OPTIMIZATION OF PHYSICAL AND NUTRITIONAL FACTORS FOR SYNTHESIS OF LIGNIN DEGRADING ENZYMES BY A NOVEL STRAIN OF *Trametes versicolor*

Hafiz Muhammad Nasir Iqbal, Muhammad Asgher,* and Haq Nawaz Bhatti

This paper reports the production of ligninase enzymes by a new strain of *Trametes versicolor* IBL-04 producing a novel pattern of ligninolytic enzymes with highest MnP activities followed by LiP and laccase. In previous studies *Trametes versicolor* has been reported to produce higher activities of MnP, followed by laccase and LiP. Lignocellulosic substrates including wheat straw, rice straw, banana stalks, corncobs, corn stover, and sugarcane bagasse were used in solid state fermentation (SSF) for the production of ligninases including peroxidase (LiP), manganese peroxidase (MnP), and laccase by *Trametes versicolor* IBL-04. Maximum production of MnP (998 U/mL), LiP (620 U/mL), and Laccase (49.7 U/mL) was observed after 5 days in the SSF medium containing 5g rice straw (60% w/w moisture) in still culture SSF. Moisture, pH, temperature, inoculums size, additional carbon and nitrogen sources, and surfactants had a significant influence on ligninase synthesis by the fungus. Production of ligninases was substantially enhanced by optimizing SSF production process. Maximum MnP (1775 U/mL), LiP (1663 U/mL), and laccase (99 U/mL) were produced when rice straw (5g) at 66.6 % moisture (w/w) receiving 5ml inoculum was incubated at pH 4.0 and 30^{o}C in the presence of maltose (1% w/w) as carbon source, urea (0.2% w/w) as nitrogen source and 1*mM* Tween-80 (0.3 ml) as surfactant.

Keywords: Trametes versicolor IBL-04; Lignocellulosic residues; Ligninases; Process optimization; Solid state fermentation; Still culture

Contact information: Industrial Biotechnology Laboratory, Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad, Pakistan. Corresponding Author, Telephone# +92-41-9200161 Ext.3312 Fax# +92-41-9201103. E.mail: mabajwapk@yahoo.com*

INTRODUCTION

Extracellular and non-specific lignin mineralizing enzymes (LMEs) including lignin peroxidases (LiPs), manganese peroxidases (MnPs), and laccases are secreted by white rot fungi (WRF) in secondary metabolism. LMEs have numerous commercial applications, including delignification of lignocellulosic biomass for fuel ethanol production, food, brewery and wine, animal feed, denim stone washing, laundry detergents, paper and pulp industries, and in bioremediation of chemical pollutants (Robinson et al. 2001; Rodríguez-Couto and Sanroman 2005; Rodríguez-Couto and Sanroman 2006; Papinutti and Forchiassin 2007). LiPs are heme proteins with high oxidation potential and are capable of oxidizing phenolic and non-phenolic substrates (Shrivastava et al. 2005). MnPs are glycoproteins with an iron protoporphyrin IX

prosthetic group (Baborova et al. 2006; Urek and Pazarlioglu 2005) that are capable of oxidizing phenolic substrates. However MnPs are unable to oxidize non-phenolic substrates, although they have the capability of depolymerizing synthetic or natural lignin in vitro (Elisashvili et al. 2001; Mester et al. 2001; Vikineswary et al. 2006). Laccases are N-glycosylated blue multi-copper oxidases (Wells et al. 2006; Asgher et al. 2008) that are versatile mineralizers of lignin as well as a variety of recalcitrant aromatic compounds including synthetic dyes (Murugesan et al. 2006; Salony and Bisaria 2006; Zouari-Mechichi et al. 2006; Quaratino et al. 2007).

Lignocellulosic agro-wastes comprising over 60% of the existing plant biomass are a potential renewable resource for biofuels, biofertilizers, animal feed, and chemical feedstock if these can be economically hydrolyzed by chemical and/or biological methods (Tengerdy and Szakacs 2003). White rot fungi (WRF) are efficient degraders of the major components of the major components of lignocellulosic materials, including lignin, cellulose, and hemicellulose by synthesizing ligninase, cellulase, and xylanase enzymes, respectively (Papinutti and Forchiassin 2007; Ruhl et al. 2008). Crop residues (straws, corn by-products, bagasse, etc.) are valuable raw materials for fermentative production of industrial enzymes (Wong and Saddler 1993; Bhat 2000; Pointing 2001; Reddy et al. 2003). Fungal solid state fermentation (SSF) is carried out using moist solid substrates as nutrient source and/or an inert anchorage for fungal mycelia growth (Moo-Young et al. 1983; Pandey et al. 1999).

