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OPTIMIZATION OF PHYSICAL AND NUTRITIONAL FACTORS FOR ENHANCED PRODUCTION OF LIGNIN PEROXIDASE BY *GANODERMA LUCIDUM* IBL-05 IN SOLID STATE CULTURE OF WHEAT STRAW

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ABSTRACT

Microorganisms are commonly used in biotechnological and environmental processes through exploitation of their natural catalytic activities. An indigenous white rot fungus *Ganoderma lucidum* IBL-05 was used in solid state fermentation (SSF) of wheat straw for lignin peroxidase (LiP) production. The SSF process for LiP production was improved by optimizing some physical and nutritional parameters at pre-optimized pH 4.5 and temperature 35^{o}C. By optimization different physical and nutritional factors, LiP production by the fungus was substantially improved to 1019 IU/mL after 48h in nutrient Medium III with 60% moisture level, 5mL inoculum size, glucose and urea as carbon and nitrogen sources in 15:1 C/N ratio, 1mL of 2mM Zn^{2+}as metal ion and 1mL of 4mM 4-MMA as mediator. Surfactants like Tween-80, Tween-20 and SDS suppressed LiP synthesis by *G. luucidum* IBL-05.

Keywords: *Ganoderma lucidum* IBL-05, Wheat straw, Lignin peroxidase (LiP), solid state fermentation, optimization

INTRODUCTION

White rot fungi are eukaryotic microorganisms belonging to the basidiomycetes group of fungi that can degrade cellulose and lignin by a fascinating developmental process of fruiting bodies formation during their growth on dead trees (Sun and Cheng, 2002; Mtui, 2009). These are the unique microorganisms having exclusive complex enzymatic machinery for the degradation of as lignin and halocellulose components as a source of carbon and energy along with the removal of polysaccharides and hence total biomass breakdown usually occurs (Madhavi *et al*., 2009). Lignin degradation by WRF takes place during secondary metabolism and (Cabana *et al*., 2007) by the action of extracellular ligninolytic enzymes produced typically under nutrient and nitrogen -deficient conditions. The ligninolytic enzymes have very low substrate specificity, enabling them to mineralize a wide variety of recalcitrant xenobiotic compounds and organopollutants having structural similarity with the lignin (Hatakka, 2001; Hofrichter, 2002).

WRF can be divided in to three groups on the basis of their ligninolytic enzyme systems: 1. LiP-, MnP- and laccase-producing, 2. MnP- and laccase-producing and 3. LiP- and laccase-producing fungi (Forgacs *et al*., 2004; Baldrian and Snajdr, 2006). Lignin peroxidases (LiPs) (E.C.1.11.1.14) are extracellular glycosylated heme proteins that catalyze the H_2O_2 dependent one-electron oxidation of a variety of lignin-related aromatic structures (Asgher *et al*., 2008; Dashtban, 2010). LiPs also strongly catalyze the H_2O_2-dependent oxidation of phenols (e.g. guaiacol, vanillyl alcohol, catechol, syringic acid, acteosyringone), aromatic amines, aromatic ethers, polycyclic aromatic hydrocarbons and depolymerization of a various non-phenolic lignin compounds (diarylpropane) and β-*O*-4 non-phenolic lignin like compounds (Wong, 2009). The crystal structure of LiP molecule has demonstrated that the heme group is embedded inside the protein and it can contact to the outer environment through a channel. Although the size of the channel is not bigger enough to tolerate the large polymer lignin to access the heme group but small molecules of substrates can find an appropriate binding site (Piontek *et al*., 2001).

Pakistan has a vast resource of the agrocellulosic waste including corn cobs wheat straw, corn stover, sugarcane bagasse, rice straw and banana stalks. Being wastes or by-products from agriculture production, these substrates are available as low cost and are attractive raw materials for the production of ligninases and cellulosic enzymes (Gassara *et al*., 2010). Wheat straw stem and leaf parts of the wheat plants contains nearly 31% hemicelluloses, 7% lignin and 36% cellulose, (Niladevi, 2009) and has gross energy for fungal growth. It also contains certain amount of soluble carbohydrates and inducers, necessary for enzyme induction production (Mckean and Jacobs, 1997), and therefore, appears as a perspective substrate for lignolytic enzymes production.

Ganoderma lucidum is biotechnologically important white rot fungus due to its abilities to degrade lignin (Gottlieb *et al*., 1998). Due to its exclusive lignin mineralizing enzymes (LMEs) *G. lucidum* is considered as an extraordinary organism which can degrade the lignin to carbon dioxide and water (Paterson, 2007). The selection of the *G. lucidum* strain is based on its rapid

growth rate on solid media which is associated with the degradation of wide variety of hard woods (Horvath *et al*., 1993). Recently, different strains of *Ganoderma* have been used in bioremediation studies and for the production of lignolytic enzymes (Paterson, 2006; Murugesan *et al*., 2007). Considering the LMEs producing potential of *G. lucidum* and low cost avaialability of wheat straw, the present work was planned to develop a hyper-producing SSF process for extracellular LiP production through optimization of different physical and nutritional factors

MATERIALS AND METHODS

Lignocellulosic inducer substrate: Wheat straw was obtained from Students Farms, University of Agriculture, Faisalabad. It was dried (50^{o}C) to constant weight in oven and ground to get different particle sizes (0.3-2.00 mm mesh). The substrate was then stored in moisture free air tight plastic jars for subsequent use in a fermentation medium.

