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Utilization of agro-wastes for production of ligninolytic enzymes in liquid state fermentation by *Phanerochaete Chrysosporium*-Ibl-03

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Abstract

Enzymatic delignification of agro-wastes using *Phanerochaete chrysosporium*-IBL-03, a White Rot Fungus (WRF), was investigated in present study. The production of lignin degrading enzymes lignin peroxidase (LiP) and manganese peroxidase (MnP) by *P. chrysosporium*-IBL-03 was screened from various agro-wastes (corn stover, corn cobs, sugarcane bagasse, wheat straw, rice straw and banana stalk) through liquid state fermentation. Wheat straw waste was found to be best for the production of enzymes. Furthermore, various fermentation variable such as carbon and nitrogen sources, carbon: nitrogen ratio, mediators and metal ions were optimized for maximum enzymes production and wheat straw (2.5g), pH (4.5), incubated period (6 days), temperature (35ºC) in the presence of beef extract as nitrogen source and molasses as carbon source in 25:1 ratio were recorded to be best for maximum activity. At optimized conditions, the highest yield of 993.9±18.4 U/mL (MnP) and 1215.15±13.5U/mL (LiP) was achieved. Wheat straw wastes served as excellent sources for the production of MnP and LiP which indicate promising scope of wheat straw utilization for industrial and biotechnological applications for the production of ligninases at large scale.

Key words: Lignin, agro-waste, Ligninases, white rot fungi, fermentation, optimization.

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1. Introduction

Ligninolytic enzymes produced by white rot fungi are of vital importance in many industries including pulp and paper manufacturing, textile, and petrochemical industries. Lignocelluloses wastes (LCW) refer to plant biomass wastes and accounts for more than 60% of the total biomass produced on earth [1, 2] which include wood residues (including sawdust and paper mill discards), grasses, waste paper, agricultural residues (including straw, stover, peelings, cobs, stalks, nutshells, non-food seeds, bagasse, domestic wastes (lignocellulose garbage and sewage), food industry residues and municipal solid wastes [3, 4, 5]. Lignin is the second most abundant renewable biopolymer in nature after cellulose and most abundantly in plant cell wall, imparting rigidity and protecting the easily degradable cellulose from attack by pathogens [6]. Owing to its complicated structure, lignin is highly resistant to microbial degradation and its association with cellulose and hemicelluloses polysaccharides also imparts degradation resistance to these polymers [7].

Two families of ligninolytic enzymes are widely considered to play a key role in enzymatic lignin degradation: Phenol oxidase (laccase) (EC 1.10.3.2), and peroxidases including lignin peroxidase (LiP) (EC 1.11.10.14) and manganese peroxidase (MnP) (EC 1.11.1.13) [8, 9]. *Phanerochaete chrysosporium* (*P. chrysosporium*) a white-rot Basidiomycete, is able to degrade and metabolize polymeric lignin as well as a broad range of recalcitrant organo-pollutants [10, 11, 12] and attracts a lot of attention in the research field of environmental cleanup and consequently, it holds enormous potential in practical applications as a bioremediation agent [13, 14]. *Phanerochaete chrysosporium* produces a potent lignin-degrading system that oxidizes lignin completely to carbon dioxide, however, the biochemical processes involved are not completely understood [15, 16]. *Phanerochaete chrysosporium* has become the most commonly used organism due to its good ligninolytic properties, fast growth and easy handling in culture. This organism has now become a model for many studies on bioremediation of pollutants [17].

In present study, the production of lignin degrading enzymes was investigated by *P. chrysosporium*-IBL-03 using agro-based waste material through liquid state fermentation. For maximum production of enzymes, various independent variables substrates, nitrogen and carbon sources, effect of carbon: nitrogen ratio effect of mediators and metal ions were investigated.