Trametes versicolor is an efficient ligninolytic enzyme producer, especially high MnP and laccase in high activities under optimum growth conditions (Pandey et al. 2000). The simultaneous occurrence of high ligninolytic enzymes and hydrogen peroxide-producing activities in the fungal genome, essential for peroxidase activity and pollutant degradation, makes *T. versicolor* an attractive fungus for diverse biotechnological and environmental applications. It has been reported that there is variability between strains of *Trametes* (*Coriolus*, *Polylorus*) *versicolor* that reflects the heterogeneity of this strain. *Trametes versicolor* secretes extracellular LiP, MnP, and laccase that together cause the lignin degradation (Waldner et al. 1988; Rogalski et al. 1991). Hossain and Anantharaman (2006) reported that *Trametes versicolor* secretes all three lignilytic enzymes (LiP, MnP and laccase) with maximum activities of 495, 440, and 410 U/mL for LiP, MnP, and laccase, respectively, and lignin containing agro-waste had enhancement effects on ligninolytic enzymes production (Ardon et al. 1998).

This paper reports the results of a study carried out to investigate the potential of an indigenous novel strain *Trametes versicolor* IBL-04 isolated in Pakistan for ligninase production in SSF using lignocellulosic substrates.

EXPERIMENTAL

Lignocellulosic Substrates

Wheat straw and rice straw were procured from Student research Farms, University of Agriculture, Faisalabad (UAF), Pakistan. Banana stalks and sugar cane bagasse were collected from a local fruit market and Crescent Sugar Mills, Faisalabad, respectively. Corn stover and corncobs were obtained from CPC-Rafhan maize products,

Faisalabad, Pakistan. The substrates were sliced into small pieces, oven dried (50 °C), and ground to 40 mm mesh particle size.

Fungal Culture and Inoculum Preparation

The pure culture of indigenous strain *Trametes versicolor* IBL-04 available in Industrial Biotechnology Laboratory, UAF was used as ligninase producer. Aqueous spore suspension was prepared by growing the fungus in inoculum medium. Inoculum medium was the Kirk's nutrient medium (Tien and Kirk 1988) supplemented with Millipore filtered sterile glucose (1%).

The medium was sterilized (121 °C) in a labora-tory scale autoclave (Sanyo, Japan) for 15 minutes. After cooling to room temperature, a loopful of spores of *Trametes versicolor* IBL-04 from PDA slant was transferred into the broth under sterilized conditions in laminar air flow (Dalton, Japan). The inoculated flask was shaken (120rpm) at 30 °C for 5 days in an orbital shaker (Sanyo-Gallemkemp, UK) to get $1x10^6$ to $1x10^8$ conidia /mL (Kay-Shoemake and Watwood 1996).

Solid State Fermentation

Ligninase production was carried out in 500 mL Erlenmeyer flasks. Triplicate flasks contained 5 g substrate moistened (60% w/w moisture) with Kirk's medium (pH 4.5), unless otherwise stated. The SSF flasks were autoclaved (120 °C) and inoculated with 5 mL homogeneous conidial suspension in laminar air flow. The inoculated flasks were kept at 30°C in a temperature controlled incubator (EYLA SLI-600ND, Japan) for stipulated time period under still culture conditions.

Harvesting and Enzyme Extraction

At the end of stipulated fermentation time, distilled water (100 mL) was added to the fermented biomass, and the flasks were shaken at 120 rpm for 30 minutes (Gomes et al. 2009). The contents were filtered through Whatman No.1 filter paper and washed thrice with distilled water. As the ligninolytic enzymes are extracellular enzymes, these were extracted by adding enough distilled water, followed by shaking. The filtrates were centrifuged at 10,000 rpm for 5 minutes and carefully collected supernatants were used for enzyme activity determinations.

Optimization of Culture Conditions

The effects of varying moisture levels (50 to 83%), pH (3 to 5), incubation temperatures (25 to 40°C), additional carbon sources (glucose, fructose, sucrose, maltose, and molasses), additional nitrogen sources (urea, yeast extract, beef extract, peptone, and ammonium sulphate), 1 *m*M Tween-80 (0.1-0.5 mL) and inoculum size (1-7mL) were investigated on the production of ligninases in SSF of rice straw.

A classical method of optimization was followed, varying the parameters one by one in a series of experiments and maintaining the previously optimized at constant level. The effects of different carbon and nitrogen sources were studied in one experiment using a Completely Randomized Design to accommodate the corresponding interactions.