White rot fungus and inoculum development: The indigenous white rot fungus *Ganoderma lucidum* IBL-05 previously isolated from decaying wood (Fig 1) and available in the Industrial Biotechnology Laboratory, Department of Chemistry and Biochemistry, University of Agriculture Faisalabad was used for LiP production in SSF of wheat straw. Inoculum medium (100 ml) was the Kirk's basal nutrient medium (Tien and Kirk, 1998) receiving 2 g glucose supplement. pH of the inoculum medium was adjusted to pH 4.5 using M NaOH /M HCL solutions. The medium was autoclaved at 121°C for 15 min. and inoculated with slant culture of *Ganoderma lucidum* IBL-05 transferred aseptically with the help of inoculation loop in laminar air flow (Dalton, Japan). After inoculation, the flask was incubated at 35^{o}C in shaking incubator (Sanyo-Gallenkemp, Japan) at 120 rpm for 5 days to get homogenous spore suspension (1×10^{6} – 10^{8} spores/ml). The spore count in the inoculums was performed using hemocytometer by the method of Kolmer (19590.

Fig 1. *G. lucidum* growing on natural decaying wood

Solid state fermentation of wheat straw: Triplicate flasks contained 5g wheat straw moistened to 60% moisture (w/w) by adding Krik's basal nutrient medium of pH 4.5 (Tien and Krik, 1998). The flasks were sterilized in laboratory scale autoclave (Sanyo, Japan), allowed to cool at room temperature and inoculated with 3 mL homogeneous inoculum of *G. lucidum* IBL-06. The inoculated flasks were incubated to ferment at 35^{o}C in a still culture incubator (Sanyo, Japan) without shaking for stipulated time period. The triplicate flasks were harvested by adding 100 mL distilled water and shaking the flasks (150 rpm) for half an hour to extract extracellular enzymes. The fermented cultures were filtered through Whatman No. 1 filter paper (125 mm) and residues were discarded. The filtrates were centrifuged (3000 × g, 10 min, 4^{o}C) to remove any fungal pellets and cell debris. The supernatants were carefully decanted and used as crude enzyme extracts for determining the activities of LiP.

Lignin peroxidase assay: Lignin peroxidase activity was determined by the method of Tien and Kirk (1983). Oxidation rate of veratryle alcohol to veratraldehyde was followed in 0.2mM sodium acetate buffer of pH 3 in the presence of 0.1mM H_2O_2. One unit of enzyme activity was defined as the amount of veratraldehyde (µmol) released per ml enzyme solution per minute.

Optimization of LiP production in SSF: The LiP production process in SSF using wheat straw as substrate was optimized by varying different physical and nutritional factors such as particle size of substrate, basal nutrient medium, moisture level, inoculum size, additional carbon and nitrogen sources, and C: N ratio. The effects of varying concentrations of metal ions,

mediators and surfactants on LiP synthesis *G. lucidum* were also investigated under pre-optimized conditions of pH 4.5 and temperature 35^{o}C. The Classical Strategy of optimization was adopted; varying one variable at a time in triplicate flasks using Completely Randomized Design (CRD). The data was analyzed by Analysis of Variance (ANOVA). The data values are the means ± S.E. (standard error) of triplicate runs. Different letters in tables indicate significant differences according to Tukey's test ($P \leq 0.05$). In subsequent trials, the previously optimized parameters were maintained at optimum level.

RESULTS AND DISCUSSION

To enhance the production of LiP by *G. lucidum*, the solid state fermentation process using wheat straw as substrate was optimized by varying different physical and nutritional factors. The results have been described and discussed under the following sub-headings

Particle size of the substrate: Triplicate flasks containing wheat straw (5 g) of different particle sizes (0.3mm, 0.5 mm, 0.7 mm, 0.9 mm, 1 mm, 2mm) were processed in SSF. The results showed that *G. lucidum* IBL-05 produced maximum LiP activity (288.9 IU/ml) when grown on wheat straw having particle size of 0.5 mm (Fig.1). It was observed that LiP production initially increased with increase in particle size of wheat straw but enzyme production deceased with substrate particle size bigger than 0.5 mm. Particle size of the substrate is very important for fungal growth and production of enzymes in SSF. In SSF cultivation of fungi, the availability of surface area plays a vital role for microbial attachment, mass transfer of various nutrients and substrates, subsequent growth of microbial strain and enzyme production that in turn depends on the particle size of the substrate/support matrix (Beeraka *et al*., 2008). Sindhu *et al*, (2009) reported maximum enzyme production with particle size of 450-500 µ and enzyme activity was decreased with further reduction in particle size. However, lowest enzyme activity was attained with the substrates containing particles bigger than 850 µ. Shrivastava *et al*. (2010) used locally collected wheat straw with uniform particle size (1.5-2.0 cm), dried at 60°C before SSF .

Basal nutrient medium: To find out a simple and economical basal nutrient medium, the Krik's basal nutrient medium and three modified simpler media were tried for LiP production by *G. lucidum* IBL-05. The maximum LiP (336.9 IU/mL) activity was obtained when M-III medium was used (Fig.2). The production of LiP in all the four media increased with time and maxium LiP activity was harvested after 10 days of cultivation. M-III medium contained urea as a nitrogen source and lower concentrations of other nutrients that may be essential for the fungal growth. It was a simple and economical medium as compared to Kirk's medium (M-I) containing costly ingredients like varatryl alcohol and tween-80. The basal nutrients present in the medium are the minimum requirements for fungal growth in SSF. The expression of ligninolytic enzymes by fungi is affected by nutrients present in liquid media used to moist the solid substrates in SSF (Baldrain, 2003) that usually contain inorganic metal ions and micronutrients supplemented with a nitrogen source (Baldrain, 2006).