2. Material and Methods

2.1. Agro-Wastes Collection And Pretreatment:

Lignocellulosic residues such as wheat straw, rice straw, corn stover, corncobs, sugar cane bagasse and banana stalk obtained from different local places of Faisalabad like wheat straw and rice straw were obtained from Student Research Farms, University of Agriculture, Faisalabad (UAF), Pakistan. Banana stalks and sugar cane bagasse were collected from local fruit market and Crescent Sugar Mills, Faisalabad, respectively. Corn stover and corncobs were obtained from CPC-Rafhan products, Faisalabad, Pakistan. The collected material were chopped into pieces, dried, ground to powder (40 mm particle mesh) and stored in air tight plastic polyethylene bags. These raw materials were used as substrate for production of ligninolytic enzymes by using *Phanerochaete chrysosporium*-IBL-03.

2.2. Liquid State Fermentation/ Screening

Homogenous spore suspension of *P. chrysosporium*-IBL-03 prepared (3-5 days) in Kirk's basal nutrient medium supplemented with 1% millipore filtered sterile glucose solution [18] and spore size of $1x10^6$ to $1x10^8$ spores /mL was used [19]. For fermentation, triplicate experiments were performed, the inoculated flasks were kept at 30ºC for 1-10 days under continuous shaking condition containing lignocellulosic residues. Samples were harvested after every 24 hour for successive 10 days to select the best substrate and activities of ligninases were determined by standard methods at the end of each experiment. In the second phase of study, after selecting the most suitable substrate for enzyme synthesis the independent variables such as carbon and nitrogen sources, carbon: nitrogen ratio, mediators (veratryl alchohol, $MnSO_4$, ammonium oxalate, H_2O_2 and ABTS) and metal ions ($CuSO_4$, $CaCl_2$, $FeSO_4$, $ZnSO_4$, and KCl) were optimized to achieve the maximum yield of ligninases at pH 4.5 and temperature 35ºC.

2.3. Analytical Methods

2.3.1. Enzymes Activities

After stipulated period of time, the culture flasks were harvested. The contents of fermented flasks were filtered through whattman No. 1 filter paper and filtrates were centrifuged at 1000 x *g* for 10 min. The supernatants were assayed for ligninase enzymes, i.e. lignin peroxidase (LiP) and Mn-peroxidase (MnP) by Tien and Kirk, (1988) and Wariishi et al, (1992) methods respectively, synthesized by *Phanerochaete chrysosporium*-IBL-03 in LSF[18, 20].

3. Results and Discussion

3.1. Screening of Lignocellulosic Substrates:

For the production of Ligninolytic enzymes by white rot fungus *P. chrysosporium*-IBL-03, six different lignin containing substrates (corn stover, corn cobs, sugarcane baggase, wheat straw, rice straw and banana stalk) were used for the production of lignin degrading enzyme through liquid state fermentation. *P. chrysosporium*-IBL-03 showed the maximum ligninases activity of MnP (732.1±12.1 U/mL) and LiP (790.0±18.9 U/mL) on wheat straw on 6^{th} day of incubation while Laccase was not detected in any of the fermented substrate culture (Table 3.1) [21].

3.2. Effects of Carbon and Nitrogen Sources

To investigate the effect of carbon and nitrogen sources, the experiment was planned in different combinations to get the maximum enzyme yield. Carbon sources such as molasses, glucose, fructose, maltose, glycerol and different inexpensive nitrogen sources like ammonium sulphate, peptone, urea, beef extract and yeast extract were used in different interaction in LSF of wheat straw to get maximum production under pre-optimized conditions. Results of enzyme assay showed that the combination of molasses and beef extract furnished the maximum MnP (782.2±22.1U/mL) and LiP (951.8±23.1U/mL) production (Table 3.2). After selection of best carbon and nitrogen combination i.e. molasses and beef extract, for varying C:N ratio for maximum ligninases production by *P. chrysosporium*in LSF under pre-optimized conditions was studied. There was an increase in enzyme production by increasing C:N ratios from 10:1 to 30:1 and maximum activity was observed in medium containing 25:1 ratio. The biomass dry weight was also found to be significant for the production of MnP and LiP up to the 25:1 (C: N) ratio. Then a further increase in C: N ratio caused decrease in biomass weight suggesting inhibition of fungal growth as well as MnP and LiP production (Figure 3.2). Statistical analysis of data revealed a significant ($P \leq 0.05$) effect of varying incubation for ligninases production. Results of DMR test showed that the differences between means of enzyme activities under different treatments were significant ($P \leq 0.05$).

