HYPERPRODUCTIVITY OF EXTRACELLULAR ENZYMES FROM INDIGENOUS WHITE ROT FUNGI (*P. chrysosporium* IBL-03) BY UTILIZING AGRO-WASTES

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An indigenous locally isolated white rot fungal strain *Phanerochaete chrysosporium* IBL-03 was investigated for the hyper-production of ligninolytic enzymes from different agro-industrial wastes including wheat straw, rice straw, banana stalks, corncobs, corn stover, and sugarcane bagasse as substrate material in still culture fermentation technique. Screening experiments were performed at 30^{o}C from 1 to 10 days and maximum enzyme activities were recorded after the 5th day of incubation on banana stalk. *P. chrysosporium* IBL-03 produced highest activities of lignin peroxidase (LiP) and manganese peroxidase (MnP) but no laccase activity was detected in any fermented culture media. Production of ligninolytic enzymes was substantially enhanced through the optimization process. When banana stalk at 66.6 % moisture level and pH 4.5 was inoculated with 5mL spore suspension of *P. chrysosporium* IBL-03 at 35^{o}C in the presence of molasses (1%) as carbon source, ammonium sulfate (0.2%) as nitrogen supplement, (1%) Tween-80 (0.3 mL) as surfactant and mediators ($MnSO_4$ and veratryl alcohol) enhanced the LiP and MnP production up to 1040 and 965 (U/mL), respectively.

Keywords: Phanerochaete chrysosporium IBL-03*; Extracellular enzymes; Agro-wastes; SSF; Optimization*

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INTRODUCTION

White rot fungi (WRF) are among the most robust micro-organisms, having the ability to degrade the major components of lignocellulosic sources, cellulose, hemicelluloses, and lignin as available hydrolyzable nutrients efficiently through their nonspecific and non stereo-selective extracellular enzyme system (Papinutti and Forchiassin 2007; Ruhl et al. 2008). Despite the remarkable collection of lignocellulolytic micro-organisms, only a few of them have been identified as efficient and extensive lignin degraders (Akin et al. 1995). Two families of ligninolytic enzymes are widely considered to play a vital role in the enzymatic degradation: phenol oxidase (laccase) and peroxidases (lignin peroxidase and manganese peroxidase) (Hammel et al. 2008).

The ligninolytic enzyme complexes mostly known as lignin modifying enzymes (LME) of WRF vary significantly in their composition. LMEs, especially peroxidases, have also been found to be formed by some other WRF, such as *Phanerochaete chrysosporium*, where lignin-degrading enzymes have received substantial consideration. In *P. chrysosporium* and *T. versicolor*, lignin peroxidases have been found only during secondary metabolism, a physiological state linked with the lignin-degrading (Levin et al.

2008). Ligninolytic enzymes of WRF find a wide range of commercial applications, including degradation of dyes, bioremediation of industrial effluents, lignin hydrolysis for ethanol production (Stoilova et al. 2010), clarification of musts and wines, improving whiteness of conventionally bleached cotton (Lorenzo et al. 2006), bio-stoning of denims (Pazarlioglu et al.2005), and bio-bleaching and bio-pulping in the pulp and paper industry (Hakala et al. 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; Vikineswary et al. 2006). MnPs are glycoproteins that are considered unable to oxidize non-phenolic substrates, although they have the capability to depolymerize synthetic or natural lignin in vitro (Baborova et al. 2006; Vikineswary et al. 2006). Laccases are N-glycosylated blue multi-copper oxidases (Asgher et al. 2008) that are versatile mineralizers of lignin and also for a variety of synthetic dyes (Murugesan et al. 2006; Zouari-Mechichi et al. 2006; Quaratino et al. 2007).

P. chrysosporium is among the most potent of lignin-degrading microorganisms that produce peroxidases extracellularly with powerful capacity. This paper reports the results of a study carried out to investigate the potential of an indigenous white rot fungal strain *P. chrysosporium* IBL-03 locally isolated in Pakistan for ligninolytic enzymes production using agro-industrial wastes and industrial by-products by keeping in view the wide spread industrial applications of ligninolytic enzymes.