Enzyme Activity Assays

Enzyme activities of supernatants collected at the end of each optimization step were determined using a spectrophotometer (T60, PG Instruments, UK). LiP activity was determined by the method of Tien and Kirk (1988). The oxidation rate of veratryl alcohol to veratraldehyde was followed at 310 nm (ε 310 9,300 M^{-1} cm^{-1}) in succinate buffer of pH 3 in the presence of H_2O_2. Blanks contained 100 µL of distilled water instead of enzyme aliquots. The absorbance of each sample was taken at 0 min., and after a 10 min. interval, MnP was assayed by the method of Wariishi et al. (1992). $MnSO_4$ was added to sodium malonate buffer in the presence of H_2O_2. Manganic ions Mn^{+3} form a complex with malonate, which absorbs at 270 nm (ε 270 11,590 M^{-1} cm^{-1}). Laccase activity was measured by the method of Wolfenden and Wilson (1982). The oxidation of ABTS was followed at 420 nm (ε420 36,000 M^{-1} cm^{-1}) in a reaction mixture containing 1mL of 0.3 mM ABTS in 1mL sodium malonate buffer (50 mM, pH 4.5) and 100 µL of enzyme solution. The activities were expressed as U/mL.

Statistical Analysis

Fermentation experiments and enzyme assays were performed in triplicate, and results have been presented as mean ± S.E. (standard error). The S.E. values have been displayed as Y-error bars in figures. The data on enzyme activities was statistically analyzed by Analysis of variance (ANOVA) under Completely Randomized Design (CRD), and treatment means were compared ($P \leq 0.05$) by applying Duncan's Multiple Range (DMR) test (Steel et al. 1997).

RESULTS AND DISCUSSION

Screening of Fungus on Lignocellulosic Substrates

Trametes versicolor IBL-04 was grown for ten days in SSF medium using different substrates for the production of LiP, MnP, and laccase. After every 24 h, the triplicate flasks were harvested and analyzed for lignolytic enzymes. Maximum production of MnP (998 U/ml), LiP (620 U/mL), and laccase (49.7 U/mL) was noted after five days in the medium containing rice straw as substrate, followed by banana stalk (8 days) and wheat straw (6 days), respectively (Table 1). Banana stalk and wheat straw also supported fungal growth, but the time taken for ligninase synthesis was longer. WRF synthesize ligninases in secondary metabolism. The time taken by WRF for ligninase synthesis is dependent on the length of lag phase and primary metabolism on a particular substrate that varies with chemical composition of different lignocellulosic residues. *Trametes versicolor* IBL04 gave a novel pattern of lignolytic enzymes showing highest MnP activities, followed by LiP. In previous studies (Pandey et al. 2000) *Trametes versicolor* has been reported as a producer of higher MnP and laccase activities, rather than LiP.

There is variability between strains of *Trametes* (*Coriolus*, *Polylorus*) *versicolor* that reflects the heterogeneity of this enzyme source. *Trametes versicolor* secretes extracellular LiP, MnP, and laccase that cause the lignin degradation (Waldner et al. 1988; Rogalski et al. 1991). *Trametes versicolor* has been found to secrete maximum

activity of LiP, followed by MnP and Laccase (Hossain and Anantharaman 2006), and lignin containing agro-waste had enhancement effects on ligninolytic enzymes production (Ardon et al. 1998).

Table 1. Activities of Ligninases Produced by *Trametes versicolor* IBL-04 on different Lignocellulosic Substrates