Moisture level: The substrate was moistened with different volumes of M-III nutrient medium to adjust initial moisture contents to varying levels (% w/w). With an increase in the moisture content up to 60% (w/w), LiP synthesis by *G. lucidum* IBL-05 increased (Fig.3) and peaked (342.8IU/mL) on 10th day of cultivation. A further increase in moisture content showed decline in LiP activity. Moisture content/ water activity is one of the most critical factors in SSF. Moisture causes swelling of substrate thus facilitating better utilization of the substrate (Pandey *et al*. 1999; Prakasham *et al.,* 2006). In SSF the microbial growth and product formation occurs at or near the surface of the solid substrate particles having low moisture contents (Pandey *et al*., 1999). However, higher moisture content beyond certain limits causes decrease in porosity (gummy texture), alter the substrate particle structure and leads to poor oxygen transfer and diffusion (Sindhu *et al*., 2009). Revankar *et al*. (2007) reported 70% moisture content as optimum for the laccase production by *Ganoderma sp. u*sing wheat bran as a substrate. Peng and Chen, (2008) used steam-exploded wheat straw with initial moisture content 75% for optimum growth of *Microsphaeropsis sp* at 30^{o}C temperature for 10 days.

Inoculum size: For the optimization of inoculum size for LiP formation by *G. lucidum*, varying volumes of homogeneous spore suspension/5gm of substrate were used to inoculate the SSF medium of wheat straw. Maximum LiP activity 9357 IU/mL) activity was noted on 10th day in the SSF flasks receiving 5 mL inoculum (Fig.4). A further increase in spore density showed decline in LiP production. Length of Lag/adaptation phase in SSF is dependent on the amount of microorganism added. Usually, lower inoculum size requires longer lag times for the microbial cells to multiply to sufficient enough numbers for efficient utilization of substrate and enzyme synthesis. An increase in the inoculum size within optimum limits would ensure a rapid proliferation and biomass synthesis (Vaithanomsat *et al*., 2010**)**. However, higher inoculum volume increases the water content of SSF medium, thus creating aeration problems in SSF of rice straw where as Mehboob *et al*. (2011) reported maximum LiP synthesis

using 3 mL inoculums of *G. lucidum* IBL-05 in SSF of corn cobs.

Additional carbon and nitrogen sources: Different carbon and nitrogen additives were used to investigate their stimulating/ inhibitory influence on LiP production by *G. lucidum* under optimum conditions. It was observed that combination of glucose and urea as carbon and nitrogen source, respectively gave higher LiP activity (587.9 IU/mL) on 6th day of cultivation as compared to all other combinations (Table 1). Both the nature and quantity of available carbon and nitrogen sources influence ligninolytic enzymes production by WRF (Songulashvili *et al*., 2007). Ligninolytic enzymes by most WRF are synthesized in nitrogen limited media and their activities are suppressed by high nutrient nitrogen concentrations (Jaouani *et al*., 2006; Levin *et al*., 2010). Kanwal and Reddy (2011) used different carbon sources for ligninolytic enzymes production and found maximum growth with glucose, followed by fructose. Asgher *et al*. (2012) also reported that combination of glucose and urea was the best for maximum MnP synthesis (1288.74 U/mL). The easily oxidizable nature of glucose in comparison to the other substrates studied makes it more favorable for growth and LiP production. Organic nitrogen sources can regenerate NADH, to act as electron donor for metabolic pathways of microorganisms (Jadhav *et al*. (2008). It has also been reported that urea stimulates fungal growth when added to make up 40–50% of total nitrogen in the substrate (Raimbault and Alazard, 1980).

C: N Ratio: After selection of best carbon and nitrogen source combination, effects of varying C: N ratios on LiP production by *G. lucidum* in SSF medium of wheat straw were investigated. The selected carbon (Glucose) and nitrogen (urea) additives were added to adjust varying C: N ratios in the medium. The medium adjusted to 15:1 C/N ratio gave maximum LiP activity (657.4 IU/mL) after 6 days (Fig. 5). Further increase in C: N ratio caused decrease in LiP formation. The production of enzyme is highly dependent on C/N ratios and the effect of C: N ratio is more pronounced as compared to the effect of carbon and nitrogen sources. It is therefore mandatory to optimize C: N ratio in the medium. At lower carbon concentration, the fungi suffer from carbon limitation and do not show optimum growth and enzymes formation (Irshad and Asgher, 2011), while at a high C: N ratio (nitrogen limitation), fungal cultures produce large amounts of polysaccharides ((Xiong *et al*., 2008; Xiaoping and Xin, 2008).

Metal ions: The maximum LiP activity (1276.4 IU/mL) was obtained with 1.5 mM concentration of $ZnSO_4.7H_2O$ after 4 days, followed by 1mM $CuSO_4.5H_2O$ (1087.6 IU/mL) and 1.5 mM MnSO4. $2H_2O$ (Table 2). LiP synthesis by the fungus increased with lower concentrations of Zn^{2+} but concentrations higher than 1.5mM caused inhibition of LiP synthesis. Fe^{2+}, $Mn^{2+}$and Mg^{2+} ions were inhibitory to LiP formation at all concentrations. Different strains and species of WRF differ in their sensitivity towards metals during their growth on lignocellulosic substrates (Sathiya-Moorthi *et al*., 2007). Metals necessary for fungal growth include copper, iron, manganese, molybdenum, zinc, and nickel (Baldrian, (2003). WRF have been reported to accumulate Cd^{2+}, Fe^{2+}, Zn^{2+} and Cu^{2+} from wood in their fruit bodies, whereas Mn^{2+} and Pb^{2+} were excluded. WRF require essential metal ions like Mg^{2+}, Ca^{2+} , Mn^{2+} , Zn^{2+} or Cu^{3+} as cofactors/prosthetic groups of different metabolic enzymes but these metals are toxic when present in excess (Srinivasan and Murthy, 2000). However, Ahammed and Prema, (2002) found that Cu^{2+} inhibited LiP markedly while Ni^{2+} and K^{+} had the least inhibitory effects.