EXPERIMENTAL

Lignocellulosic Substrate

Lignocellulosic agro-industrial wastes such as wheat straw and rice straw were obtained from Student research Farms, University of Agriculture, Faisalabad (UAF), Pakistan. Banana stalks and sugar cane bagasse were obtained from the local fruit market and Crescent Sugar Mills, Faisalabad, respectively. Corn stover and corncobs were collected from CPC-Rafhan, Faisalabad, Pakistan. The substrates were crushed into small pieces, oven dried (50 ^{o}C), and ground to 40 mm mesh particle size and stored in air-tight plastic jars.

Fungal Culture and Inoculum Development

A pure culture of the indigenous strain *P. chrysosporium* IBL-03, available in Industrial Biotechnology Laboratory, UAF was used for the present study. An inoculum was prepared by growing the fungus in Kirk's basal salt medium (Tien and Kirk 1988) supplemented with Millipore filtered sterile glucose (1%). The medium was sterilized at 121^{o}C for 15 min. After cooling to room temperature, loopful spores of *P. chrysosporium* IBL-03 from Potato Dextrose Agar (PDA) slant were transferred into the broth under sterilized conditions in laminar air flow (Dalton, Japan). The inoculated flask was incubated for 5 days at 30^{o}C in an orbital shaker (Sanyo-Gallemkemp, UK) with continuous shaking position (120 rpm) to get 1x10^{6} to 1x10^{8} spores /mL (Kay-Shoemake and Watwood 1996).

Experimental Design

Ligninolytic enzymes production was carried out in 500 mL Erlenmeyer flasks. Triplicate flasks contained 5 g of substrate moistened (60% w/w) with Kirk's medium (pH 4.0).

The prepared sample flasks were autoclaved and inoculated with 5 mL of freshly prepared spore suspension of *P. chrysosporium* IBL-03. The inoculated flasks were kept at 30°C in a temperature controlled incubator (EYLA SLI-600ND, Japan) for 1-10 days under still culture conditions.

Harvesting and Enzyme Extraction Protocol

The fermented flasks were harvested for enzyme extraction after every 24h by adding distilled water (100mL) and shaking at 120 rpm for 30 min (Gomes et al. 2009). The contents were filtered through Whatman No.1 filter paper and washed three times with distilled water.

The filtrates were centrifuged at 4000×g for 5 min and carefully collected supernatants were assayed for LiP, MnP and laccase.

Optimization of SSF Culture Conditions

Maximum production of enzyme requires the optimization of different nutritional growth conditions, so an attempt was made to optimize various parameters to investigate their effect on solid state fermentation (SSF) of banana stalk (selected in screening experiment) as the most suitable substrate. A classical method of optimization was followed by varying one parameter at a time and maintaining the previously optimized at a constant level. The effects of different carbon and nitrogen sources were studied in one experiment using a Completely Randomized Design (CRD) to accommodate the corresponding interactions.

Enzyme Activity Assays

Enzyme activities of supernatants collected at the end of each optimization step were determined using spectrophotometer (T60, PG Instruments, UK) as described earlier (Iqbal et al. 2011).

LiP activity was determined by the method of Tien and Kirk (1988), while unit activity of LiP is defined as the amount of enzyme required to oxidize one μmol of VA per minute at 25°C. MnP was assayed by the method of Wariishi et al. (1992), and unit activity of MnP is defined as the amount of enzyme required to oxidize 1 μmol of Mn (II) per minute at 25°C. Laccase activity was measured by the method of Wolfenden and Wilson (1982), while unit activity of laccase is defined as 1 μmol of ABTS oxidized per minute at 25°C.

Statistical Analysis

All the experimental data was statistically evaluated according to Steel et al. (1997). The means and standard errors of means (Mean±S.E) were calculated for each treatment.

RESULTS AND DISCUSSION

Screening of Fungus on Lignocellulosic Substrates

The results of the screening trial showed that the maximum production of MnP (620U/mL) and LiP (640U/mL) was achieved in SSF using banana stalk after 5 days, followed by rice straw after 6 days of incubation (Table 1). Enzyme assays showed that laccase was not detected in any of the fermented sample flasks. Cost-effective production of ligninases is a key for successful exploitation of lignocellulosic resources as a renewable energy source. Castillo et al. (1997) also reported LiP and MnP activities in straw extracts from cultures of *P. chrysosporium* BKM-F-1767 during SSF after 6 days of incubation. Different WRF have been reported to produce maximum ligninolytic enzymes after different time periods due to genetic variation among the strains as well as in the nature and composition of the substrates used (Patel et al. 2009). A WRF *Datronia sp*. KAPI0039 produced maximum laccase and MnP after 4 and 8 days of cultivation, respectively (Vaithanomsat et al. 2010).

