production by *S. commune* IBL-06

Table 4.8 (b): Analysis of variance of the data on activities of ligninases produced by *S. commune IBL-06* with different nitrogen sources

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatments	3	28351015	28351015	9450338	420.19*	0.000
Block	6	221689	221689	36948	1.64	0.193
Error	18	404829	404829	22490		
Total	27	28977533				

* = Significant (P≤0.05)

4.1.9 Optimization of Carbon: Nitrogen ratio

After selection of best carbon and nitrogen sources, the effect of varying C: N ratio on ligninase production by *S. commune IBL-06* in banana stalk medium under optimum conditions was studied. Maximum activities of LiP (2087 IU/mL), MnP (2917 IU/mL) and laccase (340 IU/mL) were produced C: N ratio of 20:1 (Table 4.9). It was observed that ligninases production steadily increased with an increase in C: N ratio from 5:1 to 20:1 and with further increase after optimum point there was a decrease in enzyme activities and fungal biomass weight (Fig 4.9). Statistical analysis of data (Table 4.9b) revealed a significant (P≤0.00) effect of varying C: N ratios on ligninases production.

The effects of C: N ratio was more pronounced as compared to carbon and nitrogen sources. Increase in C: N ratio caused decrease in biomass weight suggesting inhibition of fungal growth by increasing the nitrogen content. Carbon and nitrogen are critical nutritional variables in the production of LiP and MnP by WRF. Excess carbon and nitrogen repress ligninolytic activities of WRF (Pascal *et al*., 1991). At low C: N ratios, the fungi are carbon starved and under high-nitrogen conditions the ligninase production is considerably reduced (Xiaoping and Xin 2008). On the other hand, at higher C: N ratios, an imbalance between very high carbon and very low nitrogen content of the medium, leads to fungal growth inhibition. .

Table 4.9 (a) Activities of ligninases and biomass weight produced by *S. commune* IBL-06 with varying carbon to nitrogen ratio*

Nitrogen sources (1g)	Ligninase activities (U/mL)			Biomass weight (g)
	MnP	LiP	Laccase	
5:1	2255±9.0E	1670±7.2E	200±5.2E	6.2±0.4F
10:1	2365±10.7C	1732±7.1D	227±5.3D	8.9±0.5D
15:1	2712±8.2B	2001±6.5B	288±4.3B	9.2±0.7C
20:1	2917±10.5A	2087±7.3A	340±6.5A	9.8±0.8A
25:1	2285±9.6D	1989±5.4C	263±9.1C	9.5±0.6B
30:1	1901±8.3F	1400±6.1F	155±4.3F	8.5±0.3E

* Medium, M-II; Moisture, 60%; pH, 4.5; temperature, 25°C; inoculum size, 3 mL; carbon source, glucose; nitrogen source, $(NH_4)_2NO_3$; incubation time, 3 days

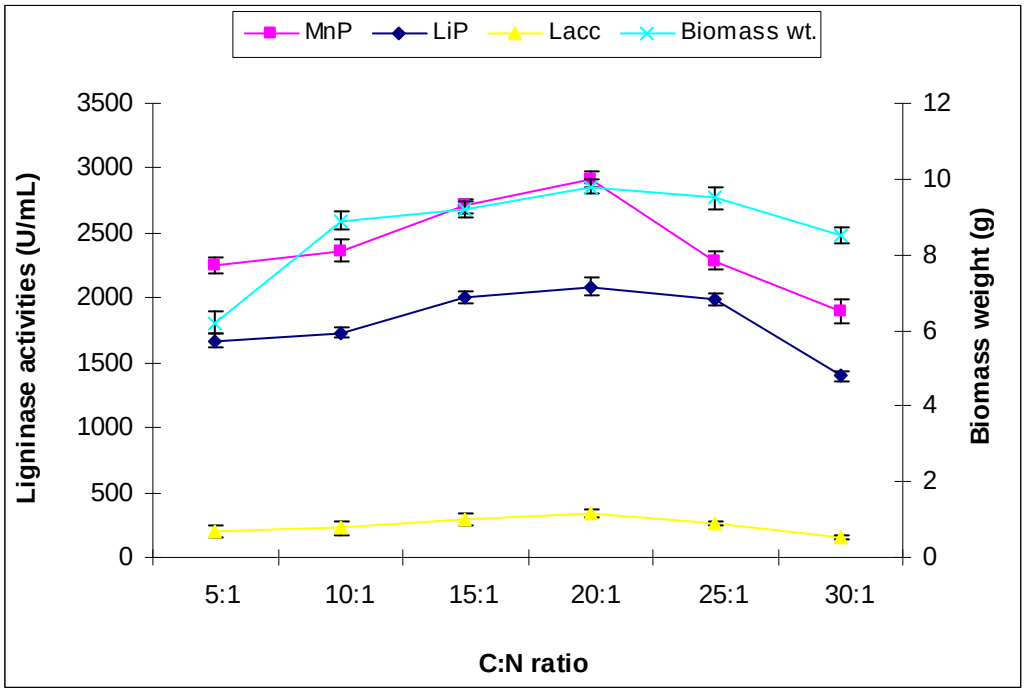


Fig. 4.9: Effect of varying C: N ratios on ligninase and biomass production by *S. commune* IBL-06

Table 4.9 (b): Analysis of variance of the data on ligninase production with varying C: N ratios

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	37859376	37859376	12619792	490.55	0.000
Block	5	330844	330844	66169	2.57	0.071
Error	15	385889	385889	25726		
Total	23	38576110				

* = Significant (P≤0.00)

4.1.10 Effect of mediators

Veratryl alcohol, $MnSO_4$, Oxalate, ABTS and H_2O_2 act as mediators of different enzymes of white rot fungi. The mediators were used to enhance liganse production by *S. commune* IBL-06. in pre-optimized fermentation medium of banana stalk. It was observed that all mediators had stimulatory effects of ligninase production by the fungus but their effects on different enzymes were highly variable. $MnSO_4$, vartaryl alcohol and ABTS were more effective for MnP, LiP and laccase production, respectively (Table 4.10a). Oxalate and H_2O_2 had almost similar impact on LiP and MnP production. (Fig.4.10). However, as MnP was the enzyme produced in higher activities as compared to other two enzymes, $MnSO_4$ was selected as the best mediator for ligninase production. Statistical analysis of of the data (Table 4.10b) revealed a highly significant (P≤0.05) effect of mediators on production of ligninase enzymes.

The natural fungal secondary metabolites veratryl alcohol (VA) acts as redox mediator of LiP (Christian *et al.,* 2005) that can also enhance its production by WRF. However, the main function of veratryl alcohol is protection of LiP from inactivation by hydrogen peroxide and veratryl alcohol is not inducer of the ligninolytic system (Kapich *et al*., 2004). Enzyme activities produced by *Phanerochaete chrysosporium* cultures in the absence of veratryl alcohol were lower than in the presence of veratryl alcohol (Ferrara *et al*., 2002). Mn^{2+} performs the role of mediator for MnP. MnP catalyzes the oxidation of Mn^{2+} to Mn^{3+} that forms complex with oxalate and other chelators that enhance the activity of MnP (Makela *et al*., 2005; Sundramoorthy *et al.,* 2005). MnP production by *Lentinula edodes* was enhanced by supplementing the on corncob solid state cultures with 5 mM $MnSO_4$ (Boer *et*

al., 2006). $MnSO_4$ has also previously been reported to increase the production of MnP by 2304 nkat/L, 1972 U/mL and 356 U/L (Rogalski *et al*., 2006; Asgher *et al*., 2010; Urek and Pzarlioglu, 2007) using different substrates in LSF and SSF. ABTS performs the role of laccase mediator in degradation of phenolic components of lignin and a range of phenolic pollutants (Liu *et al*., 2004; Quaratino *et al*., 2007).

Table 4.10 (a) Activities of ligninases and biomass weight produced by *S. commune* IBL-06 with varying mediators under optimum conditions*

Mediators (1mM, 1mL)	Ligninase activities (U/mL)			Biomass weight (g)
	MnP	LiP	Laccase	
Control	2987±14.5E	2095±13.0E	327±13.0B	7.88±0.2E
V. alcohol	2987±13.4E	2594±5.01A	313±17.0C	8.8±0.1D
$MnSO_4$	3418±11.2A	2401±10.0B	333±17.0A	11.5±0.1A
ABTS	3301±10.3B	2375±17.0D	286±15.0D	9.6±0.2B
Oxalate	3146±9.0D	1987±19.0F	237±15.0F	6.5±0.3F
H_2O_2	3277±8.7C	2378±16.0C	244±14.0E	8.9±0.4C

* Medium, M-II; Moisture, 60%; pH, 4.5; temperature, 25°C; inoculum size, 3 mL; carbon source, glucose; nitrogen source, $(NH_4)_2NO_3$; incubation time, 3 days; C:N ratio, 20:1

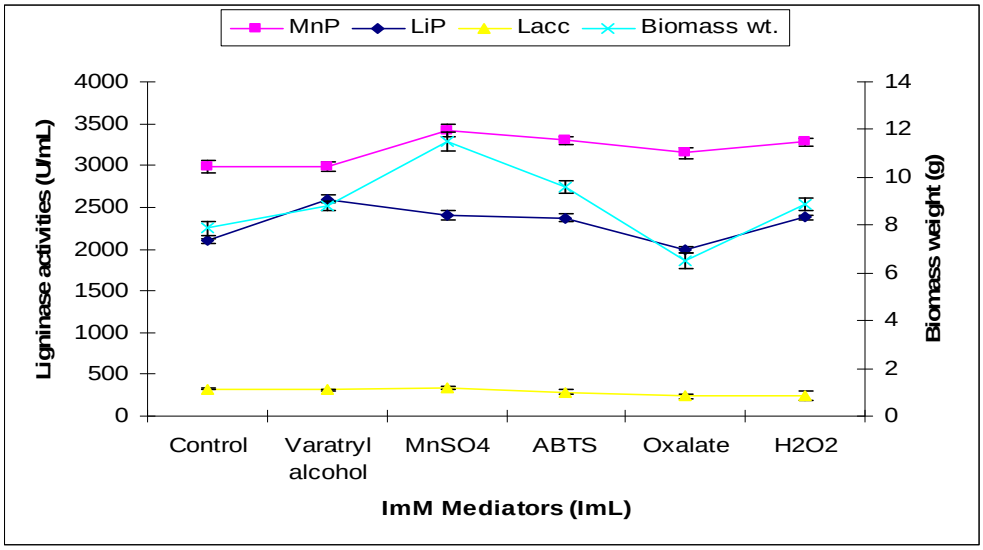


Fig. 4.10: Effect of different mediators on ligninases and biomass production by *S. commune* IBL-06

Table 4.10(b): Analysis of variance of the data on activities of ligninases produced in the presence of different mediators

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	44663090	44663090	14887697	1375.02	0.000
Block	5	104782	104782	20956	1.94	0.148
Error	15	162409	162409	10827		
Total	23	44930281				

* = Significant (P≤0.05)

4.1.11 Effects of metals ions

Various metal ions were added in the form of their salts into the optimum banana stalk medium to investigate their influence on ligninase production under optimum conditions. The optimum production of MnP (3637 IU/mL), LiP (2688 IU/mL) and laccase (331 IU/mL) was noted in the medium receiving 1mL of 1mM $MgSO_4$ in media as shown in figure 4.13. Statistical analysis of data by ANOVA (Table 4.13 b) revealed a significant (P≤0.05) effect of varying metals ion for ligninases production. Results of regression analysis under variance (Table 4.13 c in appendices) showed that the differences between means of enzyme activities under different treatments were significant (P≤0.05).

Different metal ions can enhance or inhibit the growth, cause morphological and physiological changes and may affect the reproduction of WRF (Wuyep *et al*., (2003). Different strains and species of WRF differ in their sensitivity towards metals during their growth on lignocellulosic substrates (Sathiya-Moorthi *et al*., 2007). In a recent study, the MnP production by the WRF strain *L-25* was enhanced by the addition of Mn^{+2} (Fujihara *et al*., 2010).