Substrates (5g)	Enzyme Activities (U/ml) Fermentation time (Days)										
		1	2	3	4	5	6	7	8	9	10
Sugarcane Bagasse	MnP (U/ml)	217± 0.04	232 ± 3.71	399 ±0.95	499 ±2.84	**632 ± 0.22**	525 ±5.03	436 ±6.62	322 ±2.60	312 ±6.85	258 ±3.56
	LiP (U/ml	206 ±4.98	226 ±5.12	291 ±0.87	356 ±5.28	**393 ±1.99**	292 ±5.36	188 ±3.61	152 ±1.70	129 ±0.68	113 ±0.33
	Laccase (U/ml)	6.66 ±0.00	4.85 ±0.26	9.02 ±0.06	16.7 ±1.05	**33.2 ±0.20**	18.1 ±3.60	10.4 ±1.52	29.7 ±3.97	10.9 ±3.02	9.02 ±0.06
Banana Stalk	MnP (U/ml)	221 ±1.57	214 ± 0.67	291 ±2.65	336 ±1.23	406 ±2.22	563 ±6.12	602 ±2.19	**798 ±0.31**	621 ±5.86	595 ±13.7
	LiP (U/ml	152 ±2.12	166 ±1.04	197 ±2.29	217 ±1.24	294 ±2.47	372 ±6.40	455 ±1.77	**538 ±11.60**	115 ±1.68	105 ±2.43
	Laccase (U/ml)	5.83 ±0.16	6.66 ±0.60	12.8 ±0.33	16.1 ±1.07	36.7 ±0.11	26.5 ±0.72	12.5 ±1.55	**60.7 ±2.09**	43.2 ±2.12	26.8 ±1.39
Corn Stover	MnP (U/ml)	213 ±1.22	230 ±1.26	246 ±5.18	485 ±1.60	**641 ±0.22**	588 ±14.88	445 ±5.06	348 ±3.34	215 ±0.17	194 ±5.47
	LiP (U/ml	132 ±1.85	190 ±0.00	222 ±0.83	348 ±9.24	546 ±1.63	**566 ±0.35**	396 ±2.03	325 ±0.35	233 ±0.41	198 ±9.19
	Laccase (U/ml)	3.75 ±0.91	4.91 ±0.57	7.08 ±0.51	9.13 ±0.08	**24.9 ±0.54**	19.0 ±2.13	16.2 ±2.42	15.6 ±1.04	12.6 ±0.26	10.02 ±0.55
Corn Cobs	MnP (U/ml)	138 ±0.48	228 ±1.12	263 ±1.04	330 ±3.88	435 ±0.81	**498 ±7.73**	364 ±3.59	312 ±0.08	269 ±7.88	186 ±4.79
	LiP (U/ml	221 ±2.15	262 ±1.55	291 ±0.72	375 ±2.55	**421 ±7.54**	355 ±4.00	331 ±1.94	265 ±0.28	104 ±1.96	101 ±1.18
	Laccase (U/ml)	2.22 ±0.26	3.32 ±0.43	8.45 ±0.87	10.1 ±1.89	15.1 ±0.73	**24.4 ±1.30**	13.9 ±1.47	5.96 ±0.37	4.85 ±1.06	6.24 ±2.98
Rice Straw	MnP (U/ml)	284 ±0.62	308 ±0.60	685 ±4.25	948 ±1.20	**998 ±0.32**	892 ±9.28	714 ±12.17	829 ±6.6	781 ±5.41	683 ±0.73
	LiP (U/ml	212 ±4.83	235 ±3.00	333 ±7.39	424 ±5.15	**620 ±2.04**	562 ±9.66	449 ±2.28	393 ±1.83	312 ±3.84	253 ±9.28
	Laccase (U/ml)	21.5 ±0.18	22.35 ±0.79	35.41 ±0.42	45.8 ±1.92	**49.7 ±1.13**	45.9 ±1.63	44.4 ±0.21	44.3 ±0.61	43.7 ±2.99	40.9 ±1.50
Wheat Straw	MnP (U/ml)	246 ±0.23	410 ±2.61	568 ±0.32	666 ±1.45	724 ±0.34	**762 ±7.66**	646 ±6.08	563 ±0.71	453 ±10.08	370 ±2.95
	LiP (U/ml	166 ±2.91	252 ±0.04	294 ±1.02	325 ±2.23	420 ±6.98	**501 ±1.06**	446 ±0.51	395 ±3.59	301 ±1.09	233 ±8.15
	Laccase (U/ml)	28.2 ±0.41	26.5 ±0.41	31.5 ±0.24	32.2 ±0.56	32.8 ±0.69	**35.3 ±3.18**	34.7 ±2.60	34.6 ±2.84	33.7 ±0.38	32.6 ±1.10

Optimization of Culture Conditions

Based on the results of the substrate screening trial, rice straw was selected as the best substrate for ligninase synthesis by *Trametes versicolor* IBL-04 for further optimization of the SSF production process. Effects of varying parameters were investigated, and the results have been presented under the following subheadings.

Effect of Moisture Level

Kirk's medium was added to moist rice straw with varying levels of moisture (% w/w). Rice straw was fermented at 66.6 % (w/w) moisture gave maximum MnP (1,176

U/mL), LiP (1,110 U/mL), and laccase (51.5 U/mL)) production in 5 days of incubation with *Trametes versicolor* IBL-04. The ligninase production increased with an increase in moisture level from 50 to 66.6%. However, increase in moisture above 66.6 % caused significant decrease in enzyme activities (Fig.1). Rice straw with 66.6 % (w/w) moisture gave maximum ligninase production.