Table 1. Ligninolytic Activities Produced by *P. chrysosporium* IBL-03 on Different Lignocellulosic Substrates under SSF

Substrates (5g)	Enzyme Activities (U/mL) Fermentation time (Days)										
	Enzymes	1	2	3	4	5	6	7	8	9	10
Rice Straw	MnP	358± 1.91	397± 1.2	418± 2.9	443± 1.47	499± 2.4	590± 1.3	577± 2.7	513± 1.7	377± 1.9	200± 1.1
	LiP	332± 4.5	333± 3.5	516± 2.2	572± 5.7	596± 2.9	625± 2.4	599± 1.2	571± 2.9	559± 2.7	549± 1.35
Wheat Straw	MnP	251± 1.4	375± 2.7	508± 2.3	530± 1.6	577± 3.3	441± 2.8	390± 3.4	291± 1.5	231± 2..5	221± 1.8
	LiP	139± 2.8	226± 3.5	339± 2.3	382± 3.4	460± 3.6	399± 2.5	387± 3.6	376± 3.4	350± 1.2	337± 2.6
Sugarcane Bagasse	MnP	243± 1.81	282± 1.9	419± 2.5	381± 1.3	434± 2.8	316± 1.3	292± 2.4	193± 1.7	115± 1.31	78± 1.91
	LiP	159± 1.8	175± 2.18	180± 1.2	188± 1.01	204± 2.5	283± 2.7	316± 1.2	290± 3.1	237± 2.8	209± 1.45
Banana Stalk	MnP	223± 3.4	280± 2.8	387± 1.2	490± 4.8	620± 4.5	596± 3.7	560± 5.6	486± 3.2	338± 3.1	365± 2.8
	LiP	180± 2.1	196± 2.6	288± 3.6	455± 2.6	640± 2.9	650± 4.6	628± 4.4	625± 3.2	671± 4.2	610± 4.3
Corn Stover	MnP	261± 1.6	241± 1.7	313± 3.2	337± 3.1	195± 2.5	123± 1.9	90± 1.22	70± 1.19	70± 2.5	47± 1.11
	LiP	161± 2.3	143± 2.6	283± 3.8	355± 4.2	312± 3.22	225± 4.7	220± 2.6	133± 3.5	137± 2.5	103± 2.5
Corn Cobs	MnP	176± 2.0	199± 2.6	291± 3.2	232± 3.5	270± 2.9	190± 3.3	149± 1.5	94± 1.21	57± 1.9	68± 1.21
	LiP	166± 2.2	139± 1.69	176± 4.4	261± 3.3	250± 3.2	270± 2.1	139± 1.2	151± 3.9	151± 2.3	128± 2.7

Substrate 5gm; pH 4.0; Temperature, 30°C

Optimization of SSF Culture Conditions

Effect of moisture level

Increasing the moisture level from 50 to 66.6 % causes an enhancement of fungal growth and ligninases production. Banana stalk that was fermented at 66.6% (w/w) moisture gave maximum LiP (672U/mL) and MnP (635U/mL) production after 5 days of inoculation with *P. chrsosporium* IBL-03. However, an increase in moisture level of more than 66.6 % caused a significant decrease in enzyme activities (Fig. 1). Higher and lower moisture levels were inhibitory, leading to secretion of lower activities of ligninases in secondary growth due to poor accessibility of nutrients and limited aeration (Regina et al. 2008; Shaheen et al. 2008; Bhatti and Nawaz 2009). The end product and water requirement of the fungus depends on an optimum moisture level in SSF and the water holding capacity of the substrate (Kim et al. 1985; Asgher et al. 2006).