Table 4.11 (a) Activities of ligninases and biomass weight produced by *S. commune* IBL-06 with varying metal ions under optimum conditions*

Metal ions (1mM, 1mL)	Ligninase activities (U/mL)			Biomass weight (g)
	MnP	LiP	Laccase	
Control	3414±12.2C	2232±17.1F	327±7.1C	7.6±0.8E
$CuSO_4$	3249±16.5D	2236±19.1E	376±3.2B	10.7±0.5A
$MgSO_4$	3637±14.3A	2688±16.2A	331±5.3D	10.2±0.4B
$CaCl_2$	2955±17.1F	2344±18.3D	158±9.4F	8.9±0.3D
$FeSO_4$	3163±18.2E	2537±16.1C	246±5.4E	7.5±0.1F
$ZnSO_4$	2627±17.1G	2107±17.5G	105±7.1G	5.7±0.6G
KCl	3569±11.1B	2602±19.3B	419±5.2A	9.8±0.4C

* Medium, M-II; Moisture, 60%; pH, 4.5; temperature, 25°C; inoculum size, 3 mL; carbon source, glucose; nitrogen source, $(NH_4)_2NO_3$; incubation time, 3 days; C:N ratio, 20:1; Mediator, 1mM $MnSO_4$ (1mL)

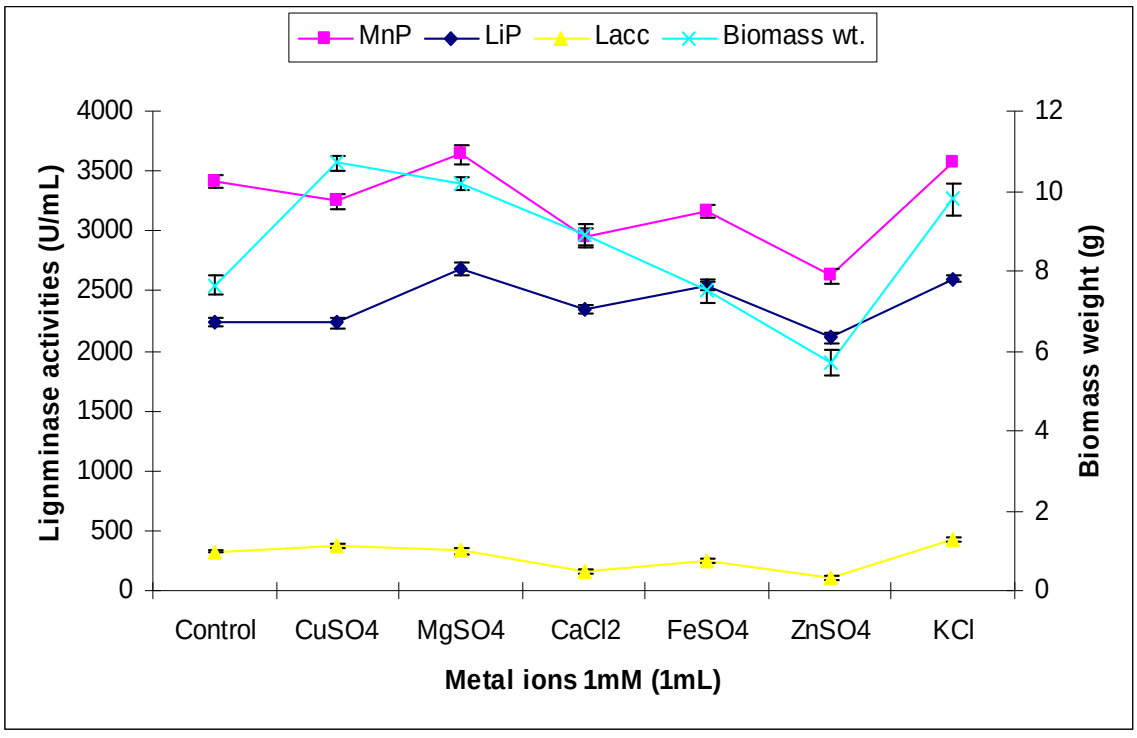


Fig. 4.11: Effect of metal ions on ligninases and biomass production by *S. commune* IBL-06

Table 4.13(b): Analysis of variance of the data on ligninase production with different metal ions

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	52529152	52529152	17509717	679.03	0.000
Block	6	602648	602648	100441	3.90	0.011
Error	18	464154	464154	25786		
Total	27	53595953				

* = Significant (P≤0.05)

4.1.12 Optimization of magnesium sulphate concentartion

Different concentrations of magnesium sulphate were used to select out the most suitable concentration for enhanced production of liginases by *S. commune* IBL-06. It was noted that 1.25 mM $MgSO_4$.$7H_2O$ showed maximum production of MnP (3714 IU/mL), LiP(2745 IU/mL) and laccase (376 IU/mL) production in 3 days under all optimum conditions (Table 4.12a). Further increase in $MgSO_4.7H_2O$ concentration decreased the liginase production as well as biomass weight (Fig.4.12). Analysis of variance of the data (Table 4.12b) revealed a significant difference in ligninase production with varying concentrations of $MgSO_4.7$ H_2O.

WRF require essential metal ions such as Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+} or Cu^{3+} as co.factors/prosthetic groups of different metabolic enzymes but these metals are toxic when present in excess (Srinivasan and Murthy, 2000). Ligninase production by *Lentinus squarrosulus* and *Psathyrella atroumbonata* was enhanced by 2-12 fold after addition of Mn^{+2} and Ca^{+2} to the lignocellulosic waste medium (Wuyep *et al*., 2003).

Table 4.12 (a) Activities of ligninases and biomass weight produced by *S. commune* IBL-06 with varying concentrations of $MgSO_4$*

Metal ions (1mM, 1mL)	Ligninase activities (U/mL)			Biomass weight (g)
	MnP	LiP	Laccase	
0.25	3099±10.1G	1987±4.7H	167±1.1G	9.0±0.2F
0.5	3312±11.1F	2145±11.6G	206±1.8F	9.5±0.2E
0.75	3516±11.3C	2345±11.2F	289±2.7D	10.2±0.4C
1	3619±10.5B	2648±12.9C	345±3.2C	10.4±0.1B
1.25	3714±14.5A	2745±15.4A	376±5.7A	10.5±0.3A
1.5	3619±11.6B	2700±12.8B	355±2.3B	9.8±0.1D
1.75	3474±14.1D	2467±13.9D	215±1.8E	8.9±0.1G
2	3455±12.2E	2368±16.9E	134±1.5H	7.1±0.1H

* Medium, M-II; Moisture, 60%; pH, 4.5; temperature, 25°C; inoculum size, 3 mL; carbon source, glucose; nitrogen source, $(NH_4)_2NO_3$; incubation time, 3 days; C:N ratio, 20:1; mediator, 1mM $MnSO_4$ (1mL); metal ion, 1mM $MgSO_4$ (1mL)

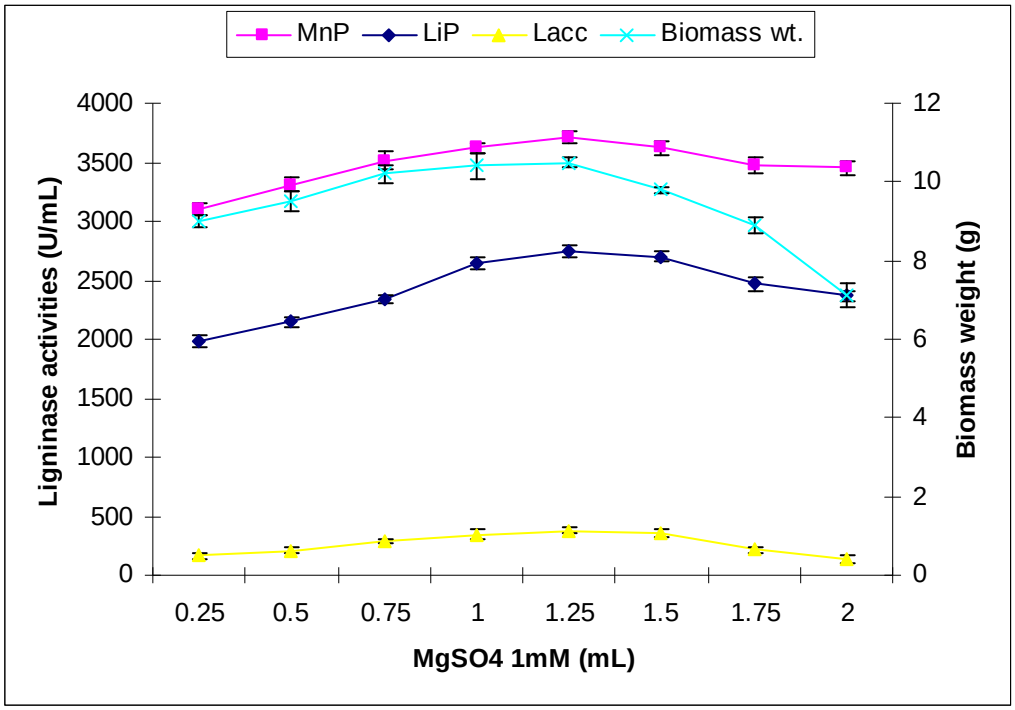


Fig. 4.12: Effect of varying concentrations of $MgSO_4$ on ligninases and biomass production by *S. commune* IBL-06

Table 4.12 (b): Analysis of variance for the on ligninase production with varying concentrations of $MgSO_4$

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	3	68211011	68211011	22737004	1435.40*	0.000
Block	7	439485	439485	62784	3.96	0.007
Error	21	332644	332644	15840		
Total	31	68983139				

* = Significant ($P \leq 0.05$)

4.2 Purification and characterization of ligninolytic enzymes

The fermented biomass produced by *S. commune* IBL-06 under optimum conditions was filtered and the filtrate was centrifuged at 3,000×g for 10 min. The supernatant was collected and used as crude enzyme extract for purification and characterization of MnP, LiP and laccase

4.2.1 Purification and characterization of MnP

4.2.1.1 Purification of MnP

The purification summary for MnP is given in table 4.13. MnP was salt out with 50% saturation with $(NH_4)_2SO_4$ to 1.4 fold purification with specific activity of 394 U/mg and yild of 60%. After Sephadex G-100 filtration (Fig. 4.13), the specific activity of MnP increased to 506 U/mg with 1.8 fold purification and 22% activity yield.

Previously, an MnP from *Phanerochaete sp* was salted out at 65% $(NH_4)_2SO_4$ saturation and MnP purification was 2.68 fold with 5.56% yield (Rajan *et al*., 2010). Hofrichter *et al*., (1999) separated the pooled ligninolytic activities and in the Sephadex column, MnP was eluted out as a single peak with tris buffer (pH 8.0) containing 0.1 M NaCl, showing that this enzyme was less anionic (Rajan *et al*., 2010). Fractionation of MnP from *P. chrysosporium* by DEAE-Sepharose, followed by ion exchange chromatography and UltragelAcA54 gel filtration chromatography resulted in 23.08% activity yield with purification factor of 5.8 (Urek and Pazarlioglu, 2004).

Table 4.13 Purification summary for MnP produced by *S. commune* IBL-06

Sr. No	Purification Steps	Total Volume (mL)	Total Enzyme Activity (IU)	Total Protein Content (mg)	Specific Activity (U/mg)	Yield (%)	Purification (fold)
1	Crude MnP	500	1857000	6628	280	100	1
2	$(NH_4)_2SO_4$ Ppt	40	1158160	2934	394	62	1.4
3	Dialysis	40	948370	2103	450	51	1.6
4	Sephadex-100	12	418546	826	506	22	1.8

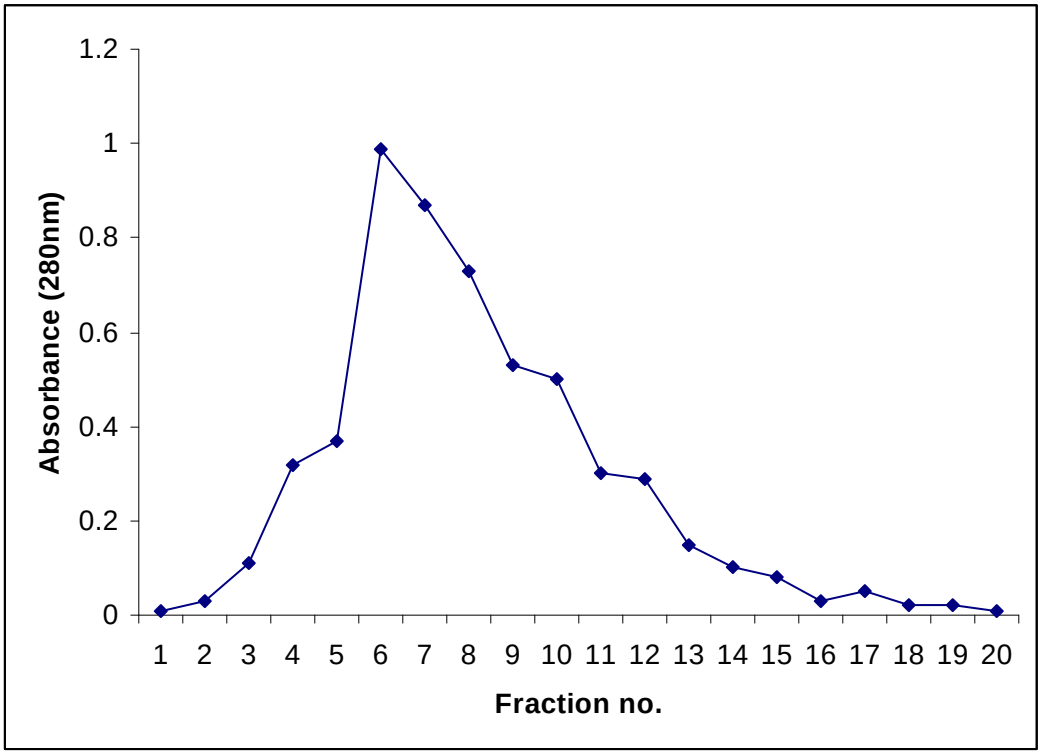
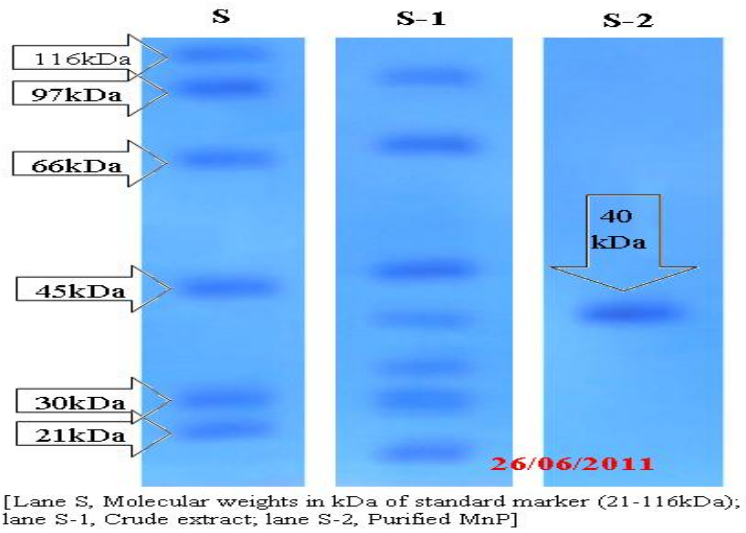