3.3. Effect Of Mediators and Metal Ions

Maximum activity of LiP was observed in medium containing veratryl alcohol and medium containing $MnSO_4$ act as inducer for MnP production (Figure 3.3.1). Along with mediator, the metal ions ($FeSO_4$**,** $CuSO_4$**,** $ZnSO_4$**,** $CaCl_2$**,** KCl) effect on enzymes production was also investigated and it was observed that medium containing Cu^{2+} ions showed the maximum enzyme activity and also biomass dry weight recorded maximum (Figure 3.3.2). Statistical analysis of data by ANOVA revealed a significant ($P \leq 0.05$) effect of mediators and metal ions for the production of enzymes.

3.4. Discussion

It is well documented that MnP, LiP and Laccase production by white rot fungi is dependent to growth conditions [21]. Limitation of carbon or nitrogen concentration in the medium is important for the onset of the production of lignin degradating peroxidases (LDPs) by *P. chrysosporium*. Either the carbohydrate- or nitrogen-limiting condition in culture resulted in an early LDPs production comparing to the nitrogen-starved cultures which illustrates that maintaining **t**he lower level of C or N source will help to trigger the early production of LDPs into the medium [22] and a number author reported that the production of ligninolytic enzymes by *P. chrysosporium* under different

culture conditions could be enhanced to varying folds. Different amounts of medium have been employed in free and immobilized culture with different C/N ratios and C/N ratio: 56/2.2 mmol/L furnished the highest MnP and LiP production [23, 24]. Similarly, the effect of different carbon-to-nitrogen (C/N) ratios and levels on lignolytic enzyme production from WRF was characterized. At the low C/N ratio, the fungus was carbon starved and did not produce extracellular polysaccharides. At a high C/N ratio, i.e. under conditions of excess carbon (nitrogen limitation) (C/N = 28/1.1 *m*M), cultures exposed to air produced large amounts of polysaccharide. Results showed that increased nitrogen concentration decreased enzyme activity. Under high-nitrogen conditions, LiP production was 1,800 U/L in cultures exposed to pure oxygen and 1,300 U/L in cultures exposed to air. The formation of manganese peroxidase was considerably reduced by a low C/N ratio [25].

Mediators act as inducers of enzyme and also enhance the growth of the fungus. Therefore, different mediators ($MnSO_4$, veratryl alcohol, ABTS (Azinobis ethylthiazolinone 6-sulfate), H_2O_2, and ammonium Oxalate) on ligninases production by *P. chrysosporium* under pre-optimized fermentation conditions were studied and it was found that veratryl alcohol (3,4-dimethoxybenzyl alcohol; VA) leads to increased LiP production. It is also reported that addition of VA along with manganese (Mn^{2+}) to the white-rot fungi culture broth could induce the production of MnP to higher levels than the non-induced cultures [26]. Gassara *et al*, (2010) also found that VA acts as inducer for the production of LiP and MnP in *P. chrysosporium* [27].