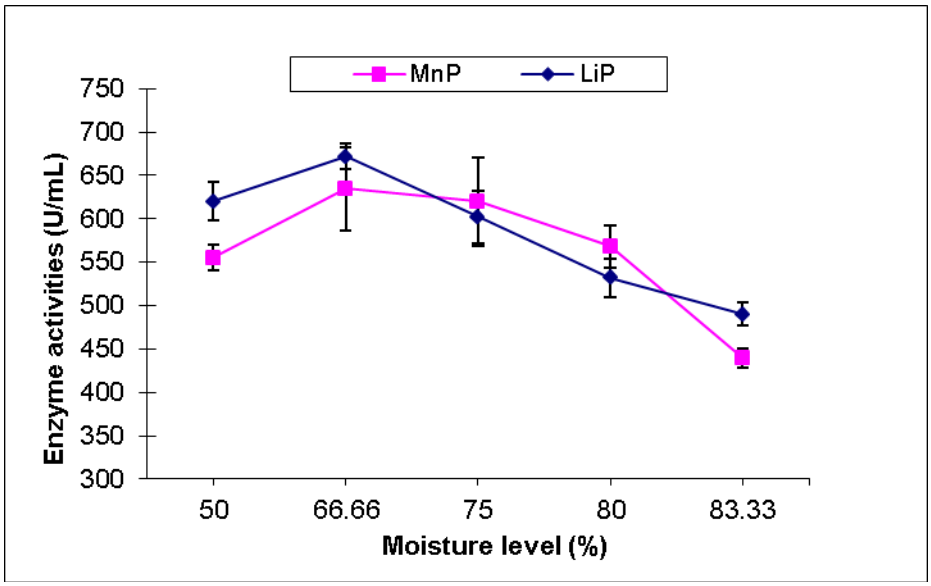

Fig. 1. Effect of varying moisture levels on ligninolytic enzymes production by *P. chrysosporium* IBL-03 in SSF of banana stalk

Effect of pH

The maximum activities of LiP (721U/mL) and MnP (688U/mL) were recorded in the SSF media processed at pH 4.5. Ligninolytic enzymes synthesis by *P. chrsosporium* IBL-03 progressively increased with an initial increase in pH and peaked in activity at pH 4.5 (Fig. 2). The pH of SSF medium had a significant influence on ligninolytic synthesis, as fungi are very sensitive to variation in pH. Radha et al. (2005) reported that the pH optimum for ligninolytic production by WRF is highly dependent on chemical composition of the substrates and fermentation media. WRF in most of the cases have shown optimum mycelial growth to produce higher activities of ligninolytic enzymes at pH 3-6 (Shin and Lee, 2000; Radha et al. 2005; Yamanaka et al. 2008). The most suitable conditions are pH around 4.5 and 32^{o}C, since both LiP and MnP exhibited great stability in these conditions (Couto et al. 2006).

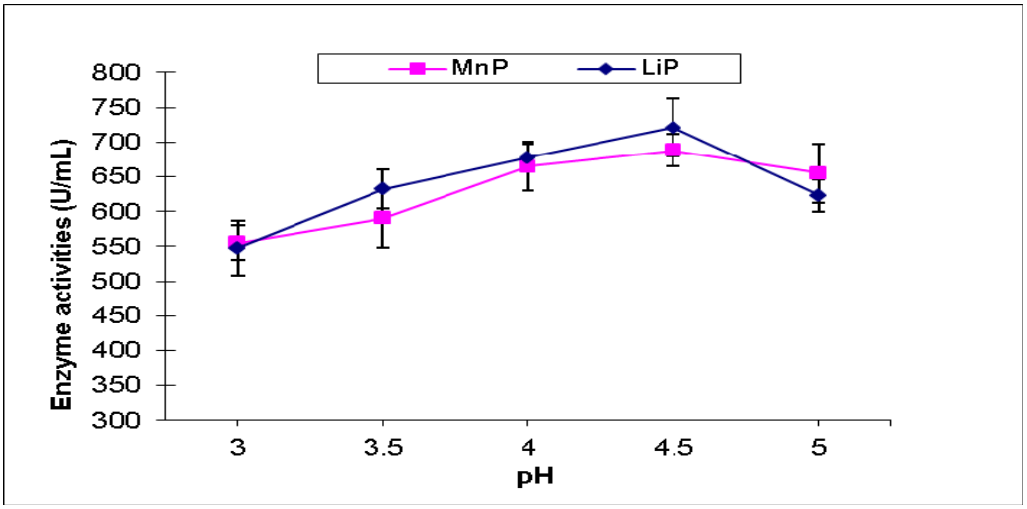

Fig. 2. Effect of varying pH on ligninolytic enzymes production by *P. chrysosporium* IBL-03 in SSF of banana stalk