Fig. 4.13 Gel filtration chromatography of MnP produced by *S. commune* IBL-06

4.2.1.2 Native and SDS-PAGE for MnP

The enzyme purified by ammonium sulphate fractionation and gel filtration was run on Native PAGE and SDS-PAGE. On Native as well as on SDS-PAGE the enzyme yielded a single band of 40 KDa molecular mass (Figure 4.14) suggesting that the enzyme was a

monomeric protein. Manganese peroxidases (MnPs) are extracellular glycoproteins with heme prosthetic groups with molecular weights varying between 32 and 75 kDa (Ürek & Pazarlioglu 2004; Baborová *et al.,* 2006). MnPs are also secreted in multiple isoforms having differences in structure and molecular masses (Hakala *et al.,* 2005; Cheng *et al.,* 2007). MnP produced by *G. Leucidum* in SSF of wheat straw also yielded two bands of 27 KDa and 43 KDa on SDS-PAGE (Stajic *et al.,* 2010). The molar masses for MnPs from *B. Pumilus* and *Paenibacillus sp* determined by SDS-PAGE were 25 kDa and 40 kDa, respectively (Oliveira *et al.*, 2009) where as MnP from *Bjerkandera Species* was also a single polypeptide of 43 KDa (Rubia *et al*., 2002). (Shin *et al*., 1997).



[Lane S, Molecular weights in kDa of standard marker (21-116kDa); lane S-1, Crude extract; lane S-2, Purified MnP]

Fig. 4.14: SDS-PAGE for MnP

(Lane MW, Molecular weights in kDa of standard marker; lane 1, standard protein markers (β-Galactosidase, 116kDa; Phosphorylase B, 97kDa; albumin, 66kDa; ovalbumin, 45kDa; carbonic anhydrase, 30kDa and trypsin inhibitor, 21kDa); lane 2, Crude extract; lane 3, Purified MnP (43kDa)

4.2.1.3 Characterization of MnP

4.2.1.3.1 Effect of pH MnP activity

The effect of varying pH on MnP activity was investigated in buffer of pH 3.0-9.0 range using $MnSO_4$ as substrate. The pH-activity profile (Fig 4.15) showed that activity of MnP peaked at pH 5. The enzyme was fairly stable at pH 5-6 after 1hour incubation. However, a further increase in pH caused a gradual deactivation of the enzyme.

Table 4.14 Effect of pH on activity and stability of purified MnP produced from *Schyzophyllum commune* IBL-06

pH	Activity and stability of purified MnP (U/mL)	
	Activity	Stability
3	562±11.2	478±2.1
4	715±10.2	679±5.1
5	877±11.4	853±3.2
6	852±15.0	818±5.1
7	693±7.1	631±2.1
8	542±11.1	441±6.8
9	363±13.0	301±2.4

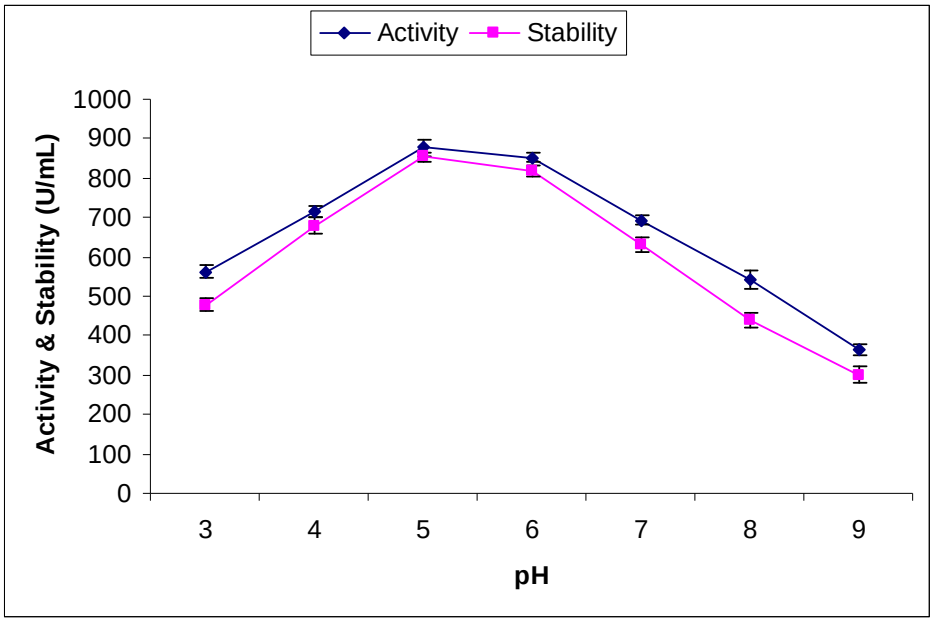


Fig. 4.15 Effect of pH on activity and stability of MnP from *S. commune* IBL-06

4.2.2.3.2 Effect of temperature on MnP activity

Temperature verses laccase activity curve (Fig. 4.16) showed an initial increase in MnP activity with temperature but at higher temperatures, the enzyme showed a rapid loss in activity due to denaturation. The optimum temperature for the $MnSO_4$ oxidation was 40°C at optimum pH but the enzyme was stable at 30-35°C.

Table 4.15 Effect of temperature on activity and stability of purified MnP produced from *S. commune* IBL-06

Temperature (°C)	Activity and stability of purified MnP (U/mL)	
	Activity	Stability
25	613±13.7	603±12.0
30	914±17.3	868±18.1
35	975±11.0	871±15.0
40	1045±13.1	801±17.1
45	1017±15.1	721±11.0
50	883±17.2	441±11.2
55	657±12.1	332±11.5
60	551±14.2	245±15.7

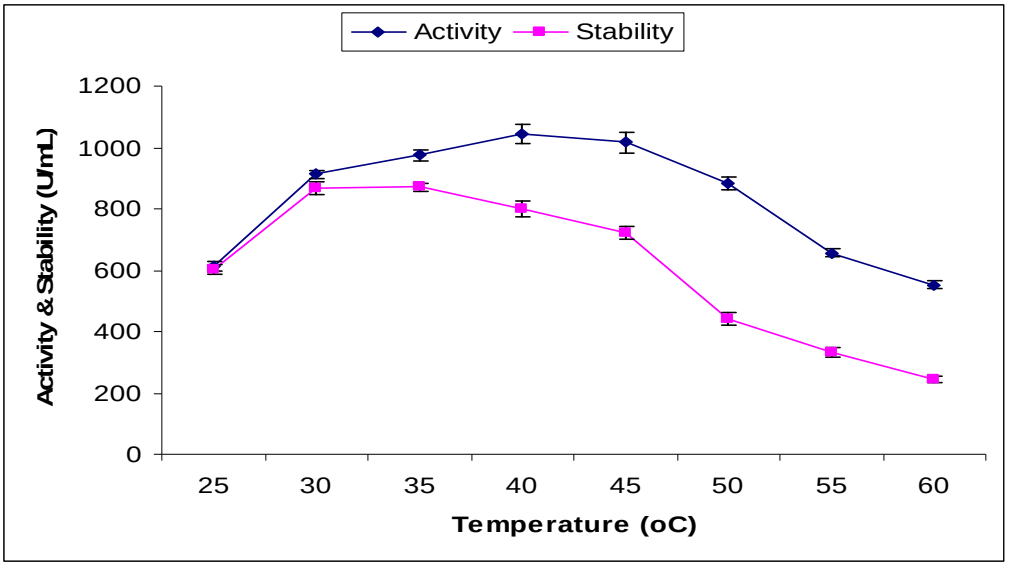


Fig. 4.16: Effect of temperature on activity and stability of MnP produced by *S. Commune* IBL-06

4.2.2.3.3 Determination of Kinetic constants K_M and V_{max}

Effect of different concentrations of $MnSO_4$ on the activity of MnP was studied and reciprocal plot of 1/S Vs 1/V was constructed (Fig. 4.17). To determine the Michaelis–Menten constants K_M and Vmax. The K_M value for MnP was 0.33mM and Vmax value was 410 mM/min.

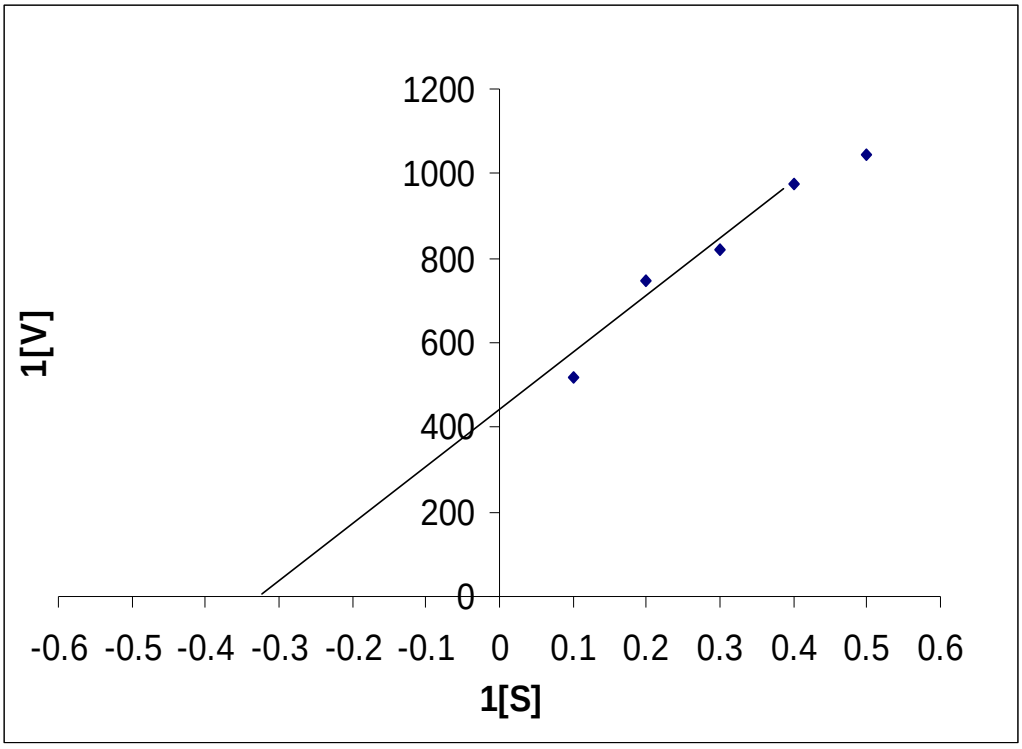


Fig. 4.17 Reciprocal plot of 1/[S] vs 1/[V] for determination of K_M and V_{max} of MnP

4.2.2.2.4 Effect of activators and inhibitors on MnP activity

The effects of varying concentrations of different activators and inhibitors such as $CuSO_4$, $CaCl_2$, EDTA, TEMED, Marcaptoethanol, $AgNO_3$ and $Pb(NO_3)_2$ on MnP activity were investigated using $MnSO_4$ as substrate. $CuSO_4$ was found to increase the activity of MnP. All other compounds were found inhibitory to MnP activity and $AgNO_3$ was the strongest inhibitor (Fig. 4.18)

Table 4.16 (a): Activity of MnP in the presence of varying concentrations of different activators/inhibitors