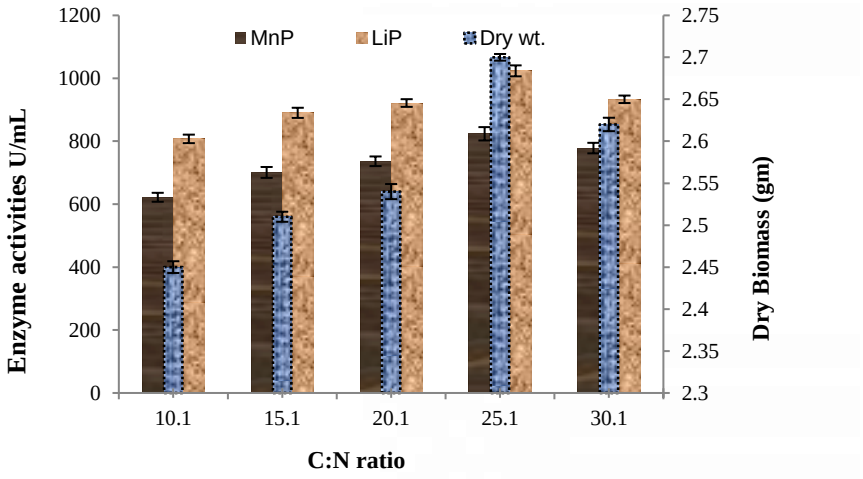

Figure 3.2: Effect of C: N ratio on ligninases production by *Phanerochaete chrysosporium*-IBL-03in LSF of wheat straw

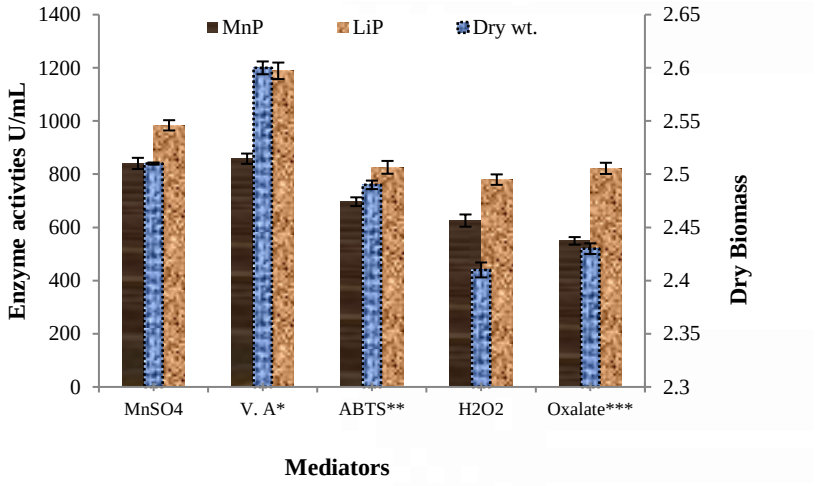

Figure 3.3.1: Effect of Mediators on ligninases production by *Phanerochaete chrysosporium*-IBL-03in LSF of wheat straw (*V.A, Veratryl alcohol; **ABTS, Azinobis ethylthiazolinone 6-sulfate; ***Oxalate, Ammonium Oxalate)

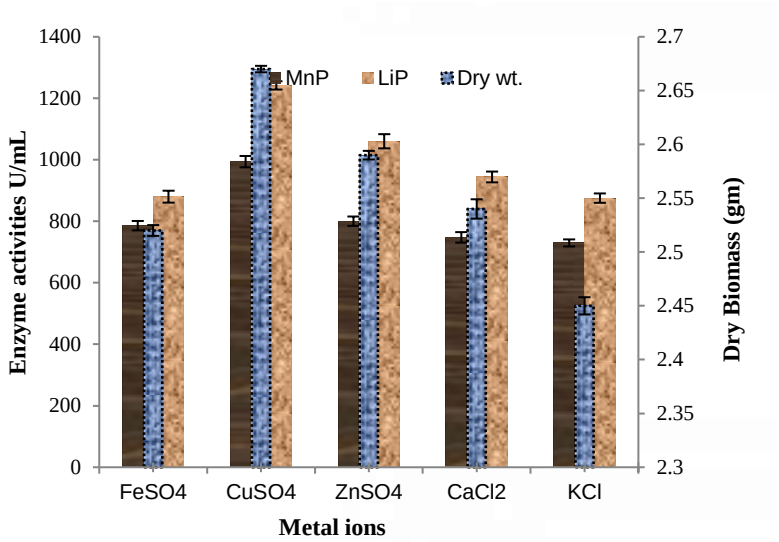

Figure 3.3.2: Effect of Metal ions on ligninases production by *Phanerochaete chrysosporium*-IBL-03in LSF of wheat straw