Effect of incubation temperature

To optimize the temperature for maximum ligninolytic enzymes formation, triplicate samples were incubated at 25, 30, 35, and 40ºC; highest enzyme activity was noted in the medium fermented at 35°C (Fig. 3). When cultivated at temperatures higher than 35°C, the ligninolytic enzymes activities were substantially decreased. Tekere et al. (2001) found that the optimum temperature for cultures of *Coriolus* (*Trametes, Poly-porus*) *versicolor* and *P. chrysosporium* for ligninase synthesis in solid-state fermentation varied between 25-37°C. A significant influence of incubation temperature on lignin-lytic enzymes of *Pleurotus* sp. and *Dichomitus squalens* and other WRF has been reported (Arora and Gill 2000; Tripathi et al. 2008). A variation in incubation temperature has a significant influence on synthesis of ligninolytic enzymes and their activities. The temperatures ranging from 25 to 37°C have been found optimum for ligninase production by different WRF (Zadrazil et al. 1996; Arora and Gill 2000; Tripathi et al. 2008).

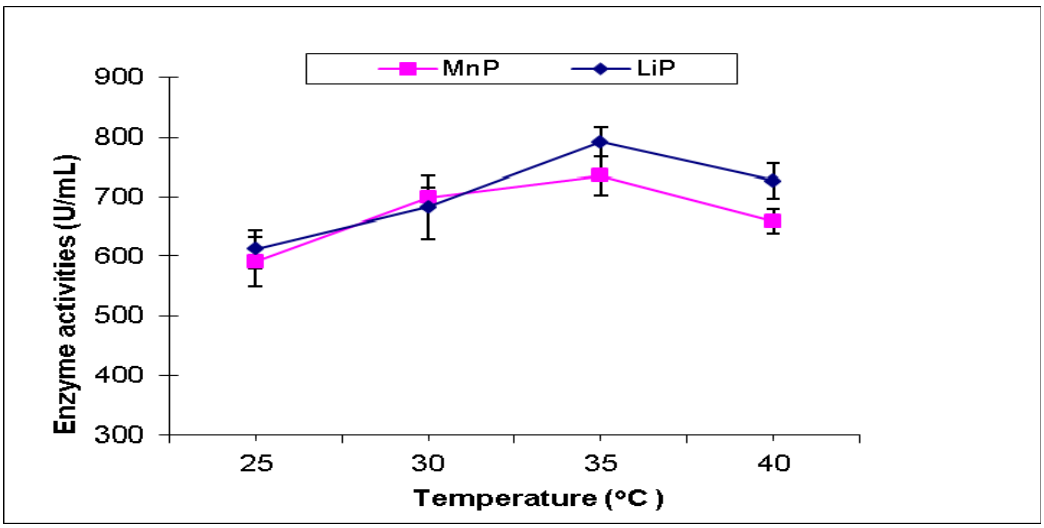

Fig. 3. Effect of varying incubation temperatures on ligninolytic enzymes production by *P. chrysosporium* IBL-03 in SSF of banana stalk

Effect of Carbon and Nitrogen Sources

To investigate the effect of additional carbon and nitrogen sources, glucose, fructose, sucrose, maltose, and molasses (1%) were used as carbon sources along with (0.2%) inexpensive nitrogen supplements like urea, yeast extract, beef extract, peptone, and ammonium sulfate that were used in different interactions for high production of ligninolytic enzymes from banana stalk. It was important to note that when carbon and nitrogen sources were added, the production pattern of enzymes changed; the combination of molasses and ammonium sulfate proved best and gave maximal production of LiP (856U/mL) and MnP (799U/mL) (Table 2). The source and concentration of carbon and nitrogen are the powerful factors in regulating the synthesis of ligninolytic 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 (Bonnarme et al. 1991). Selvam et al. (2006) reported that an easily available and utilizable additional carbon source increases the growth and enzyme activity of white rot fungi. However, different WRF strains behave differently in nitrogen enriched media. Different strains and species of WRF differ in their sensitivity towards metals during their growth on lignocellulosic substrates (Sathiya-Moorthi et al. 2007).