Activators and inhibtors	MnP activity (U/mL)				
	1 (mM)	2 (mM)	3 (mM)	4 (mM)	5 (mM)
Control	985±1.3	976±0.2	918±0.2	876±1.8	786±1.2
$CuSO_4$	1041±1.4	988±0.1	887±0.1	871±4.5	789±0.5
$MnSO_4$	1055±0.9	1051±0.5	989±2.7	897±5.5	789±3.3
$CaCl_2$	919±2.1	901±0.2	751±3.5	701±0.3	599±3.4
EDTA	743±3.1	641±0.9	622±0.4	531±0.5	377±2.9
TEMED	902±1.3	813±1.1	622±0.5	516±0.4	302±2.2
M. Ethanol	589±1.1	404±1.4	304±1.1	287±0.5	277±1.9
$AgNO_3$	725±0.4	524±1.9	421±1.7	355±0.6	260±0.1
$Pb(NO_3)_2$	647±0.3	365±2.1	346±1.3	311±0.3	200±0.6

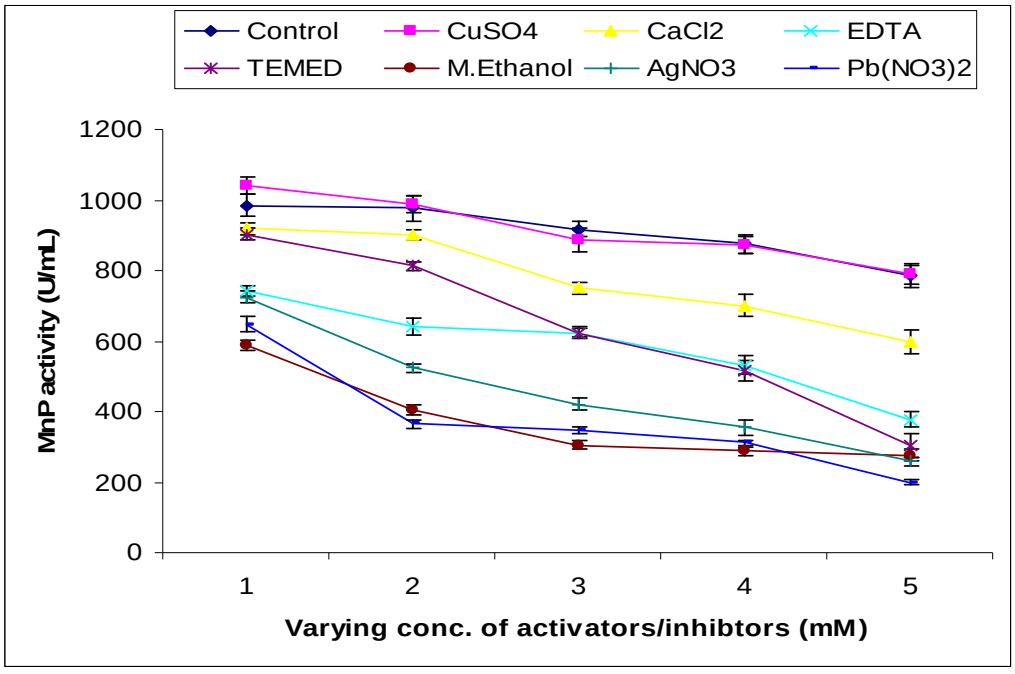


Fig. 4.18 Effect of activators and inhibitors on purified MnP produced by *S. commune* IBL-06

Overall, the MnP from *S. commune* IBL-06 had optimum pH 5 (stable at pH 5-6), optimum temperature, 40°C (stable at temperature 30 to 35°C), K_M value of 0.33mM and Vmax value of 410 mM/min for oxidation of $MnSO_4$. The enzyme was found to be activated by $CuSO_4$ and inhibited by $CaCl_2$, EDTA, TEMED, β-Marcaptoethanol, $AgNO_3$, $Pb(NO_3)_2$ (most inhibitory).

MnPs from different WRF have been reported to have molecular masses varying between 32 and 73 kDa, optimum pH of 4-7 and optimum temperature of 40-60°C (Bermek *et al*., 2004; Ürek & Pazarlioglu 2004; Hakala *et al*., 2006; Baborová *et al.,* 2006; Asgher *et al.,* 2008; Oliveira *et al.,* 2009). MnP from *P. chrysosporium* was stable in the pH range 4.5-6.0, at 25 ^oC and up to 35^0C at pH 4.5 for 1 h. and its activity was inhibited by 2mM NaN_3, ascorbic acid, β-mercaptoethanol and dithreitol. The Km values of MnP for H_2O_2 hydrogen peroxide and 2.6-dimetoxyphenol were 71.4 and 28.57 μM at pH 4.5, respectively (Urek and Pazarlioglu, 2004). The MnP2 from *Lentinula edodes* produced in SSF of corn cobs had optimum pH 4.5 and 40^oC temperature. The K_M value of MnP2 for $MnSO_4$ was $22.2\times10^{-}3$ mM and the enzyme was stable in the pH range 4.5-6.0 and at temperature up to 45 ^oC (Boer *et al*., 2006). The MnP from *Bjerkandera sp.* strain BOS55 expressed the kinetic constants K_M of 51 μM and turn over number of 59/s (Mester and Field, 1998).

P. chrysosporium MnP activity was inhibited by NaN_3, ascorbic acid, β-mercaptoethanol and dithreitol (Ürek & Pazarlioglu 2005), whereas the activity could be enhanced in the presence of cooxidants such as glutathione, unsaturated fatty acids and Tween 80 (Hofrichter, 2002; Ürek & Pazarlioglu 2005). The MnP from *Trichophyton rubrum* LSK-27 was strongly inhibited by Hg^{2+}, while Fe^{3+}, Ca^{2+} and Ni^{2+} did not cause any alteration in the activity (Bermek *et al.,* (2006).

4.2.2 Purification and characterization of LiP

4.2.2.1 Purification of LiP

The crude enzyme containing 1372500U/500 mL of LiP activity was treated with ammonium sulphate to 65 % saturation. After dialysis the enzyme was purified to 1.75 fold with specific activity of 350 U/mg (Table 4.17). The dialyzed positive fractions were pooled and run on Sephadex G-100 column that was equilibrated with in 50 mM tartrate buffer pH 4.5. The eluted fractions were monitored for absorbance at 280 nm as shown in figure 4.19. After gel

filtration the enzyme purification was 2.34 fold and specific activity of LiP was 468 U/mg with 5.2 % yield.

Table No 4.17 Purification summary for LiP produced by *S. commune* IBL-06

Sr. No.	Purification Steps	Total Volume (mL)	Total Enzyme Activity (IU)	Total Protein Content (mg)	Specific Activity (U/mg)	Yield (%)	Purification (fold)
1	Crude LiP	500	1372500	6843	200	100	1
2	$(NH_4)_2SO_4$ (65% saturation)	40	998520	3289	303	72	1.5
3	Dialysis	40	886563	2529	350	64	1.75
4	Sephadex-G100	12	69790	149	468	5.2	2.34

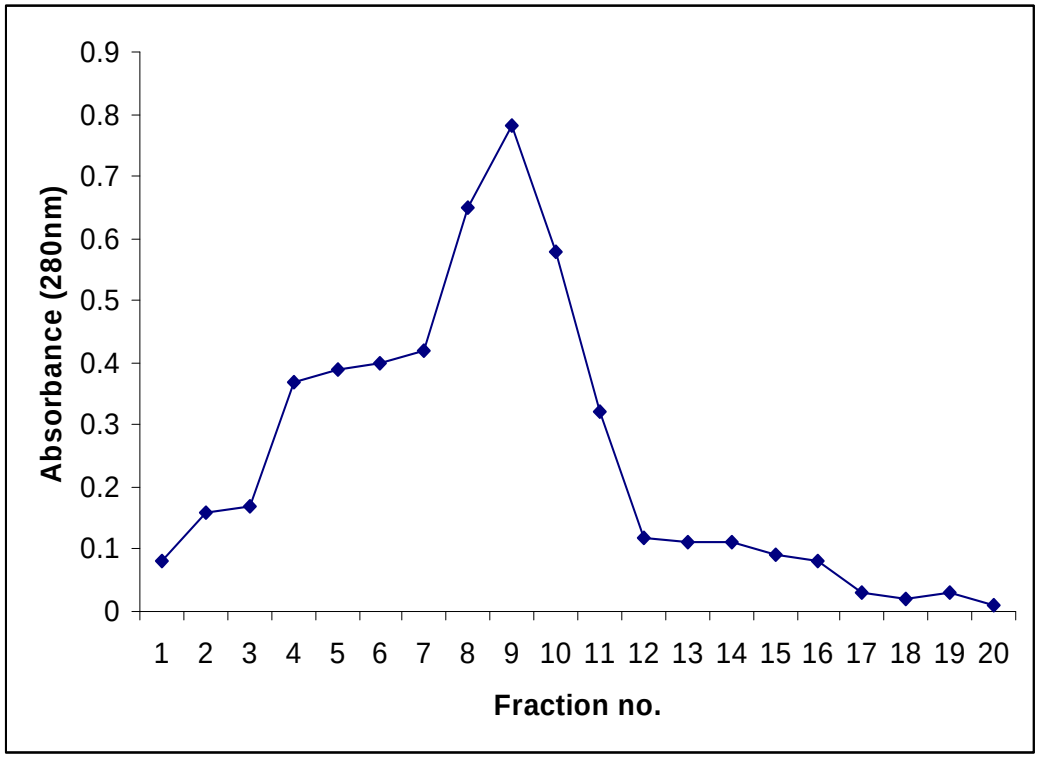


Fig. 4.19 Gel Filteration Chromatography of LiP produced by *S. commune* IBL-06

4.2.2.2 SDS-PAGE of LiP

The presence of two bands on SDS-PAGE confirmed that the enzyme was a dimeric protein having two polypeptide chains as shown in figure 4.20.

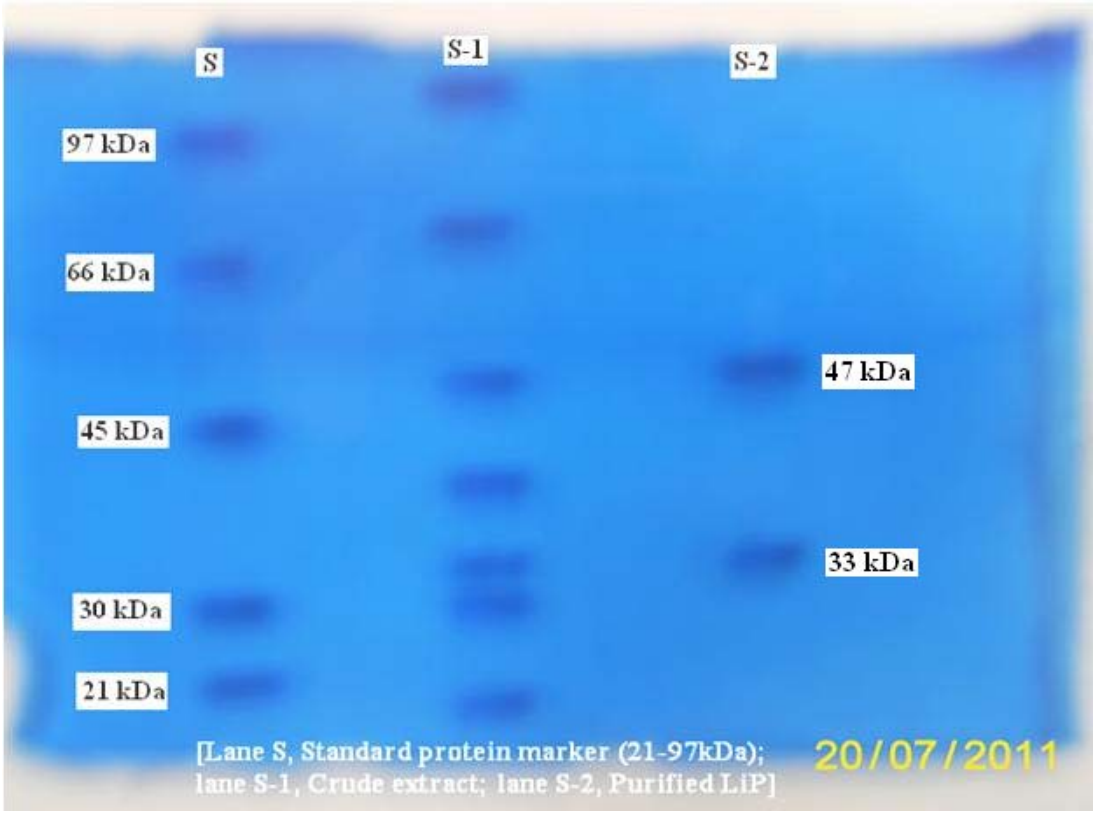


Fig. 4.20 SDS-PAGE for LiP

The procedure adopted for purification of LiP from *S. commune* IBL-06 was very simple and after ammonium sulpahte fractionation, dialysis and gel flirtation the LiP gave a single activity peak, showing that under optimum conditions only one form of LiP was produced, whereas in case of LiPs from other WRF strains multiple isozymes were reported (Tien and Kirk, 1988, Wang *et al*., 2008). An LiP isolated from *Phanerochaete sordida* YK-624. YK-LiP was purified to homogeneity by anion-exchange and gel permeation chromatography. The YK-LiP was 50 kDa molecular mass single polypeptide protein (Sugiura *et al.,* 2003). LiP from *Loweporus lividus* MTCC-1178 was purified using Amicon concentration and DEAE cellulose chromatography. The molecular weight of the purified lignin peroxidase using SDS-PAGE analysis was 40 kDa (Yadav *et al.* 2009). The molecular

masses of different isozymes of LiP purified from *Phanerocheate chryososporium* ATCC 20696 have recently been reported (Wang *et al*., 2008) to be 38 and 40 kDa, respectively. LiP isozymes from *Pleurotus sajor caju* MTCC-141 also gave 38 and 40 kDa bands, respectively (Yadav *et al.,* 2009). Thus, the molecular masses of the LiP from *S. commune* IBL-06 were in the range reported for different WRF cultures. The LiP from *Loweporus lividus* MTCC-1178 was a single polypeptide of 40 kDa on SDS-PAGE (Yadav *et al.,* 2009).