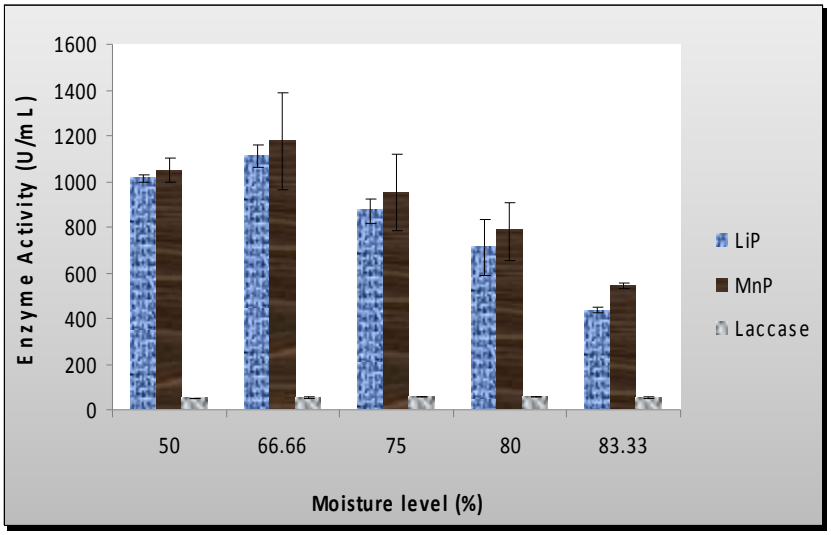

Fig. 1. Effect of varying moisture levels on ligninase production by *Trametes versicolor* IBL-04 in SSF of rice straw after 5 days of still culture incubation

Analysis of variance of the data showed significant ($p \leq 0.01$) difference between ligninase yields with varying moisture levels. Results of Duncan's Multiple Range (DMR) test revealed significant differences ($p \leq 0.05$) between all the treatment means except T_3 Higher moisture levels were inhibitory to ligninase formation. Microbial growth occurs on or near the surface of the solid substrate matrix in SSF. The optimum moisture level in SSF is governed by the water holding capacity of the substrate, the type of the end product, 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 to secretion of lower activities of ligninases in secondary growth (Rodriguez et al. 1998; Raghavarao et al. 2003; Regina et al. 2008). Low moisture contents in SSF have also been reported to decrease the enzyme formation and metabolic activities of fungi due to reduced solubility of nutrients, low substrate swelling, and higher water tension (Lonsane et al. 1992).

Effect of pH

Rice straw was moistened (66.6 % w/w) using Kirk's nutrient media of varying pH. The maximum activities of MnP (1,205 U/mL), LiP (1,110 U/mL), and laccase (52.8 U/mL) were noted in the SSF media processed at pH 4.0. Ligninase synthesis by the fungus gradually increased with an initial increase in pH and peaked at pH 4.0 (Fig. 2).

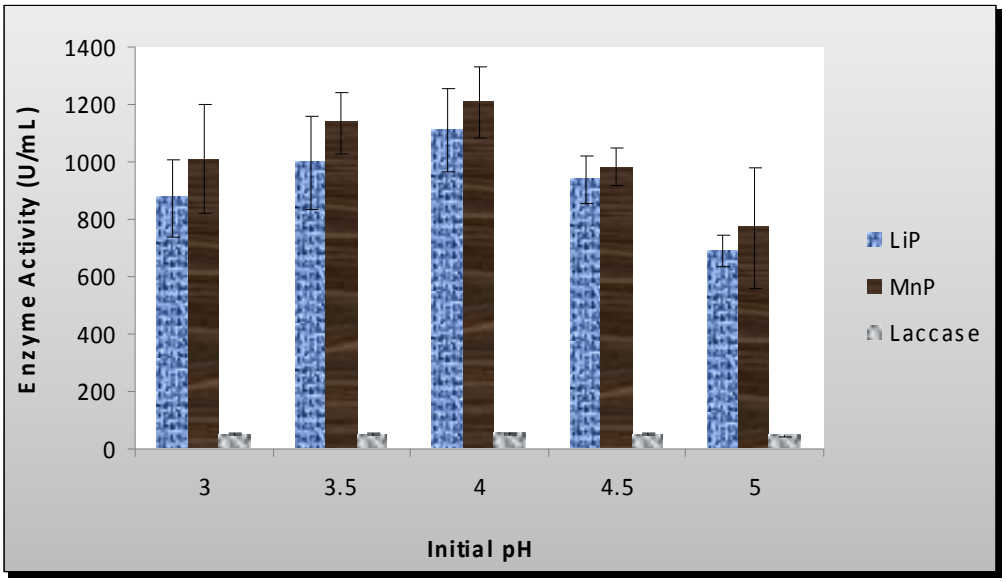

Fig. 2. Efffect of varying pH on ligninase production by *Trametes versicolor* IBL-04 in SSF of rice straw after 5 days of incubation

Statistical analysis by ANOVA showed a significant difference ($p \leq 0.01$) in ligninase yield with varying pH. Results of DMR test also revealed a significant ($p \leq 0.05$) difference between all treatments means. The pH of SSF medium had a significant effect on ligninase synthesis by the fungus. The pH optima for ligninase production by WRF are highly dependent on chemical composition of the substrates and fermentation media (Xu 1997; Radha et al. 2005). Similar to our findings, maximum LiP and MnP activities were produced by *Pleurotus ostreatus* in the pH range 4.0 to 5.0 at 25°C (Metamora et al. 2003). The optimum ligninase production by *Coriolus hirsutus* and *Trametes villosa* CCB176 has been reported at pH 4.0 and 5.0, respectively (Shin and Lee 2000; Yamanaka et al. 2008).