Table 2. Ligninolytic Activities Produced by *P. chrysosporium* IBL-03 with Different Carbon and Nitrogen Sources under Optimum Time Period

Nitrogen sources (0.2% W/W) ↓ / Enzymes →		Enzyme Activities (U/mL) Carbon sources (1% W/W) →				
		Glucose (C1)	Fructose (C2)	Sucrose (C3)	Maltose (C4)	Molasses (C5)
Urea (N_1)	MnP	331±4.0	250±4.26	365±5.65	576±2.09	489±3.16
	LiP	401±16.1	467±5.67	493±14.3	1542±6.71	575±13.7
Yeast extract (N_2)	MnP	381±6.4	402±1.63	484±5.60	321±3.22	506±0.53
	LiP	478±11	489±6.57	332±14.7	516±14.6	527±10.6
Beef extract (N_3)	MnP	448±5.3	334±3.71	469±4.46	441±1.77	511±6.51
	LiP	478±11	271±10.3	513±9.14	315±4.67	617±3.68
Bacteriological Peptone (N_4)	MnP	576±3.9	398±6.47	424±2.81	503±3.81	660±5.46
	LiP	542±4.7	356±7.03	327±11.9	480±12.0	719±1.67
Ammonium sulfate (N_5)	MnP	745±4.1	514±1.03	545±1.42	625±5.56	799±8.12
	LiP	802±5.0	612±10.0	686±10.9	702±16.81	856±15.68

Substrate 5gm; pH 4.5; Temperature, 35°C; Fermentation Time Period, 5 Days

Effect of Surfactant (Tween-80)

The effect of varying levels of 1% Tween-80 (0.1, 0.2, 0.3, 0.4, and 0.5 mL) was studied under pre-optimized conditions. It was observed that lower concentrations of Tween-80 enhanced ligninase production by *P. chrsosporium* IBL-03 in SSF of banana stalk. Flasks incubated with 0.3 mL of 1% Tween-80 showed maximum production of LiP (946U/mL) and MnP (872U/mL) (Fig. 4). Surfactants enhance enzymes production

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 enzymes from the fungal cells into the medium (Asther et al. 1987; Rodriguez-Couto et al. 2001). In our previous studies (Asgher et al. 2006; Iqbal et al. 2011), surfactants have the potential to enhance microbial growth in SSF by promoting the penetration of water into the solid substrate matrix leading to an increase in surface area.

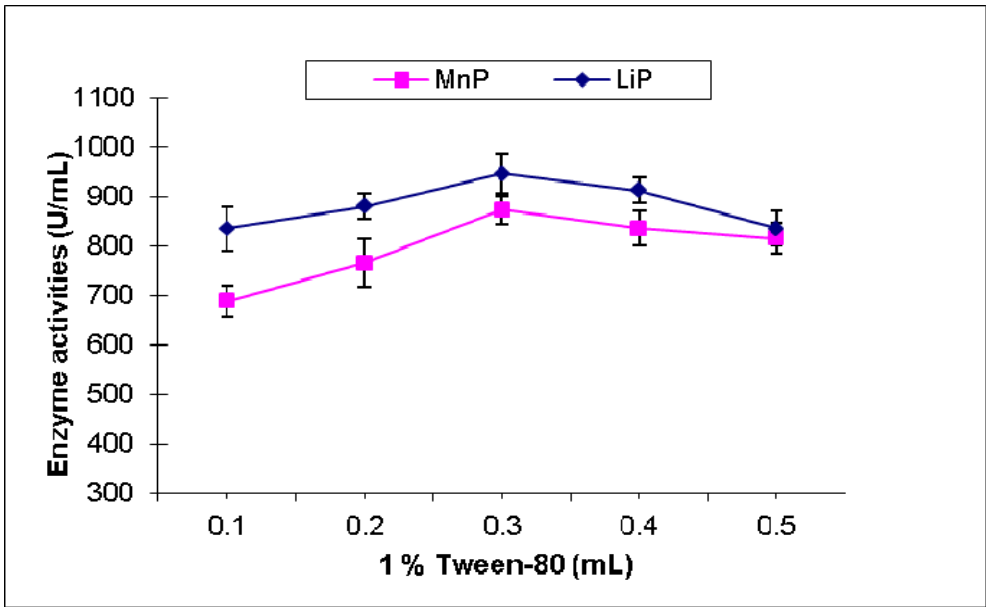

Fig. 4. Effect of varying concentrations of 1% Tween-80 on ligninolytic enzymes production by *P. chrysosporium* IBL-03 in SSF of banana stalk