Table 3.1: Activities of ligninaseenzymes produced by *Phanerochaete chrysosporium*-IBL-03 on different lignocellulosic substrates for the period of 10 days (At day sixth (06) shows maximum activity)

Substrates	Enzymes	Enzyme activities (IU/mL)					
		Incubation time (Days)					
		1	2	3	4	5	6
Wheat straw	MnP	266.2±6.3	308.5±7.1	330.2±6.2	402.8±9.6	532.4±9.8	732.1±12.1
	LiP	574.2±19.2	555.9±17.2	629.0±13.2	529.0±16.1	624.7±16.7	790.1±18.9
Rice straw	MnP	291.3±7.4	386.4±6.9	407.9±9.2	437.4±11.6	542.7±13.1	575.6±15.3
	LiP	561.3±21.2	515.1±18.1	618.2±16.9	576.3±13.8	605.4±19.6	750.5±23.5
Corn stover	MnP	292.2±10.2	312.8±9.6	352.6±9.7	386.3±7.5	481.4±5.5	546.2±9.9
	LiP	587.1±12.3	520.4±21.1	438.7±13.5	591.4±27.1	587.1±17.4	720.0±19.1
Corn cobs	MnP	229.9±5.3	240.2±6.2	306.8±5.9	300.7±8.3	312.1±21.1	314±15.6
	LiP	362.4±7.1	409.6±9.6	330.1±6.7	366.6±9.1	373.1±8.7	570.9±9.6
Bagasse	MnP	71.07±3.2	123.5±4.5	197.0±5.9	260.2±6.8	265.3±3.8	270.5±9.6
	LiP	241.9±7.9	196.8±5.6	184.9±5.1	304.3±11.1	237.6±9.3	320.4±12.0
Banana stalk	MnP	203.1±10.4	215.2±12.1	283.9±8.7	372±5.8	450.5±13.5	322.8±13.0
	LiP	404.3±12.3	427.9±17.1	461.3±10.6	483.8±19.8	470.9±6.1	309.8±11.3

Table 3.2: Activities of ligninases produced by *P. chrysosporium*-IBL-03with varying carbon and nitrogen sources

Carbon sources (1%)	ENZYMES	Enzyme activities (U/mL)				
		Nitrogen Sources (0.2%)				
		Urea (N_1)	Peptone (N_2)	Beef extract (N_3)	Yeast extract (N_4)	$(NH_4)_2SO_4$ (N_5)
Glucose (C_1)	MnP	565.7±12.9	481.4±15.3	572.6±11.9	638.2±21.3	587.7±19.1
	LiP	706.5±15.7	755.4±23.2	850.5±19.8	753.7±13.6	789.9±17.4
Fructose (C_2)	MnP	350.9±11.0	427.8±9.1	439.1±13.5	471.9±12.3	552.2±17.4
	LiP	406.5±10.9	427.9±14.2	462.4±15.6	533.3±19.8	531.1±21.2
Maltose (C_3)	MnP	544.9±14.3	529.8±12.3	539.3±21.7	555.8±17.2	528.1±13.4
	LiP	601.1±23.1	584.9±19.3	683.3±15.7	640.8±17.3	764.5±25.1
Molasses (C_4)	MnP	543.2±12.5	627.1±14.7	**782.2±22.1**	718.2±18.9	682.8±21.0
	LiP	794.6±18.8	870.9±19.8	**951.8±23.1**	851.1±20.1	798.92±25.1
Glycerol (C_5)	MnP	584.2±12.3	624.5±11.9	533.3±17.1	657.7±13.2	523.3±9.4
	LiP	517.2±18.9	637.1±17.9	588.7±19.7	786.0±21.1	609.1±13.5