Effect of Incubation Temperature

The temperature verses enzyme production profiles (Fig. 3) of *Trametes versicolor* IBL-04 revealed higher MnP, LiP, and laccase activities at 30 °C as compared to other temperatures of incubation. When cultivated at temperatures higher than 30 °C, the activities of ligninolytic enzymes of WRF were substantially decreased. Statistical analysis of data revealed significant difference ($p \leq 0.01$). When compared by DMR test, differences among all the treatment means were significant ($p \leq 0.05$).

The incubation temperature showed considerable impact on ligninase production, and 30 °C was the optimum temperature. *Coriolus hirsutus* has also been found to excrete a considerable amount of laccase and MnP at 28 ^{o}C (Koroleva et al. 2002). A significant influence of incubation temperature on ligninolytic enzymes of *Pleurotus* sp. and *Dichomitus squalens* and other WRF has been reported, and temperatures ranging from 25 to 30 °C were found optimum for ligninase production (Zadrazil et al. 1999; Arora and Gill 2000; Lang et al. 2000; Pointing et al. 2000). In a previous study (Shin et al.

1997), the highest rate of enzyme formation by *Pleurotus ostreatus* was observed at 25 °C, and rapid enzyme inactivation occurred above 35 °C. Similar to our findings, an incubation temperature of 32 °C was optimum for laccase production by *Streptomyces psammoticus*, and considerable activity was also observed at 30 °C (Niladevi et al. 2007).

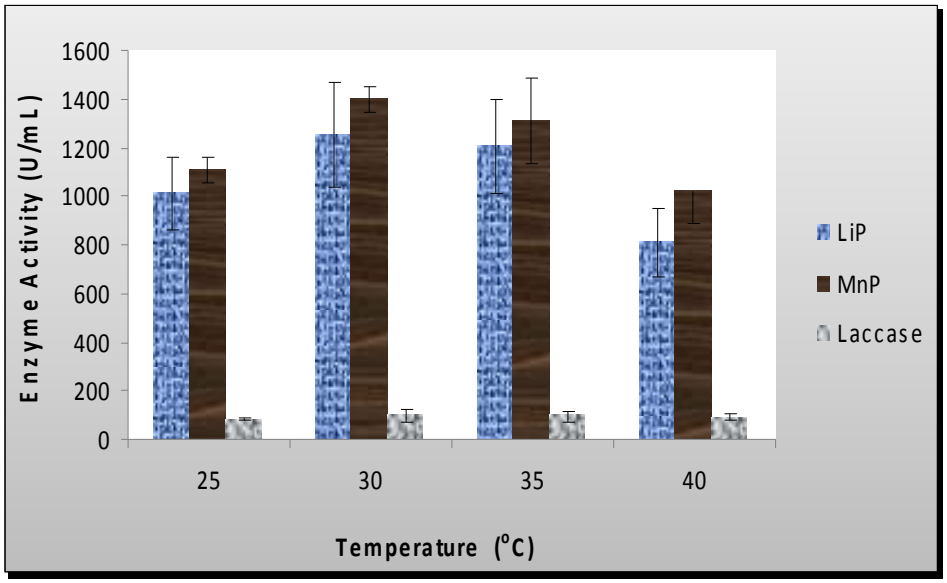

Fig. 3. Efffect of varying incubation temperatures on ligninase production by *Trametes versicolor* IBL-04 in SSF of rice straw after 5 days of still culture incubation

Effect of Carbon and Nitrogen Additives

Glucose, fructose, sucrose, maltose, and molasses (1%) were used as carbon sources along with urea, yeast extract, beef extract, peptone, and ammonium sulphate (0.2%) as nitrogen sources in the SSF medium of rice straw to study their stimulatory/ inhibitory effects on ligninase production under pre-optimized conditions. It was important to note that by the addition of carbon and nitrogen sources, the production pattern of enzymes changed; for MnP (1,576 U/mL) and LiP (1,542 U/mL) the best carbon and nitrogen source combination was C_4N_1 (maltose and urea), while for laccase (98.05 U/mL) C_1N_2 (glucose and yeast extract) was the best combination (Table 2). The enzyme profiles varied with different carbon and nitrogen source combinations. The source and concentration of carbon and nitrogen are the powerful factors regulating the synthesis of lignolytic enzymes by WRF (Mikiashvili et al. 2005; Songulashvili et al. 2007). An optimum C:N ratio is necessary for good LiP, MnP, and laccase production because some white rot fungi grow better under carbon and nitrogen limitations, but others perform better in carbon and nitrogen sufficient culture media (Bonnarme et al. 1991). Revankar and Lele (2006) obtained the highest laccase yields by using a combination of glucose and starch as carbon and yeast extract as nitrogen source.