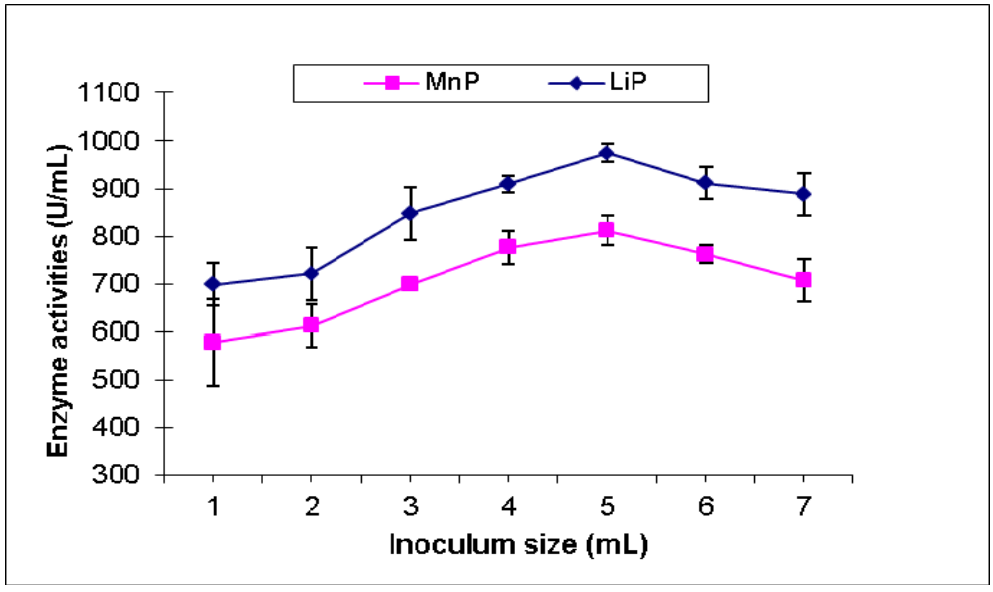

Fig. 5 Effect of varying inoculum levels on ligninolytic enzymes production by *P. chrysosporium* IBL-03 in SSF of banana stalk

Effect of Inoculum Size

The maximum ligninolytic enzymes were produced in the flasks receiving 5 mL of inoculum (Fig. 5). The production of enzymes increased with an increase in inoculum from 1 to 5mL. However, a further increase in an inoculums volume caused a decrease in enzyme production. In our previous studies (Iqbal et al. 2011), maximum ligninolytic enzyme production from lignin containing waste rice straw was achieved when fermentation media was inoculated with 5 mL freshly prepared fungal spores suspension. Lower inoculum's level may not be sufficient to promote the fungal growth resulting in longer lag phase (Sabu et al. 2005; Iqbal et al. 2011), whereas higher inoculum size causes faster depletion of available nutrients required for growth (Galhaup et al. 2002; Patel et al. 2009).

Effect of Mediators

The enzymatic analysis of extracted crude enzymes showed that different mediators have different effects on production of individual enzymes. Veratryl alcohol and $MnSO_4$ showed notably enhancing outcomes on production of LiP and MnP, respectively. The maximum activity of LiP (1040U/mL) and MnP (965U/mL) was noted after four days in the culture supernatants of flasks inoculated with veratyl alcohol and $MnSO_4$, respectively (Fig. 6), whereas ABTS had little or no affect on LiP and MnP activities. However, according to literature reported by Lu et al. (2007), ABTS acts as a mediator for ligninolytic laccase and also for a variety of pollutants.

The veratryl alcohol is natural fungal secondary metabolite that acts as a redox mediator to stimulate the LiP catalyzed oxidation of substrates (Huang et al. 2003) while $MnSO_4$ acts as mediator for MnP and causes the release of Mn^{2+} that performs the role of action. Sundaramoorthy et al. (2005) report that MnP catalyzes the peroxide-dependent oxidation of Mn^{2+} to Mn^{3+} that makes a complex with other chelators that enhance the activity of MnP.