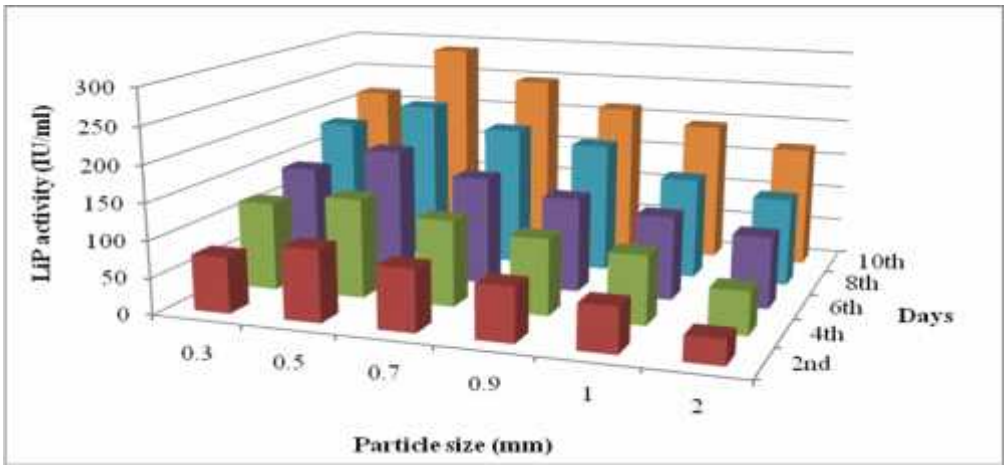

Fig.1 Effect of particle size of substrate on LiP production by *G. lucidum* IBL-05 in SSF of wheat straw*
*pH 4.5; temperature, 35^{o}C

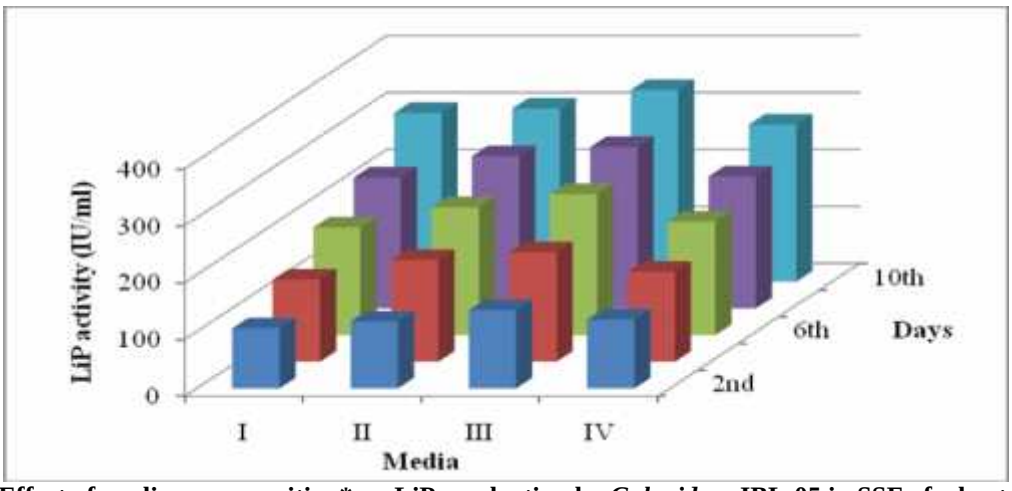

Fig.2 Effect of medium composition* on LiP production by *G. lucidum* IBL-05 in SSF of wheat straw

*pH 4.5; temperature, 35°C; Particle size, 0.5 mm

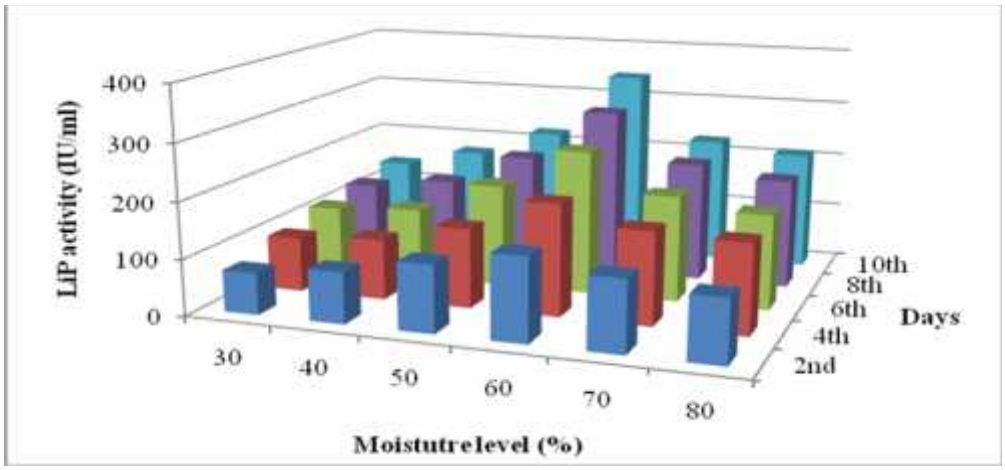

Fig.3 Effect of moisture level on LiP production by *G. lucidum* IBL-05 in SSF of wheat straw under optimum conditions*