4.2.2.3 Characterization of LiP

4.2.2.3.1 Effect of pH on LiP activity

The effect of pH on LiP activity was studied. Using varatryl alcohol as substrate the optimum pH for *S. commune* IBL-06 LiP was 5 (Table 4.18). The activity of LiP increased with an increase in pH from 3-5, and a gradually decrease in activity was noted at higher pH values (Fig. 4.21). The purified LiP showed stability in its activity within the pH range of 4.0–6.0 for 1h.

Table 4.18 Effect of pH on activity and stability of purified LiP produced from *Schyzophyllum commune* IBL-06

pH	Activity and stability of purified LiP (U/mL)	
	Activity	Stability
3	459±13.1	147±1.1
4	668±17.1	466±1.0
5	755±12.0	464±2.0
6	708±5.5	459±4.0
7	658±4.3	357±1.1
8	548±12.7	312±5.0
9	464±14.8	277±1.1

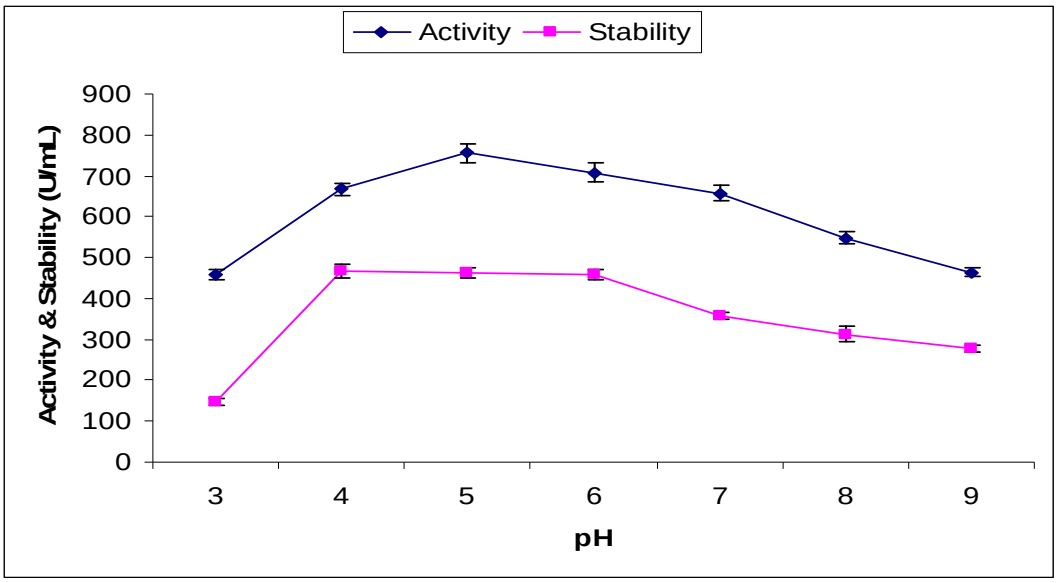


Fig. 4.21 Effect of pH on activity of purified LiP produced by *S. commune* IBL-06

4.2.2.3.2 Effect of temperature on LiP activity

The enzyme was incubated at varying temperatures for 15m min before the routine activity assay. The optimum activity of LiP on varatryl alcohol was observed at 35˚C. To investigate the stability of LiP, the enzyme was incubated at varying temperatures for 24 hrs. LiP retained good activity in temperature range of 25-35˚C but its stability decreased after incubation at higher temperatures (40, 45, 50, 55, 60˚C) as shown in figure 4.22

Table 4.19 Effect of temperature on activity and stability of purified LiP produced from *Schyzophyllum commune* IBL-06

Temperature (^{o}C)	Activity and stability of LiP (U/mL)	
	Activity	Stability
25	735±2.1	625±3.1
30	856±3.2	644±5.5
35	986±3.2	655±7.1
40	875±2.3	566±8.1
45	775±3.5	501±10
50	581±2.1	343±7.0
55	473±2.1	245±3.0
60	463±2.2	222±4.0

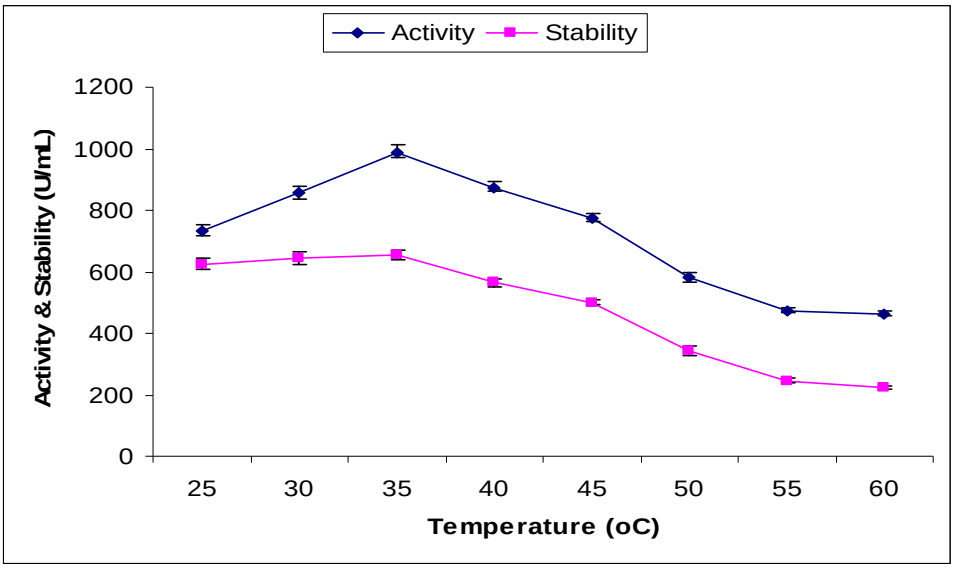


Fig. 4.22 Effect of temperature on activity and stability of purified LiP produced by *S. commune* IBL-06

4.2.2.3.3 Determination of Michaelis-Menton Constants K_M and V_{max}

The effect of varying concentrations of varatryl alcohol on LiP activity was studied and the data was used to plot a graph between 1/S and 1/Vo to determine the values of kinetic parameters. The values of K_M and V_{max} for LiP isolated and purified from solid state culture filtrates of *S. commune IBL-06* were 0.46mM and 388 mM/min, for varatryl alcohol oxidation (Fig. 4.23)

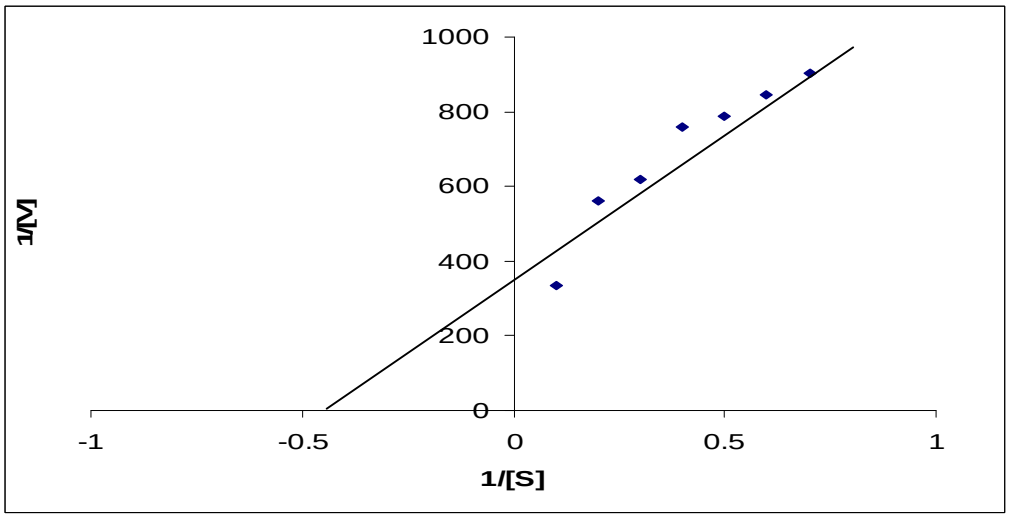


Fig. 4.23: Reciprocal plot for determination of K_M and V_{max} for LiP

4.2.2.3.4 Effect of activator/Inhibitors on purified LiP

The effect of different inorganic and organic compounds (1mM to 5 mM) on LiP activity was investigated. All concentrations of all the compounds decreased the varatryl oxidation oxidation activity of LiP (Table 4.20). EDTA, β-Marcaptoethanol and $Pb(NO_3)_2$ were found to strongly inhibit the activity of LiP as in figure 4.24.

Table 4.20 Effect of activators and inhibitors on activity of purified LiP produced from *Schyzophyllum commune* IBL-06

Activators and inhibtors	LiP activity (U/mL)				
	1 (mM)	2 (mM)	3 (mM)	4 (mM)	5 (mM)
Control	985±0.5	976±0.1	918±1.5	876±0.1	786±0.1
$CuSO_4$	825±1.3	603±1.85	601±1.2	514±0.2	413±0.5
$MnSO_4$	813±2.3	756±1.9	731±0.7	629±1.1	542±0.6
$CaCl_2$	725±0.7	701±2.1	619±0.9	413±5.2	410±0.2
EDTA	655±1.4	367±2.6	201±0.8	187±3.2	65±0.1
TEMED	778±1.8	571±3.1	306±0.6	113±1.9	109±0.1
M. Ethanol	672±0.9	411±3.2	134±0.5	98±0.7	71±0.5
$AgNO_3$	557±2.3	444±4.1	329±0.4	133±0.1	88±0.8
$Pb(NO_3)_2$	533±0.1	501±2.7	233±0.3	203±0.3	133±0.5

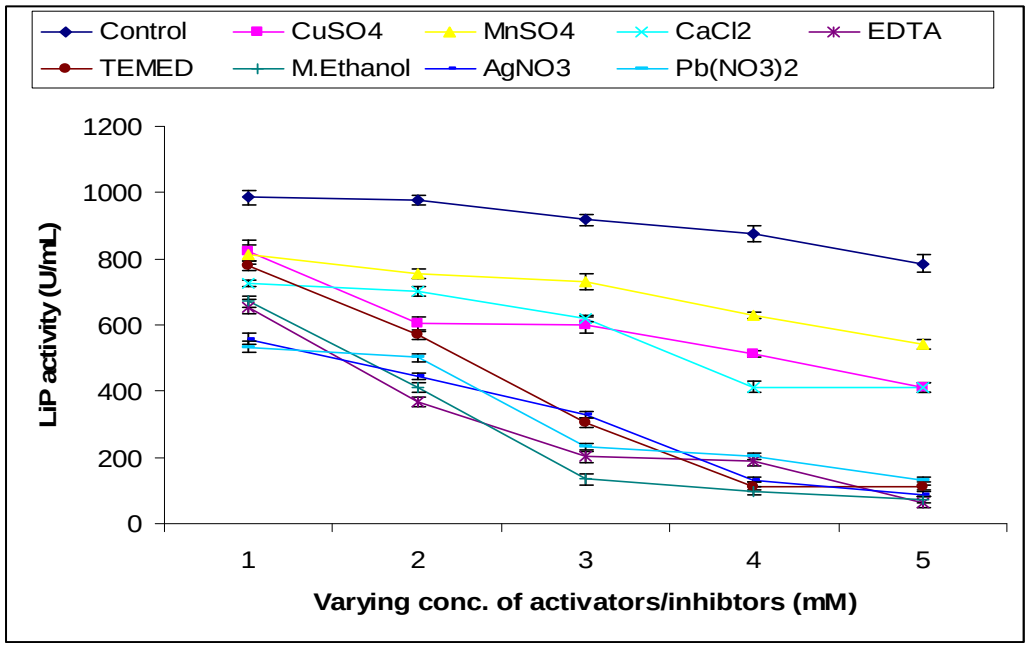


Fig. 4.24 Effect of activators and inhibitors on activity of purified LiP produced by *S. commune* IBL-06

In conclusion, LiP purified from culture filtrate of *S. commune* IBL-06 showed optimum activity at pH 5 (stable at pH 5-6) and 35°C (stable at temperature 30 to 35°C). The K_M value of LiP was 0.46mM and V_{max} was 388mM/min using varatryl alcohol as substrate. The enzyme was found to be inhibited by all organic and inorganic compounds including $CuSO_4$, $MnSO_4$, $CaCl_2$, EDTA, TEMED, β-Marcaptoethanol, $AgNO_3$, $Pb(NO_3)_2$.