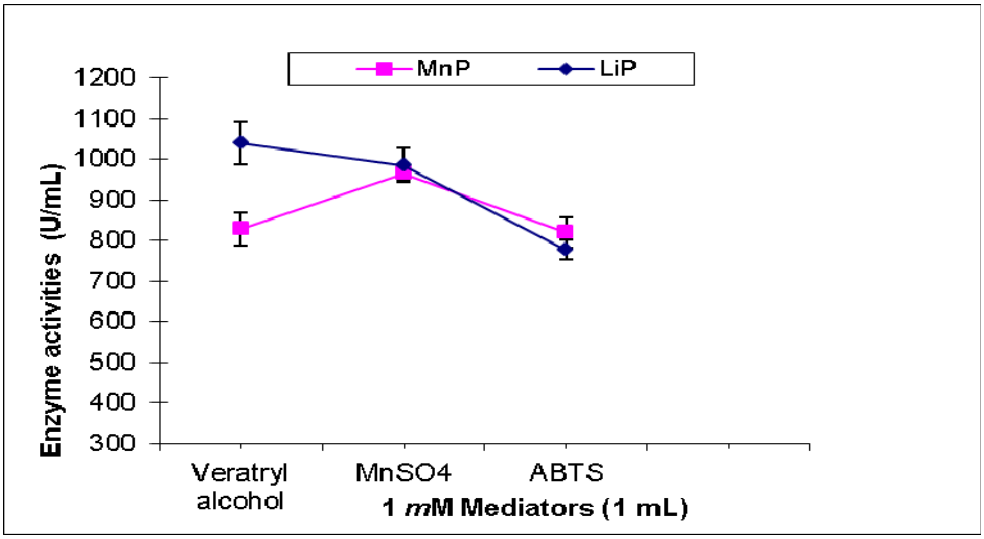

Fig. 6. Effect of different mediators on ligninolytic enzymes production by *P. chrysosporium* IBL-03 in SSF of banana stalk

Results obtained after optimization showed that *P. chrysosporium* IBL-03 had an unexpected potential to produce high amount of LiP (1040U/mL) through SSF of banana stalk than those described in literature under SSF of varying lignocellulosic substrates: 15.8U/mL from *Cunninghamella elegans* (Roushdy et al. 2011), 42U/mL from *Flavodon flavus* (Mtui and Nakamura 2008).

P. chrysosporium IBL-03 used in this study had an extraordinary capability to produce higher amounts of MnP (965U/mL) under optimum fermentation conditions than those described in the literature: 30 U/mL from *P. ostreatus* (Palmieri et al. 2000), 214.5U/mL from *Trametes trogii* (Levin et al. 2005), 0.148U/mL from *T. versicolor* (Mikiashvili et al. 2005), 25U/mL from *Flavodon flavus* (Mtui and Nakamura 2008), and 4.48U/mL from *M. racemosus* CBMAI 847 (Bonugli-Santos et al. 2010).

CONCLUSIONS

1. *Phanerochaete chrysosporium* IBL-03 showed remarkable potential for ligninolytic enzymes formation in SSF of banana stalk (an inexpensive lignocellulosic substrate) under optimized conditions with maximum activities of LiP (1040U/mL) and MnP (965U/mL) through SSF of banana stalk than those described in the literature under SSF of varying lignocellulosic substrates.
2. The promisingly high activities of LiP and MnP suggest the possibility of commercialization of the production process. However, the suitability of the enzymes for biotechnological applications can be investigated through kinetic characterization of the purified enzymes, as thermo-stability is a desired characteristic of an enzyme for its possible use in industry.
3. In conclusion, an attempt was made towards finding the best growth conditions for successful cultivation of *P. chrysosporium* IBL-03, and production of the ligninolytic enzymes, which have the most important role in the processes of biotransformation of plant raw materials in different manufacturing industries.

ACKNOWLEDGMENTS

The study was a part of a project focused on the development of ligninolytic enzymes for industrial applications. The project studies have been funded by the Higher Education Commission of Pakistan.

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Article submitted: June 18, 2011; Peer review completed: Sept. 6, 2011; Revised version received and accepted: Sept. 10, 2011; Published: Sept. 13, 2011.