Similar to mediators, the metal ion also enhanced the production of enzymes to varying folds and has been correlated with ligninolytic ability of WRF which increase the solubility and mineralization of lignin [27, 28]. The influence of Zn^{2+} and Cu^{2+} in the basal medium on mycelia growth (dry weight), activities of lignin peroxidase, manganese peroxidase, solubilization, and mineralization of lignin during a period of 3 weeks in *P. chrysosporium* strain MTCC-787 was studied. Highest mycelia growth was obtained at 0.6 µM Zn^{2+} and 0.4 µM Cu^{2+} levels. Enzyme activities were found to increase up to the highest levels of both the trace elements [28].

4. Conclusion

The results revealed that the production of lignin degrading enzymes from agro-based wastes by *Phanerochaete chrysosporium*-IBL-03 through liquid state fermentation significantly depends on the culture conditions. Promising enzyme activity found with wheat straw also suggest the application of this substrate to large-scale processes in order to produce high amounts of ligninolytic enzyme having numerous applications which would be a cheap source of enzymes production and utilization of agro-based waste biomass as well.

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References

[1] Y. Eynollahi., M. B. Khalkhali and F. Tavankar. (2013). Study of physiochemical properties of heartwood and sapwood of Oak and Maple trees in the Caspian forest, Iran**.** Int. J. App. Sci. and Eng. Res. 2(5): 487-493.

[2] G. Y. S. Mtui. (2009). Recent advances in pretreatment of lignocellulosic wastes and production of value added products. Afr. J. Biotechnol. 8 (8): 1398-1415.

[3] B. C. Qi., C. Aldrich, L. Lorenzen and G. W. Wolfaardt. (2005). Acidogenic fermentation of lignocellulosic substrate with activated sludge. Chem. Eng. Communications. 192(9): 1221-1242.

[4] A. Roig., M. L. Cayuela and M. A. Sanchez-Monedero. (2006). An overview on olive mill wastes and their valorisation methods. Waste Manag. 26(9): 960-969.

[5] G. Rodriguez., A. Lama, R. Rodriguez, A. Jimenez, R. Guillena and J. Fernandez-Bolanos. (2008). Olive stones an attractive source of bioactive and valuable compounds. Bioreasour. Technol. 99(13): 5261-5269.

[6] J. H. Grabber. (2005). How do lignin composition, structure and cross linking affect degradability? A review of cell wall model studies, Crop sci. 45: 820-831.

[7] A. Hatakka., L. Viikari and A. Kantelinen. (2001). Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. FEMS Microbiol. Reviews. 13: 125-135.

[8] P. J. Hoegger., A. Majcherczyk, R.C. Dwivedi, K.I. Svobodov, S. Kilaru and U. Kues. (2007)

Enzymes in wood degradation, wood production, wood technology and Biotechnological impacts. Molecular Wood Biotechnology, Institute of Forest Botany, Georg-August-University, Göttingen. Pp 383-413.

[9] D. O. Krause., S. E. Denman and R. I. Mackie. (2003). Opportunities to improve fibre degradation in the rumen: microbiology, ecology and genomics. FEMS Microbiol. 797: 1-31.

[10] M. H. Gold and M. Alic. (1993). Molecular biology of the lignindegrading basidiomycete *Phanerochaete chrysosporium*. Microbiol. Rev. 57(3): 605–622.

[11] P. Kersten and D. Cullen. (2007). Extracellular oxidative systems of the lignin-degrading Basidiomycete *Phanerochaete chrysosporium*. Fungal Genet. Biol. 44: 77–87.

[12] C. A. Reddy and T. M. D. Souza. 1994. Physiology and molecular biology of the lignin peroxidases of *Phanerochaete chrysosporium*. FEMS Microbiol. Rev. 13: 137–152.