Table 2. Activities of Ligninases produced by *Trametes versicolor* IBL-04 with different Carbon and Nitrogen Sources*

Nitrogen sources (0.2% W/W)		Enzyme Activities (U/ml) Carbon sources (1% W/W)				
		Glucose (C_1)	Succrose (C_2)	Fructose (C_3)	Maltose (C_4)	Molasses (C_5)
Urea (N_1)	MnP	1331±4.0	1250±4.26	1365±5.65	1576±2.09	1489±3.16
	LiP	1401±16.1	1467±5.67	1493±14.3	1542±6.71	1475±13.7
	laccase	71.24± 4.35	76.66± 0.53	73.75±0.20	84.58±0.14	74.02± 1.57
Yeast extract (N_2)	MnP	1381±6.4	1402±1.63	1484±5.60	1321±3.22	1406±0.53
	LiP	1478±11	1189±6.57	1332±14.7	1516±14.6	1407±10.6
	laccase	98.05±3.42	75.97± 0.78	80.82± 0.48	86.80±0.56	91.05± 2.65
Beef extract (N_3)	MnP	1448±5.3	1334±3.71	1469±4.46	1441±1.77	1411±6.51
	LiP	1478±11	1271±10.3	1513±9.14	1315±4.67	1427±3.68
	laccase	80.97±0.41	70.96± 1.56	71.66± 1.53	65.00±1.31	84.16± 1.58
Peptone (N_4)	MnP	1576±3.9	1398±6.47	1424±2.81	1503±3.81	1360±5.46
	LiP	1542±4.7	1356±7.03	1327±11.9	1480±12.0	1519±1.67
	laccase	80.13± 0.46	80.05± 0.62	59.02± 4.78	76.24± 0.74	92.07± 2.10
Ammonium sulphate (N_5)	MnP	1476±4.	1421±1.03	1500±1.42	1460±5.56	1396±8.12
	LiP	1152±5.	1329±10.	1251±10.9	1439±16.81	1497±15.68
	laccase	68.46± 0.30	74.99± 0.27	81.24± 2.28	68.88± 2.12	90.13± 1.84

* Fermentation time, 5 days

Effect of Surfactant (Tween-80)

The effect of varying levels of 1mM Tween- 80 (0.1, 0.2, 0.3, 0.4, 0.5 mL) was studied under pre-optimized conditions. It was observed that lower concentrations of Tween-80 enhanced ligninase production by *C. versicolor* in SSF of rice straw (Fig. 4). Flasks receiving 0.3 mL of 1mM Tween-80 showed maximum production of MnP (1,756 U/mL), LiP (1,605 U/mL), and laccase (95.55 U/mL). Analysis of variance of the data showed significant ($p \leq 0.01$) difference between ligninase yields with varying concentrations of Tween-80. Differences among different treatment means were also significant ($P \leq 0.05$) except T_4. Surfactants enhance enzymes production and secretion by promoting the penetration of water into the solid substrate matrix and by increasing the surface area for microbial growth (Jager et al. 1985). Tween-80 has been reported to transform the cell membrane structure and promote the permeation of ligninases from the fungal cells into the medium (Asther et al. 1987; Rodriguez-Couto et al. 2001). In our previous studies (Asgher et al. 2006), addition of 0.1 ml of 1mM tween-80 to SSF medium of corncobs substantially enhanced LiP formation by *P. chrysosporium*.