LiP purified from different WRF cultures have been reported to possess molecular masses in the range of 37-50 kDa (Asgher *et al*., 2006; Hirai *et al*., 2005). pH and temperature optima for LiPs from different strains also vary significantly. The optimum activities of various WRF LiPs vary between pH 2-5 and 35-55 ^oC temperatures, respectively (Yang *et al*., 2004; Asgher *et al.,* 2007; Snajir and Baldrian, 2007). Lignin peroxidase from *Loweporus lividus* MTCC-1178 was purified using Amicon concentration and DEAE cellulose chromatography. The Km values for veratryl alcohol and H_2O_2 for the purified enzyme were 58 and 83 mM, respectively with pH and temperature optima of 2.6 and 24 ^oC, respectively (Yadav *et al.,* 2009). For LiP from *Pleurotus sajor-caju* MTCC-141 the Km values for veratryl alcohol, n-propanol, and H_2O_2 were 57 µM, 500 µM, and 80 µM, respectively (Yadav *et al*., 2009). The pH and temperature optima were 3 and 30^oC, respectively and the enzyme was uncompetitively inhibited by sodium azide with KI value of 4mM

EDTA inhibits LiP and the mechanisms of inhibition are different for different substrates (Chang and Bumpus, 2001; Aust *et al.,* 1989; Shah and Aust, 1993). Lignin peroxidase activity was inhibited about 90% by potassium cyanide and sodium azide, and the chelating agent, EDTA (Jeon *et al.,* 2002; Michels and Gottschalk, 1994). A cationic surfactant cetyltrimethylammonium bromide (CTAB) modifies the LiP conformation and lowers K_M and increases Vmax of LiP (Liu *et al*., 2003). With the addition of 2mM TEMED (N-N-N'-N'-tetramethylenediamine) and 2 mM EDTA 79 and 95% inhibition of *P. chrysosporium* LiP was noted (Chang and Bumpus, 2001). However, the non-competitive inhibition was reversed in the presence of Zn(II).

4.2.3 Purification and characterization of Laccase

4.2.3.1 Purification of laccase

Laccase activity of crude extract was 183500U/500 mL with specific activity of 158U/mg The purification steps for laccase are given in table 4.24. Laccase was salt out with ammonium sulphate saturation of 50%. After dialysis and gel filtration the enzyme was purified to 3.95 fold with specific activity of 158 U/mg. A single peak obtained in gel filtration profile (Fig. 4.21) showed that laccse was a single enzyme activity and there was no isozyme of laccase.

Table No 4.21 Purification summary of Laccase

Sr. No	Purification Steps	Total Volume (mL)	Total Enzyme Activity (IU)	Total Protein Content (mg)	Specific Activity (U/mg)	Yield (%)	Purification (fold)
1	Crude Lac	500	183500	4535	40	100	1
2	$(NH_4)_2SO_4$ Ppt	40	88750	855	104	48	2.6
3	Dialysis	40	79166	718	110	43	2.75
4	Sephadex-100	12	6031	38	158	3	3.95

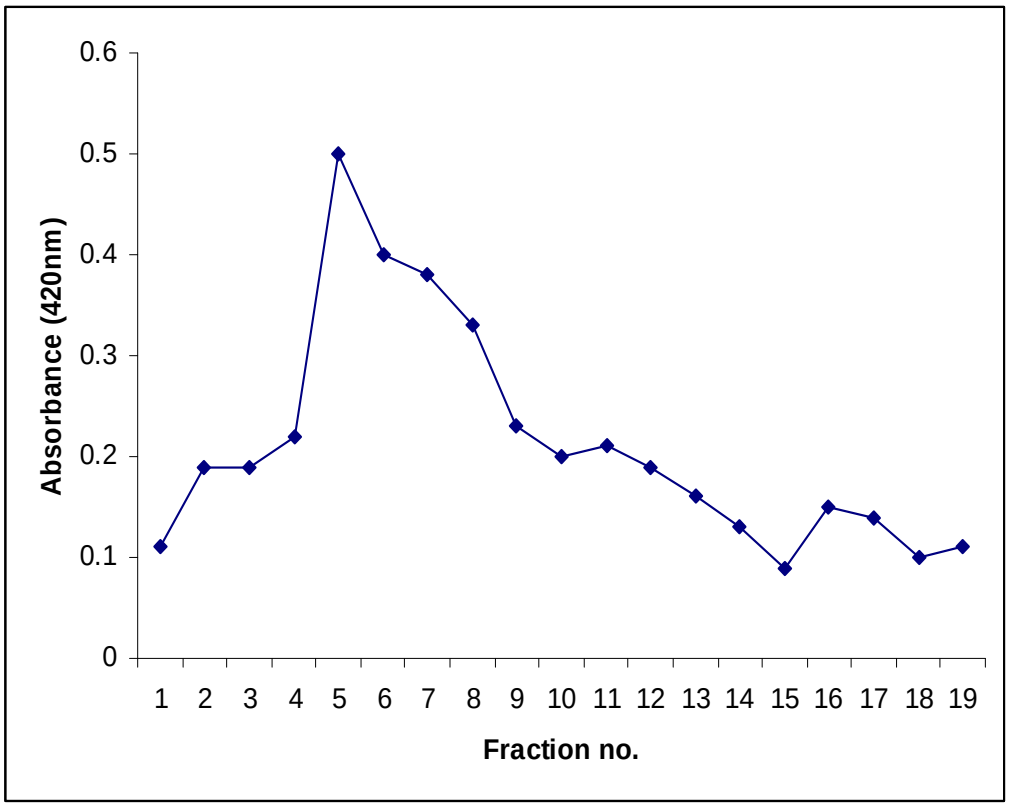


Fig. 4.25 Gel filteration chromatography of laccase produced by *S. commune* IBL-06

4.2.3.2 **Native and SDS-PAGE**

The purified laccase resolved on SDS-PAGE was a homogenous monomeric protein as indicated by a single band corresponding to 63 kDa relative to the standard molecular weight markers (Fig.4.26). A single band on SDS-PAGE showed that the enzyme is a single polypeptide protein.

A-el-Gammal *et al*., (2001) and Mtui and Nakamura (2008) also achieved fractionation and isolation of laccses by 50 and 80% $(NH_4)_2SO_4$ saturation, respectively followed by chromatographic purification techniques for recovery of pure ligninolytic enzymes. The molecular mass obtained for laccase from *S. commune IBL-06* was in the range of 55-90 kDa already reported for laccases from other sources (Ryan *et al.,* 2003; Yaropolov *et al.,* 1994; Anne-Marie *et al.,* 2004; Murugesan *et al*., 2006; Salony *et al*., 2006; Zouari-Mechichi *et al*., 2006; Quaratino *et al*., 2007).

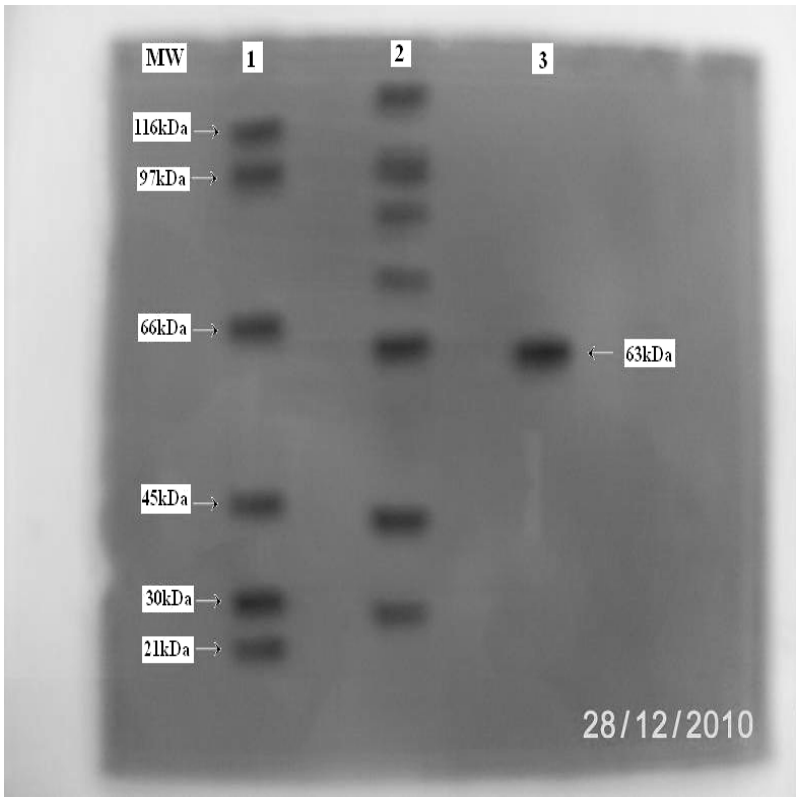


Fig. 4.26 SDS-PAGE of laccse

(Lane MW, Molecular weights in kDa of standard marker; lane 1, standard protein markers (β-Galactosidase, 116kDa; Phosphorylase B, 97kDa; albumin, 66kDa; ovalbumin, 45kDa; carbonic anhydrase, 30kDa and trypsin inhibitor, 21kDa); lane 2, Crude extract; lane 3, Purified extra cellular laccase (63kDa)

4.2.3.3 **Characterization of Laccase**

4.2.3.3.1 **Effect of pH on partially purified lacasse**

Laccse activity was studied at varying pH (pH 3-7). The optimum pH of partially purified laccase produced by *S. commune* IBL-06 was 6.0 for ABTS oxidation (Table 4.22). A furter rise in pH was found to deactivate the laccse protein. The purified laccase remained stable within the pH range of 6.0-7.0 after 1h incubation (Fig. (4.27). The enzyme retained only 22% of its activity at pH 9 after 24 h incubation at 35°C.

Table 4.22 Effect of pH on activity and stability of partially purified Laccase produced from *Schyzophyllum commune* IBL-06

pH	Activity and stability of purified laccase (U/mL)	
	Activity	Stability
3	83±2.1	88±1.7
4	125±7.0	145±5.1
5	195±1.5	170±1.8
6	243±9.1	181±2.3
7	231±3.2	180±2.1
8	207±7.1	166±1.1
9	107±5.3	155±1.6

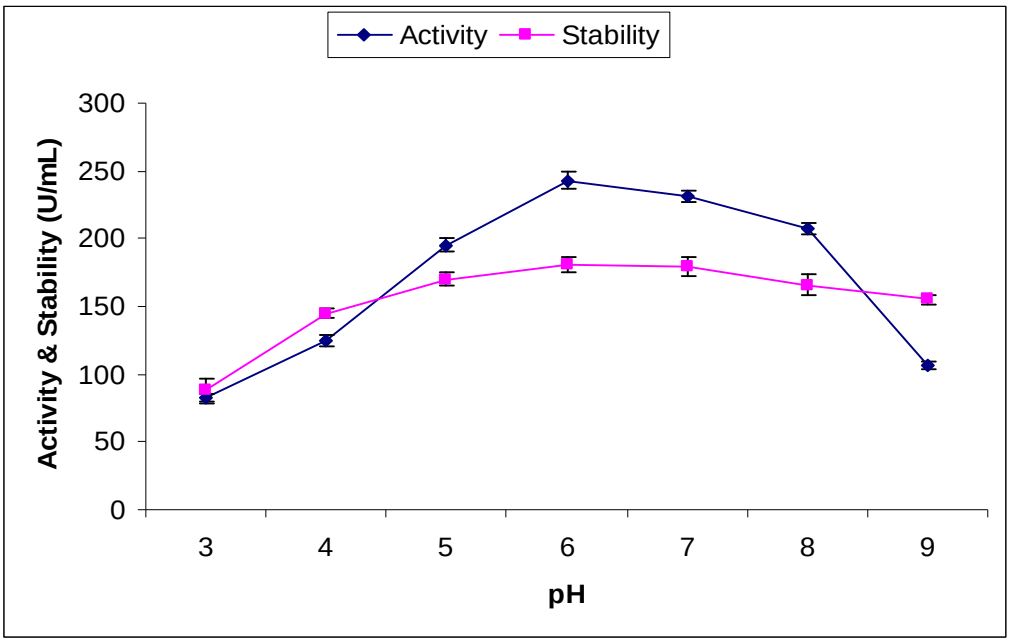


Fig. 4.27 Effect of pH on activity and stability of partially purified laccase produced by *S. commune IBL-06*

4.2.3.3.2 Effect of temperature on partially purified lacasse

The optimum activity of purified lacasse was observed at 40^{o}C (Table 4.28). An initial increase in temperature increased the enzyme activity, possibly by enhancing the kinetic energy of the molecules and increasing the interaction between enzyme active site and

interacting groups of the substrate. The enzyme was stable at 30-35°C for I hour. It retained only 33% of its activity at 60˚C after incubation for an hour as shown in figure 4.26.