*pH, 4.5: temperature, 35°C: particle size, 0.5 mm; nutrient medium, M-III

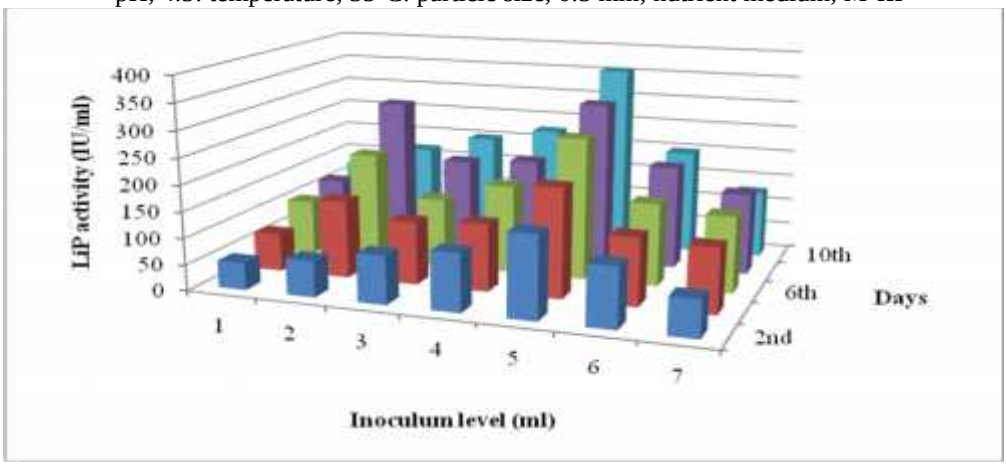

Fig.4 Effect of inoculum size on LiP production by *G. lucidum* IBL-05 in SSF of wheat straw under optimum conditions*

*pH 4.5; temperature, 35°C; Particle size, 0.5 mm; nutrient medium, M-III; moisture level, 60%

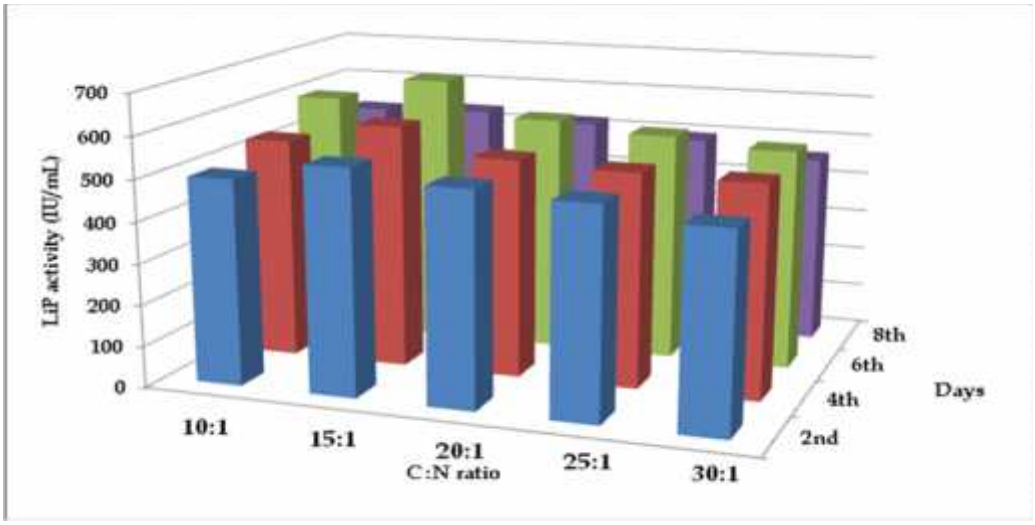

Fig.5 Effect of C:N ratio on LiP production by *G. lucidum* IBL-05 in SSF of wheat straw under optimum conditions*

*pH, 4.5; temperature, 35°C; particle size, 0.5 mm; nutrient medium, M-III; moisture level, 60%; inoculums, 5mL; carbon source, glucose; nitrogen source, urea

Table 1. Activities of lignin peroxidase produced by *G. lucidum* IBL-05 with varying combinations of Carbon & nitrogen sources under optimum conditions.