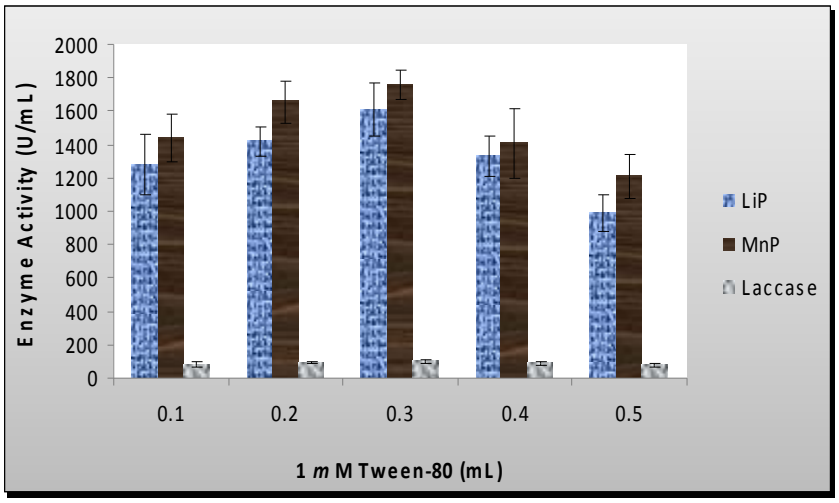

Fig. 4. Effect of varying concentrations of 1mM tween-80 on ligninase production by *Trametes versicolor* IBL-04 in SSF of rice straw after 5 days of still culture incubation

Effect of Inoculum Size

Growth media containing 5g rice straw with optimum (66.6 %) moisture content were inoculated with varying volumes of inoculum and incubated under optimum conditions. The maximum ligninases were produced in the flasks receiving 5 mL inoculum (Fig. 5).

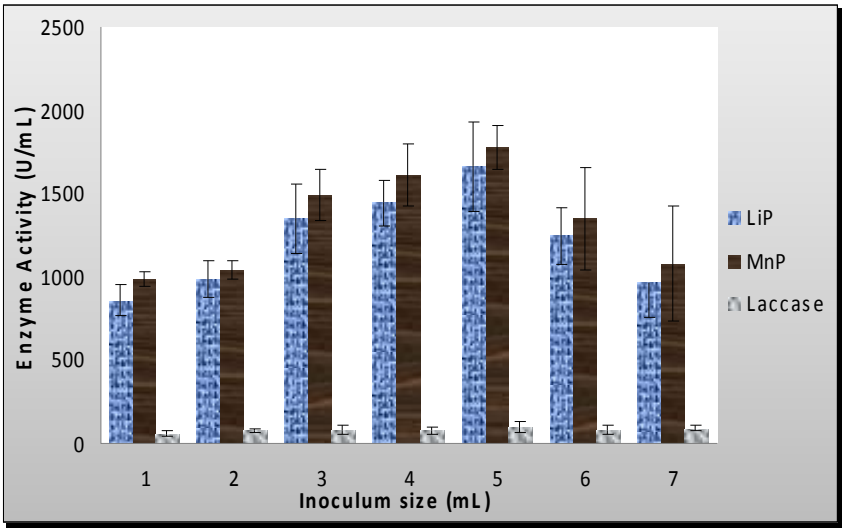

Fig. 5. Effect of varying inoculum levels on ligninase production by *Trametes versicolor* IBL-04 in SSF of rice straw after 5 days of still culture incubation

Statistical analysis showed a significant ($p \leq 0.01$) difference in the production of ligninase enzymes with varying inoculum size, and all the treatment means were found to have significant differences ($p \leq 0.05$) when compared with DMR test. The production of enzymes increased with an increase in spore density from 1 to 5 mL. However, a further increase in spore density caused lower enzyme production. Optimum spore density is important for the SSF process. Lower inoculum levels may not be sufficient to initiate the

growth of microorganism, resulting in a longer lag phase. On the other hand, higher inoculum size increases spore density, causing faster depletion of available nutrients (Galhaup et al. 2002; Patel et al. 2009). Higher inoculum volumes also increase the water content, thus decreasing aeration in solid substrate matrix and consequently inhibiting fungal growth and enzyme formation (Galhaup et al. 2002).

CONCLUSIONS

The fungus produced maximum activities of MnP (1775 U/mL), LiP (1663 U/mL), and laccase (99 U/mL) when rice straw (66.6 % moisture, pH 4.0) containing maltose (1% w/w) as carbon source, urea (0.2% w/w) as nitrogen source, and 1*mM* Tween-80 (0.3 ml) as surfactant was fermented at 30 ^{o}C for 3 days. Previously, Koroleva et al. (2002) reported maximum laccase activity of 80 U/mL produced by *Coriolus hirsutas* 075 after 3 days of inoculation. Hossain and Anantharaman (2006) reported that *Trametes versicolor* secretes 495, 440 and 410 U/mL of LiP, MnP and laccase, respectively. Koroleva et al. (2002) reported maximum laccase activity of 80 U/mL produced by *Coriolus hirsutas* 075 after 3 days of inoculation. It can therefore, be concluded that *Trametes versicolor* IBL-04 showed tremendous potential for ligninolytic enzymes synthesis in SSF of rice straw. The promisingly high activities of MnP and LiP suggest the possibility of commercialization of the production process. The suitability of the enzymes for industrial applications can be assessed through the study of their kinetic and thermostabilty characteristics.

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