Table 4.23 Effect of temperature on activity and stability of partially purified Laccase produced from *Schyzophyllum commune* IBL-06

Temperature (°C)	Activity and stability of purified laccase (U/mL)	
	Activity	Stability
25	185±1.1	121±3.4
30	211±5.1	266±1.1
35	280±1.1	267±5.2
40	285±4.0	255±2.1
45	211±3.1	203±2.7
50	175±2.1	95±1.9
55	155±1.0	77±1.3
60	135±1.0	33±1.1

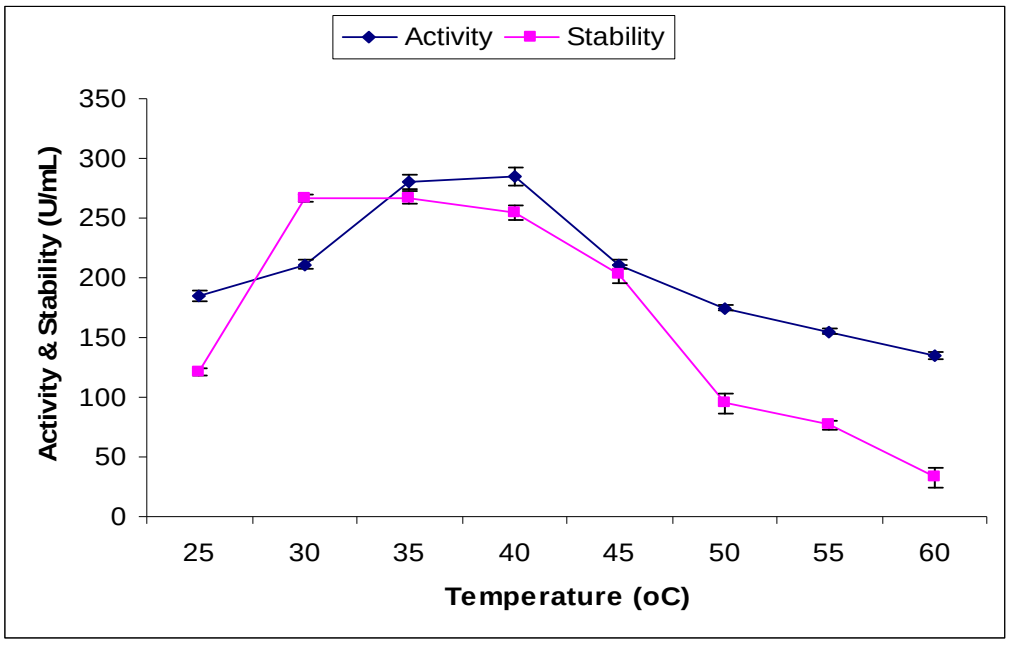


Fig. 4.28 Effect of temperature on activity and stability of purified laccase produced by *S. commune IBL-06*

4.2.3.3.3 Determination of Kinetic constants K_M and V_{max}

The K_M and V_{max} values for purified laccase were determined using varying concentrations of ABTS as substrate. Enzyme activities were measured under standard assay conditions and results were used to construct reciprocal plot using Line-Weaver and Burk equation. Reciprocal of laccase activity (1/[V]) in U/mL was plotted against reciprocal of substrate concentration (1/[S]) in µM (Fig.4.29). The values of kinetic parameters K_M and V_{max} for purified laccase were 0.025mM and 80mM/min respectively using ABTS as substrate.

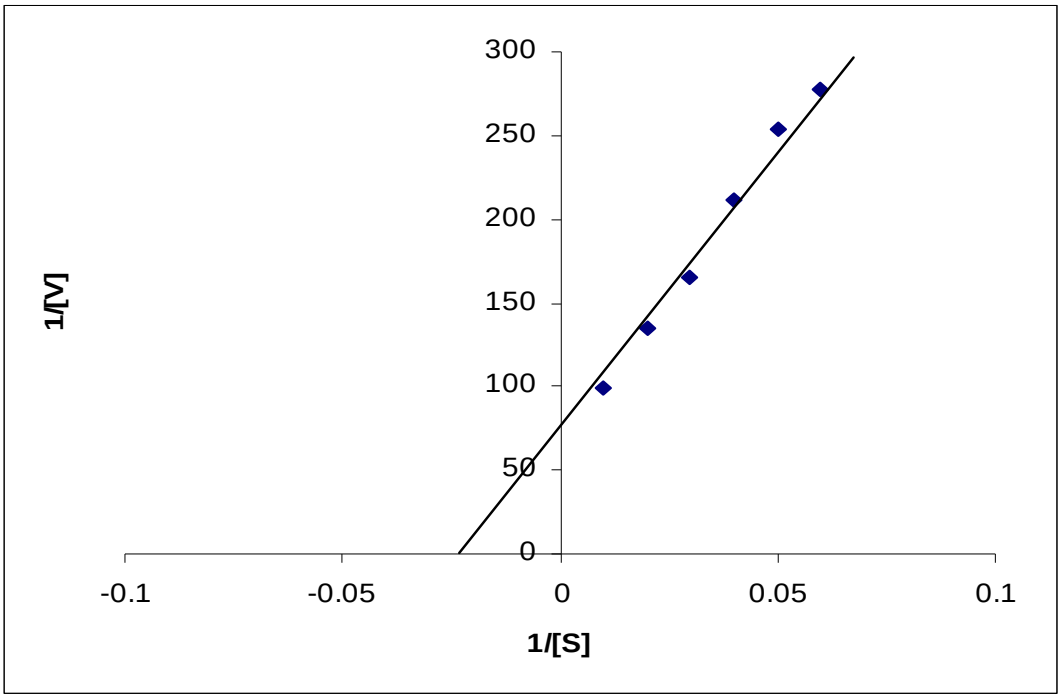


Fig. 4.29 Reciprocal plot for determination of K_M and V_{max} for laccase

4.2.3.3.4 Effect of organic and inorganic activators/inhibitors

Among the various ions and organic compounds were used, only 1mM $CuSO_4$ increased the activity of laccase (Table 4.24). While $AgNO_3$, TEMED, marcaptoethanol there was almost 25% reduction in laccase activity. The inhibitory effect of $MnSO_4$ was non-significant on laccase activity.

Table 4.24 Effect of activators and inhibitors on partially purified Laccase produced from *Schyzophyllum commune* IBL-06

Activators and inhibtors	Laccase activity (U/mL)				
	1 (mM)	2 (mM)	3 (mM)	4 (mM)	5 (mM)
Control	290±0.5	280±1.1	278±0.1	277±1.3	254±2.7
$CuSO_4$	311±0.3	315±1.2	296±0.1	270±2.1	266±3.1
$MnSO_4$	286±0.6	280±1.3	255±0.3	235±5.1	221±4.1
$CaCl_2$	255±0.6	235±0.7	200±0.5	185±1.1	132±0.1
EDTA	215±0.7	201±0.8	167±0.6	154±1.7	14±1.1
TEMED	201±0.1	176±0.6	91±0.7	89±0.1	41±0.1
M. Ethanol	109±0.2	56±0.02	33±0.5	12±0.1	6±0.03
$AgNO_3$	97±0.3	77±0.01	22±0.03	13±0.1	3±0.01
$Pb(NO_3)_2$	142±0.5	113±0.3	58±0.01	50±0.1	39±0.01

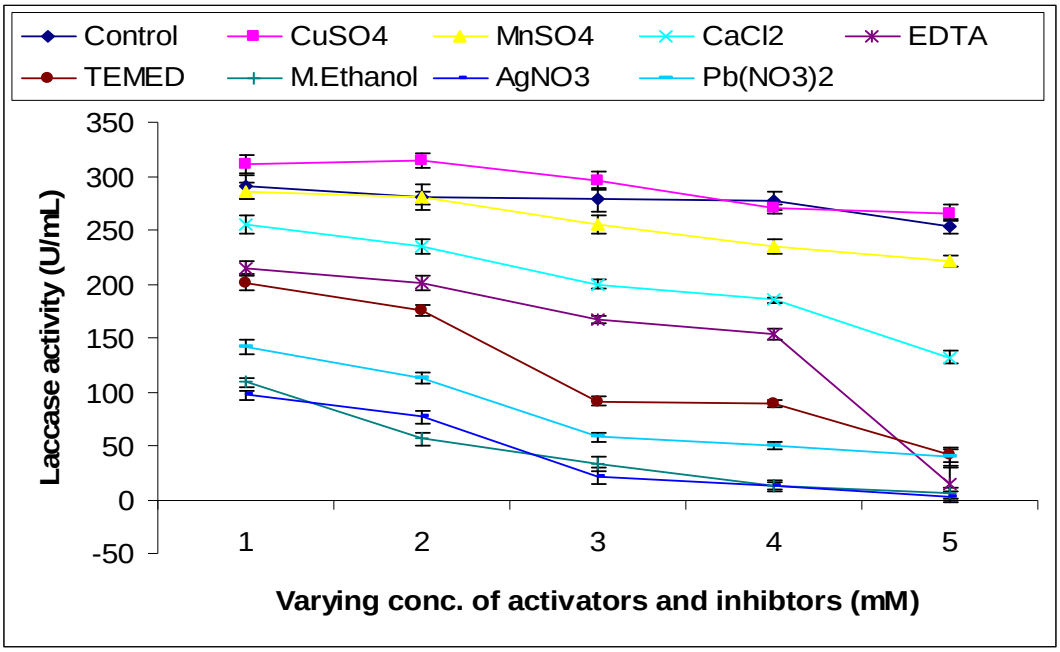


Fig. 4.30 Effect of metals ions and organic compound on purified partially laccase produced by *S. commune* IBL-06

Laccases from different WRF have been reported to show optimum activities in the pH range of 2-10 and temperature range of 40-65 ^{o}C (Murugesan *et al*., 2006; Quaratino *et al*., 2007; Asgher *et al*., 2008). Two laccase isozymes from *Trametes trogii* had optimum pH 2.0 & 2.5 and temperature optimum around 50^{o}C. *Cerrena unicolor* 137 laccase displayed optimum activity at 60^{o}C temperature around and *Panus tigrinus* laccse isozymes showed similar optimum pH 7.0 and 60-65°C temperature (Cadimaliev *et al*., 2005; Mäkelä *et al*., 2006; Michniewicz *et al*. 2006; Zouari-Mechichi *et al.,* 2006).The enzyme purified from culture filtrate of *Lentinula (Lentinus) edodes* was completely stable in a large pH range (4.0–6.0) and presented an optimum pH value of 4.5 (Boer *et al.,* 2004). An other laccase from *Mauginiella sp* had optimal activity at pH 2.4 and remained reasonably stable within the pH range of 4.0–8.0 after 24-h incubation (Palonen *et al.,* 2003). Recently a crude laccase produced by *Trametes versicolor* having optimum pH 4.5 and 45ºC optimum temperature has been reported (Stoilova *et al.,* 2010).

The relationship between rate of reaction and concentration of substrate depends on the affinity of the enzyme for its substrate expressed as K_M (Michaelis constant) of the enzyme. However, a laccase from *Cerrena maxima* had a K_M value of 65 µM for ABTS and that from *Coriolus hirsutus* had 62.9 µM for guaiacol (Koroljova *et al.*, 1999). Three substrates ABTS, DMP and syringaldazine wewre used for determining the Km for laccase (Litthauer *et al.,* 2007) and Km for ABTS was significantly higher as compared to other substrates. The activation of laccase by Cu^{2+} may be due to the filling of type-2 copper binding sites with copper ions (Sadhasivam *et al*., 2008; Nagai *et al*., 2002). However, *Sinorhizobium meliloti* CE52G laccase was inhibited to different extents by $Fe^{3+,}Mn^{2+}$, and $Cu^{2+,}$ suggesting that these metal ions interfere with the oxidation of the classical organic laccase substrates [58]. EDTA is an inhibitor of metallo-enzymes including laccases due to its property of forming inactive complexes with inorganic prosthetic groups/cofactors of the enzyme (Sadhasivam *et al.,* 2008). However, this is not true for all laccases; *Marasmius quercophilus* and *Sinorhizobium meliloti* CE52G laccases are not significantly affected by EDTA (Rosconi *et al*., 2005; Dedeyan *et al*., 2000). Similar finding on laccse activity inhibition by NaCN (1.0 mM), $FeCl_3$ (1.0 mM), $CuCl_2$ (10.0 mM), mercaptoethanol (0.1 mM) and reduced glutathione (0.1 mM) and $MnCl_2$ have already been reported (Stajic *et al.,*

2006). EDTA and cystein were also found as inhibitors of laccase activity while strong inhibition was observed with sodium azide *(*Anni-Marri *et al*., 2004 *;* Ryan *et al*., 2003*).*

4.3 Application of ligninases for decolorization of dyes and Industrial effluents

4.31. Decolorization of textile dyes

In the present study, the ability of the ligninase enzymes produced by *S. commune IBL-06* for decolorization of different textile dyes was investigated. The crude extract of ligninolytic enzymes was used because purification is a time consuming procees involving costly chemicals and columns. To develop a cost effective and economically feasible technology, there is no need to purify the enzymes for application in textile industries. The varying volumes of crude ligninase extract were added to 100mL solutions of individual dyes prepared in distilled water and the flasks were incubated for 1hour at 35^{o}C. The enzyme extract caused maximum decolourization of Novasol direct blue dye to 80%, followed by Novasol direct yellow dye to 60%, Novasol direct red to 38% and Novasol direct black to 37% (Table 4.25). By increasing the volume of enzyme extract, the dye decolorization was enhanced in all cases (Fig. 4.23).