C-N Sources	LiP Activity (IU/ mL) Time (day)				
	2	4	6	8	10
Control	156.68 ± 2.6J	207.6 ± 2.2F	269.9 ± 2.8D	310.5 ± 1.6B	357 ± 0.6A
C1N1	389.5 ±3.1X	423.3 ±1.4P	487.1 ±3.4H	515.9 ±1.3D	535.6 ±2.4B
C1N2	411.7 ±1.6R	469.9 ±2.8K	499.8 ±2.9F	368.2 ±2.6AA	344.2 ±2.7AF
C1N3	485.7 ±2.6I	528.9 ±1.1C	587.1 ±1.6A	518.9 ±3.7D	478.6 ±1.57J
C1N4	344.4 ±1.2AF	393.6 ±2.4W	443.2 ±2.7N	413.7 ±1.6R	367.6 ±2.9AA
C1N5	327.6 ±2.6AJ	343.9 ±1.2AF	378.1 ±1.1Y	303.1 ±3.3AN	256.7 ±1.5AU
C2N1	396.5±3.4V	466.8 ±2.6L	497.2 ±2.1F	367.3 ±1.7AA	341.2 ±3.5AG
C2N2	479.6 ±1.2J	492.2 ±3.8G	535.6 ±2.6B	417.1 ±1.2Q	269.4 ±2.4AS
C2N3	336.7 ±1.3AH	363.4 ±1.3AB	391.1 ±1.9 W	296.6 ±3.7AO	214.0±2.9AY
C2N4	372.7 ±2.6Z	411.4 ±1.5R	441.3 ±1.6N	327.9 ±2.8AJ	268.1 ±1.7AS
C2N5	249.4 ±3.5AU	318.8 ±1.7AL	358.7 ±1.7AC	278.3 ±3.4AQ	221.9 ±2.5AX
C3N1	443.8 ±1.5N	480.6 ±3.7J	506.2 ±1.7E	352.0 ±1.3AD	369.9 ±1.6AA
C3N2	437.9 ±1.7O	452.8 ±2.9M	482.9 ±2.6I	411.1 ±1.7R	346.7 ±3.8AE
C3N3	463.0 ±3.5L	485.5 ±1.8I	516.0 ±1.9D	341.1 ±2.4AG	271.4 ±1.8AR
C3N4	230.2 ±1.1AW	335.7 ±3.5AI	265.6 ±3.8AS	184.9 ±3.6BB	118.7 ±3.3BF
C3N5``	337.6 ±1.2AH	398.0 ±2.6U	358.7 ±1.6AC	214.0 ±3.2AY	167.5 ±1.8BD
C4N1	386.4 ±2.8X	415.0 ±1.2Q	439.9 ±2.4O	367.5 ±1.4AA	232.7 ±1.7AW
C4N2	411.6 ±1.2R	452.8 ±2.3M	339.5 ±2.9AH	271.1 ±2.7AR	165.7 ±3.6BD
C4N3	423.7 ±1.6P	472.1 ±2.7K	489.7 ±1.4H	376.8 ±1.3Y	217.7 ±1.7AY
C4N4	433.8 ±1.4O	489.7 ±1.8H	401.5 ±2.1S	351.2 ±2.2AD	195.0 ±3.9BA
C4N5	321.0 ±1.1AL	207.6 ±3.8AZ	211.3 ±1.0AZ	178.5 ±3.5BC	114.4 ±1.6BF
C5N1	388.6 ±3.6X	414.8 ±2.0Q	399.6 ±1.9T	285.1 ±3.3AP	229.5 ±3.5AW
C5N2	400.6 ±1.4S	359.6 ±2.6AC	285.1 ±1.6AP	232.2 ±1.9AW	213.3 ±1.7AY
C5N3	414.4 ±1.37Q	488.4 ±1.5H	488.7 ±3.7H	335.8 ±2.1AI	244.0 ±2.6AV
C5N4	326.0 ±2.9AK	347.1 ±1.8AE	313.1 ±1.6AM	261.3 ±3.4AT	160.3 ±1.2BE
C5N5	308.8 ±1.8AN	330.2 ±2.2AJ	298.0 ±1.4AN	243.8 ±1.1AV	122.5 ±2.5BF

*pH, 4.5; temperature, 35°C; Particle size, 0.5 mm; medium, M III; moisture level, 60%; inoculum size, 5mL

Table 2. Activities of LiP produced by *G. lucidum* IBL-05 with varying concentrations of different metal ions under optimum conditions*

Metals ions salts	Conc (mM)	LiP activity (IU/mL)		
		Days		
		2	4	6
Control		544.6 ± 2.8E		657.4 ± 2.7A
$Fe_2(SO_4)_3.7H_2O$		534.0 ± 1.5AJ	709.5 ± 3.9R	722.6 ± 1.1P
$CuSO_4.5H_2O$		489.2 ± 1.8AM	685.2 ± 3.2U	719.5 ± 3.3P
$MnSO_4.H_2O$	0.01	582.6 ± 1.3AH	680.9 ±1.5V	705.0 ± 1.7R
$MgSO_4.7H_2O$		580.5 ± 1.2AH	632.2 ± 2.3AA	686.8 ± 1.7U
$ZnSO_4.7H_2O$		698.5 ± 1.3T	721.1 ± 3.5P	759.3 ± 2.7K
$Fe_2(SO_4)_3.7H_2O$		596.7 ± 0.8AF	713.3 ± 1.3Q	766.6 ± 1.8I
$CuSO_4.5H_2O$	**0.05**	592.7 ± 1.3AG	719.1 ± 1.6P	747.7 ± 3.9M
$MnSO_4.H_2O$		616.6 ± 1.3AC	722.2 ±2.32P	747.0 ± 2.29M
$MgSO_4.7H_2O$		624.7 ± 1.8AB	671.0 ± 1.3W	784.5 ± 2.1G
$ZnSO_4.7H_2O$		728.8 ± 1.7O	788.1 ±2.6F	796.6 ± 1.9E
$Fe_2(SO_4)_3.7H_2O$		616.2 ± 2.7AC	742.6 ± 1.6N	790.9 ± 2.3F
$CuSO_4.5H_2O$		659.3 ± 3.3X	763.0 ± 3.5J	798.7 ± 1.9E
$MnSO_4.H_2O$	**1.00**	641.8 ± 2.7Z	778.1 ± 3.8H	703.9 ± 3.9S
$MgSO_4.7H_2O$		638.9 ± 3.5Z	730.5 ± 1.2O	796.4 ± 1.5E
$ZnSO_4.7H_2O$		756.5 ± 2.3K	818.4 ± 1.9D	842.6 ± 1.3C
$Fe_2(SO_4)_3.7H_2O$		626.9 ± 2.9AB	658.8 ± 3.7X	550.8 ± 1.1AL
$CuSO_4.5H_2O$	**1.50**	626.9 ± 3.9AB	701.1 ± 1.2S	654.1 ± 1.8Y
$MnSO_4.H_2O$		581.0 ± 1.1AH	618.1 ± 1.4AC	667.5 ± 1.4W
$MgSO_4.7H_2O$		711.7 ± 3.3Q	748.9 ± 2.0M	718.8 ± 3.6P
$ZnSO_4.7H_2O$		783.6 ± 1.2G	856.4 ± 1.4B	816.5 ± 2.9D
$Fe_2(SO_4)_3.7H_2O$		499.5 ± 1.3AL	524.2 ± 2.8AK	585.8 ± 1.8AH
$CuSO_4.5H_2O$		613.9 ± 2.1AD	660.2 ± 1.7X	602.6 ± 2.6AE
$MnSO_4.H_2O$	**2.00**	730.0 ± 1.2O	753.8 ± 2.4L	786.7 ± 2.5F
$MgSO_4.7H_2O$		547.5 ± 1.6AI	666.9 ± 2.9W	602.4 ± 2.3AE
$ZnSO_4.7H_2O$		798.8 ± 2.6E	877.7 ± 2.3A	828.3 ± 1.9D