[13] M. D. Cameron., S. Timofeevski and A. D. Aust. (2000). Enzymology of *Phanerochaete chrysosporium* with respect to the degradation of recalcitrant compounds and xenobiotics. Appl. Microbiol. Biotechnol. 54: 751–758.

[14] D. Wesenberg., I. Kyriakides and S. N. Agathos. (2003). White-rot fungi and their enzymes for the treatment of industrial dye effluents. Biotechnol. Adv. 22: 161–187.

[15] L. Zacchi., G. Burla, D. Zuolong and P. J. Harvey. (2000). Metabolism of cellulose by *Phanerochaete chrysosporium* in continuously agitated culture is associated with enhanced production of lignin peroxidase. J. Biotechnol. 78: 185–192.

[16] B. D. Faison and T. K. Kirk. (1985). Factors Involved in the Regulation of a Ligninase Activity in *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 49(2): 299-304.

[17] M. Asgher., M. J. Asad and R. L. Legge. (2006). Enhanced lignin peroxidase synthesis by *Phanerochaete Chrysosporium* in solid state bioprocessing of a lignocellulosic substrate. World J. Microbiol. Biotechnol. 22: 449–453.

[18] M. Tien and T. K. Kirk. (1988). Lignin peroxidases of *Phanerchaete chrysosporium*. Methods in Enzymol. 161: 238-249.

[19] J. L. Kay-shoemake and M. E. Watwood. (1996). Limitation of the lignin peroxidases system of the white rot fungus, *Phanerochaete chrysosporium*. Appl. Microbiol. Biotechnol. 46: 438-442.

[20] H. Wariishi., K. Valli and M. H. Gold. (1992). Manganese (II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*, kinetic mechanism and role of chelators. J. Biol. Chem. 267: 23688-23695.

[21] J. Rogalski., J. Szczodrak and G. Janusz. (2006). Manganese peroxidase production in submerged cultures by free and immobilized mycelia of *Nematoloma frowardii*. Biore. Technol. 97: 469-476.

[22] D. Singh and S. Chen. (2008). The white-rot fungus *Phanerochaete chrysosporium*: conditions for the production of lignin-degrading enzymes. Appl. Microbiol. Biotechnol. 81: 399–417.

[23] X. Xiong., X. Wen, Y. Bai and Y. Qian . (2008). Effects of culture conditions on ligninolytic enzymes and protease production by *Phanerochaete chrysosporium* in air. J. Environ. Sci. 20(1):94-100.

[24] P. Wang., H. Xiaoke, C. Sean, B. Maria, S. L. Ken and H. Huey-Min. (2008). Effects of culture conditions on the production of ligninolytic enzyme by white rot fungi *Phanerochaete chrysosporium* (ATCC 20696) and separation of its lignin peroxidase. World J Microbiol Biotechnol. 24:2205–2212.

[25] X. U. Xiaoping and W. Xin. (2008). Effects of culture conditions on ligninolytic enzymes and protease production by *Phanerochaete chrysosporium* in air. Chinese Academy of Sciences, Beijing, China. 19: 17-25.

[26] M. A. Khiyami., A. L. Pometto and W. J. Kennedy. (2006). Ligninolytic Enzyme Production by *Phanerochaete chrysosporium* in Plastic Composite Support Biofilm Stirred Tank Bioreactors. J. Agric. Food Chem. 54: 1693-1698.

[27] F. Gassara., S. K. Brar, R. D. Tyagi, M. Verma and R.Y. Surampalli. (2010). Screening of agro-industrial wastes to produce ligninolytic enzymes by *Phanerochaete chrysosporium.* Biochem. Eng. J. 49: 388–394.

[28] V. Singhal and V. S. Rathor. (2001). Effects of Zn^{2+} and Cu^{2+} on grpwth, lignin degradation and ligninolytic enzymes in *Phanerochaete chrysosporium.* World J. Microbiol. Biotechnol. 17: 235-240.