Table 4.25 Decolourization of textile dyes by ligninase extract produced by *S. commune* IBL-06

Ligninase extract (mL)	Dye decolourization (%)			
	Textile dyes			
	Novasol direct Black	Novasol direct Yellow	Novasol direct Blue	Novasol direct Red
2	20±0.08	19±0.9	22±1.1	17±2.1
4	29±0.3	28±0.8	47±1.1	29±2.1
6	33±1.3	57±2.1	77±2.7	37±3.5
8	44±0.01	66±0.05	80±0.03	46±0.09

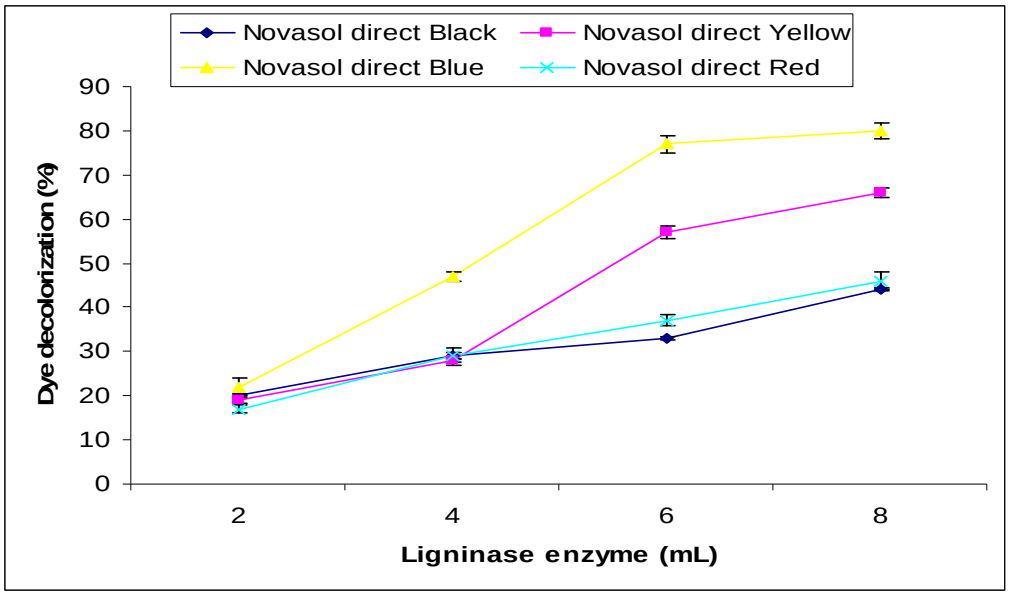


Fig. 4.31 Effect of varying volumes of ligninases extract to decolourized the textile dyes after 1hour incubation.

4.3.2 Decolourization of effluents from textile industries

Crude ligninase extract (2-8 mL) produced by S. commune under optimum conditions was incubated for 1hour with waste waters collected from different industries. The enzyme mixture (8mL) was found to maximally decolourize the MGT effluent to 80%, followed by Crescent (55%), Arzoo (50%) and Chenab (39%) textile industry effluents as shown in table 4.26. The ffluent decolorization increased with increase in volume of enzyme extract and maximum effluent color loss was noted with 8mL enzyme extract.

Table 4.26 Decolourization of textile industrial effluent by Partially purified ligninases produced by *S. commune* IBL-06

Ligninase extract (mL)	Effulent decolourization (%)			
	Chenab	Arzo	Cresent	Magna
2	28±1.1	22±2.1	18±0.1	35±4.1
4	38±1.7	25±1.9	24±3.1	40±4.1
6	39±0.5	44±0.8	46±2.1	75±2.1
8	42±1.8	52±2.1	60±3.1	80±4.5

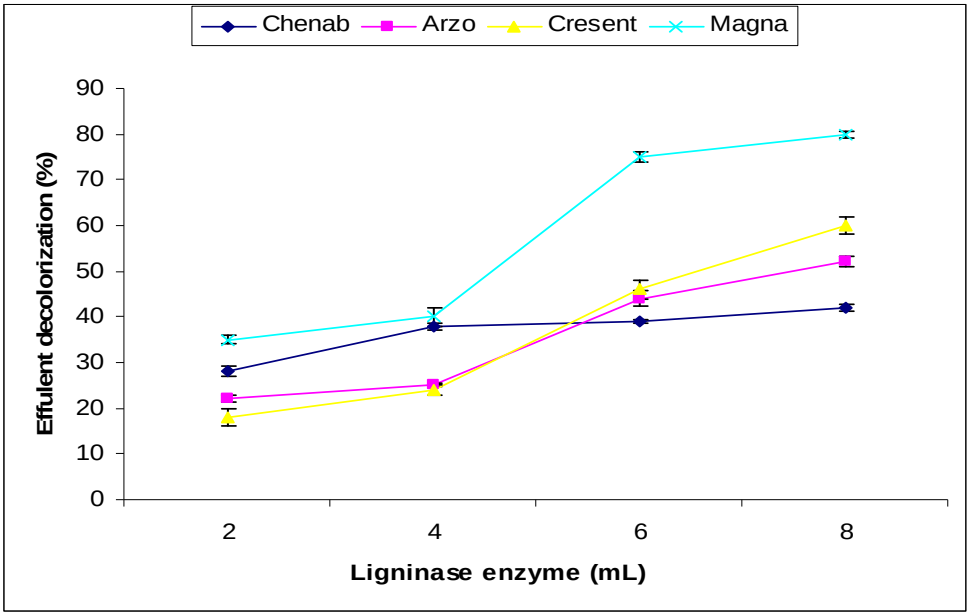


Fig. 4.32 Effect of varying volume of ligninases extract on decolorization of textile industry effluents

White rot fungi grown in synthetic textile dye solutions and industrial effluents take more time to decolorize the effluents as compared to isolatedenzymes and enzyme extracts because the fungi have to pass through lag phase before they grow and secrete ligninolytic enzymes for dye degradation. Where as the enzymes added directly into the dye solutions and effluents start acting on the dye present in effluents immediately and take less time for color removal. The ability of the white-rot fungi to degrade dyes has been directly correlated to its ability to synthesisze ligninolytic enzymes including LiP, MnP and laccase that are involved in oxidation of dyes and other organic pollutants.The difference in the extent of decolourization of different dyes varies with chemical structures of the dyes and composition of the enzyme extract. In a previous study *Coriolus versicolor* has been found to decolorize Arzoo textile industry effluents to 84% in 3 days (Asghar *et al.*, 2009). The variation in effluent composition is responsible for the difficulty of decolorization of a particular effluent (Toh *et al.*, 2003; Maas and Chaudhary, 2005).

CHAPTER 5

SUMMARY

Ligninolytic enzymes produced by white rot fungi have numerous important applications in different industries. Each application of enzymes in particular industrial process requires enzymes with different characteristics. Keeping in view the many industrial applications of ligninolytic enzymes there is a dire need to explore more and more sources of enzymes with suitable characteristics for different aplications. In this research project, the potential of an indigenous strain *S. Commune* IBL-06 for the production of ligninolytic enzymes was investigated. In a preliminary time course study, the fungus was found to secrete reasonably high activities of MnP, LiP and laccase in solid state culture using banana stalk as substarte. MnP was produced in highest activities, followed by LiP and laccase.

To further enhance the ligninase production capability of *S. Commune* IBL-06 different physical parameters (incubation time, moisture level, pH, temperature, inoculums size) and nutritional factors (carbon and nitrogen sources, carbon: nitrogen ratio, mediators and metal ions) were optimized. By optimization of different parameters the enzymes production was significantly enhanced. The maximum productivities (IU/mL) of ligininases from *S. Commune* IBL-06 in SSF were 3745 IU/mL of MnP, 2700 IU/mL of LiP and 345 IU/mL of Laccase in 3 days incubation time at pH 4.5 and 35°C temperature with inoculum size 3mL and moisture content 60%, C: N ratio 20:1(glucose and ammonium nitrate as carbon and nitrogen supplements), 1mM $MnSO_4$ as mediator, 1mL and 1.25mM $MgSO_4$.$7H_2O$a, 1mL.

After optimization of fermentation parameters, the enzymes produced under optimum conditions were purified by $(NH_4SO_4)_2$ precipitation, dialysis and Sephadex G-100 gel filtration chromatography. The purified enzymes were run on Native and SDS-PAGE to check their purity and molecular masses relative to standard protein markers run parallel with the enzyme samples. The enzymes were then characterized through kinetic studies by investigating the effects of pH, temperature, substrate concentration (determination of K_M and V_{max}) and activators/inhibitors on activities of individual enzymes.

MnP was salt out at 90% saturation with ammonium sulphate, was dialyzed and purified to by gel filtration chromatography to 3.1fold with a yield of 4.2%. On Native Page,

the the enzymes gave a single band, where as on denaturing SDS-PAGE, two bands of 30 and 20 kDa appeared on the gel showing that the enzyme was an oligomeric protein made up of two polypeptide chains. The optimum pH and temperature of purified MnP were 5 and 40°C. The purified enzyme had K_M valuesof 0.29 mM showing high affinity for ABTS as a substrate with V_{max} of 450 mM/min. The enzyme was found to be activated by $CuSO_4$ but was inhibited by $MnSO_4$, $CaCl_2$, EDTA, TEMED, β-Marcaptoethanol, $AgNO_3$, $Pb(NO_3)_2$.

For LiP, 2.34 fold purification was achieved after $(NH_4)_2SO_4$ precipitation (65% saturation), dialysis and gel filtration. The molecular weight of the LiP was 43KDa single band on Native as well as SDS-PAGE suggesting that the enzyme was a monomeric protein. The optimum pH of LiP was 5.0, optimum temperature was 35°C, K_M value was 0.5 mM and Vmax was 400 mM/min. The enzyme was found to be inhibited by all organic and inorganic compounds including $CuSO_4$, $MnSO_4$, $CaCl_2$, EDTA, TEMED, β-Marcaptoethanol, $AgNO_3$, $Pb(NO_3)_2$

Laccase was salt out with ammonium sulphate saturation of 50%. After dialysis and gel filtration the enzyme was purified to 3.95 fold with specific activity of 158 U/mg. On both Native and SDS-PAGE, the enzymes displayed a single band of apprimately 63 kDa. Purified laccase had optimum pH 6.0 and optimum temperature 40°C. The values of kinetic parameters K_M and V_{max} for purified laccase were 0.025mM and 80mM/min respectively using ABTS as substrate. The laccse activity was found to be enhanced by 2mM $CuSO_4$, and was inhibited by $MnSO_4$, $CaCl_2$, EDTA, TEMED, β-Marcaptoethanol, $AgNO_3$, $Pb(NO_3)_2$.

Crude ligninase extract (2-8 mL) was added to 100 mL solutions of different dyes prepared in 50mM succinate buffer of pH 4 and to practical effluents collected from different textile units (adjusted to pH 4). The flasks were incubated for 24 hours. The enzyme extracts decoulorized Novasol direct blue dye to 80%, followed by Novasol direct yellow dye to 60%, Novasol direct red to 38% and Novasol direct black to 37%. The effluent from Magna textile industry was maximally decolorized t0 87% in 24 hours, flowed by effluents from Cresent, Arzoo and Chenab textile industries.

In conclusion, *S. Commune* IBL-06 produced high activities of MnP and LiP with lower laccase activities. MnP, LiP and laccase from *S. commune* IBL-06 had lower K_m and higher V_{max} values as compared to most of the previously reported enzymes. However, optimum pH and temperatures and thermostabilities were comparable to most of the

ligninases isolated and characterized from different WRF strains. When used for decolorization of textile dyes and industrial effluents, the enzymes showed very encouraging results in 24 hours as compared to using white rot fungi that have been reported to take many days to achieve complete decolorizatiuon of textile dyes and textile industry effluents.

In future studies the activities and thermostabilities of the enzymes can be improved by immobilization of individual enzymes using different solid supports or by entrapment in hydrophobic gels, making them more suitable catalysts for industrial applications. Immobilization is preferred on all other technique because immobilization modifies the activity, selectivity and equipped permanence of enzymes. Solid support like xerogels, sand, clay or soil are required for the attachment of enzyme, pose no environmental risk and beneficial for industrial application.Immobilization is an excellent approach to exploit the enzymes at industrial scale as immobilized enzymes are more vigorous, more stable and have ability to catalyze the reactions in wide environmental conditions.

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