*pH, 4.5; temperature, 35^{o}C; particle size, 0.5 mm; nutrient medium, M-III; moisture level, 60%; inoculum, 5mL; glucose, 1 %; urea, 0.2%

Effect of mediators: To enhance the production of LiP by *G. lucidum* under optimum conditions, the mediators including H_2O_2, Veratryl Alcohol, Oxalate, 1, 4-Dimethoxybenzene and 4-methoxy mandelic acid were added to the SSF medium of wheat straw in varying concentrations. All the mediators were found to enhance LiP production but enhancing effects were highly variable. By adding the mediators, there was a reduction in incubation time from 4 to 2 days to get optimum LiP yield. 4-methoxy mandelic acid at 4mM concentration showed the maximum LiP (1019.6 IU/mL) formation on 2nd day, followed by 3mM VA and 2mM H_2O (Table 3).Different natural and synthetic organic and inorganic compounds perform the role of mediators in LiP catalysis (Asgher *et al*., 2012). Mediators are a group of low molecular weight organic compounds (Gochev *et al*., 2007) that can diffuse far away from the mycelium to sites that are difficult to reach by the enzyme itself (e.g., the lignin macromolecule inside the plant cell wall) (Camarero *et al*., 2004), and enhance the range of substrates and efficiency of degradation of the recalcitrant compounds by several fold. LiP alone is practically not able to oxidize 4-methoxymandelic acid, 4-MMA to anisaldehyde, but these oxidations take place with good efficiency in the presence of VA (Arora and Gill, 2001; Gassara *et al*., 2010). An additional observation is that the fungal secondary metabolites such as 1,4-dimethoxybenzene (14 DMB) (Baciocchi *et al*., 2002) and 2-chloro-1,4-dimethoxybenzene (2Cl- 14 DMB) can replace the function of VA in increasing the efficiency of LiP-catalysed oxidations (Husian and Husain, 2008). These findings also provide the new evidence for the possible role of fungal metabolites other than VA as redox mediators of LiP in lignin degradation.

Effect of surfactants: All the surfactants were found to inhibit the synthesis of LiP by *G. lucidum* IBL-05 at all concentrations (Table 4). Contrary to our findings**,** it has been reported in literature that surfactants are growth enhancing agents in SSF for the production of LiP. However, the response of certain microorganisms to a surfactant will depend on several factors, such as cellular ultrastructure, surfactant concentration, bioavailability, and environmental and culture conditions (Van Hamme

et al., 2006; Bustamante *et al*., 2010). Surfactants are organic molecules with a polar or ionic hydrophilic group and a non polar or hydrophobic chain, known as the head and tail groups, respectively that increase the surface area for growth of micro-organisms and subsequently affect enzyme secretion (Christofi and Ivshina, 2002). Garon *et al*. (2002) evaluated the toxicity of SDS, Triton X-100, and TW 80 on fungal strains and results showed growth inhibition by SDS (anionic surfactant), whereas Triton X-100 and TW 80 (nonionic surfactants) were well tolerated at the doses evaluated in most of the tested fungi. This negative effect can be explained by disruption of the cell membranes through interactions with structural lipid components (Laha and Luthy, (1992).

Table 3. Activities of lignin peroxidase produced by *G. lucidum* IBL-05 with varying concentrations of different mediators under optimum conditions*.

Mediators	Conc (mM)	LiP activity (IU/mL)		
		Days		
		2	4	6
Control		798.8 ±2.6E	877.7 ±2.3A	828.3 ±1.9D
H_2O_2		630.8 ± 1.7Q	657.6 ± 1.1O	586.3 ± 3.9T
VA		803.7 ± 1.9I	793.4 ± 3.8I	518.3 ± 2.7V
4MMA	**1**	832.1 ± 3.9H	784.7 ± 3.7J	561.1 ± 3.1U
OA		489.1 ± 3.5X	437.2 ± 1.4AB	353.5 ± 3.5AH
1,4 DMB		511.6 ± 2.8V	427.6 ± 1.1AC	215.5 ± 3.8AM
H_2O_2	**2**	663.3 ± 1.7O	639.2 ± 3.5P	426.6 ± 1.2AC
VA		869.4 ± 2.2F	819.7 ± 1.5H	505.2 ± 2.4W
4MMA	**2**	885.2 ± 1.4D	851.5 ± 3.6G	626.3 ± 2.6Q
OA		636.6 ± 2.7P	413.1 ± 2.7AD	395.2 ± 1.9AE
1,4 DMB		448.2 ± 1.0Z	365.0 ± 2.5AG	134.4 ± 2.7AO
H_2O_2		459.5 ± 1.9Z	411.0 ± 2.8AD	371.1 ± 3.6AF
VA		885.8 ± 1.1D	722.9 ± 2.8M	616.7 ± 1.5R
4MMA	**3**	939.8 ± 1.8C	872.7 ± 1.3E	603.5 ± 2.4S
OA		671.2 ± 3.3N	376.3 ± 1.7AF	233.8 ± 3.8AK
1,4 DMB		596.2 ± 1.9S	411.4 ± 1.5AD	220.8 ± 2.4AM
H_2O_2		392.0 ± 1.7AE	368.2 ± 2.9AF	231.6 ± 3.5AL
VA		853.3 ± 3.5G	751.1 ± 1.1L	597.4 ± 1.82S
4MMA	**4**	1019.6 ± 2.5A	974.4 ± 1.8B	710.3 ± 1.82M
OA		653.4 ± 3.8O	319.3 ± 3.6AI	238.6 ± 3.9AK
1,4 DMB		440.3 ± 3.6AA	313.0 ± 3.3AI	297.6 ± 1.6AJ
H_2O_2		365.0 ± 3.7AG	237.7 ± 3.3AK	187.3 ± 2.9AN
VA		769.0 ± 2.6K	674.9 ± 1.8N	470.1 ± 1.6Y
4MMA	**5**	796.9 ± 1.9I	570.2 ± 2.3U	352.3 ± 2.7AH
OA		587.3 ± 2.5T	314.3 ± 3.7AI	178.8 ± 2.2AN
1,4 DMB		420.8 ± 2.2AC	492.4 ± 3.5X	438.2 ± 2.9AB

*pH, 4.5; temperature, 35^oC; particle size, 0.5 mm; nutrient medium, M-III; moisture level, 60%; inoculum, 5mL; carbon source, glucose; nitrogen source, urea; C: N ratio, 15:1; $ZnSO_4$. $7H_2O$, 4mM

Table 4. Activities of lignin peroxidase produced by *G. lucidum* IBL-05 with varying concentrations of different surfactants under optimum conditions*

Surfactants (10%)	Conc. (1mM, mL)	LiP activity (IU/mL)		
		Days		
		2	4	6
Control		1019.6 ±2.5A	974.4 ±1.8B	710.3 ±1.82M
Tween 20		663.4 ± 2.4G	733.7 ± 1.4E	441.1 ± 2.2Q
Tween 80		773.2 ± 3.5D	555.4 ± 1.9M	289.9 ± 1.6AF
SDS	**0.5**	669.3 ± 1.7G	475.6 ± 1.5P	335.6 ± 2.6AC
Triton x		575.8 ± 1.5L	323.7 ± 1.8AD	206.8 ± 1.8AJ
Tween 20		786.1 ± 1.8C	818.9 ± 4.1B	375.5 ± 4.5Y
Tween 80		792.3 ± 3.9C	807.1 ± 1.5B	264.2 ± 3.5AG

SDS	**1**	569.2 ± 1.8L	490.3 ± 2.5O	358.4 ± 2.0AA
Triton x		570.5 ± 1.4L	583.2 ± 2.4K	379.1 ± 2.6X
Tween 20		728.8 ± 1.7E	628.1 ± 2.7I	718.2 ± 3.5F
Tween 80		624.9 ± 2.7I	643.9 ± 1.4H	321.6 ± 1.8AE
SDS	**1.5**	431.4 ± 1.8S	473.4 ± 1.9P	413.1 ± 2.0U
Triton x		247.3 ± 2.6AH	444.4 ± 3.7Q	398.1 ± 3.4W
Tween 20		542.9 ± 2.9N	593.4 ± 1.8J	852.6 ± 3.3A
Tween 80		446.6 ± 1.4Q	419.4 ± 1.1T	326.9 ± 3.9AD
SDS	**2.0**	367.7 ± 2.5Z	448.2 ± 2.9Q	402.3 ± 2.9V
Triton x		240.3 ± 3.9AH	337.8 ±3.4AC	136.6 ± 2.9AL
Tween 20		336.4 ± 3.8AC	415.1 ± 1.4U	447.2 ± 2.7Q
Tween 80		382.8 ± 2.3X	280.8 ± 2.8AF	187.9 ± 1.6AK
SDS	**2.5**	346.2 ± 2.6AB	433.6 ± 1.3R	374.7 ± 1.8Y
Triton x		227.3 ± 1.4AI	334.8 ± 1.8AC	124.2 ± 2.6AL

*pH, 4.5; temperature, 35^{o}C; particle size, 0.5 mm; nutrient medium, M-III; moisture level, 60%; inoculum, 5mL; carbon source, glucose, nitrogen source, urea; C: N ratio, 15:1, $ZnSO_4$. $7H_2O$, 4mM; 4MMA, 4mM

Conclusions: The indigenous strain *G. lucidum* IBL-05 synthesized substantial LiP enzyme in SSF of easily available and cost effective substrate wheat straw. The production of LiP could be significantly improved by using a cheaper basal nutrient medium M-III and optimizing physical and nutritional factors for fungal growth. In future studies, the activities and thermostabilities of LiP can be improved by immobilizing the enzyme using different solid supports to make it a suitable catalyst for industrial applications.

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