Study of Production of Lignin Degrading Enzymes by Fungal Cultures and their Ability to Decolorize Selected Dyes

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Abstract - The production of the lignin degrading enzymes namely Laccase, Manganese Peroxidase (MnP) and Lignin Peroxidase (LiP) of selected white-rot fungus were examined in this study. Three fungal cultures were studied, two of which belong to Ganoderma sp. & one belongs to Schizophyllum sp. After screening the cultures for the presence of the enzymes Laccase by Plate Assay techniques, quantitative analysis of all the three enzymes was done by Spectrophotometric method. Production was studied using liquid media. The effect of pH & different carbon sources namely Sugarcane bagasse, Rice bran and Wheat bran was examined on the production of these enzymes in liquid media. Effect of 2, 5-xylidine on Laccase production was also assessed. As it is these enzymes that help in decolourization / degradation of dyes, the crude enzyme solution was also investigated for its ability to decolorize selected dyes. All the cultures showed diverse enzyme production of all the three enzymes in different media & pH. All the selected dyes were decolorized by the crude enzyme extract in varying degrees. This study demonstrated that media manipulation can change the enzyme production in the fungal cultures. By optimizing the media condition we can maximize the desired enzyme production. The study also shows that rather than using fungi, is possible to directly use crude enzymes for decolourization of dyes & probably treat industrial effluents.

Index - Terms: Laccase, Manganese Peroxidase (MnP), Lignin Peroxidase (LiP), Reactive dyes, Effluent, Mycelia, Ganoderma sp., Schizophyllum sp.

I. INTRODUCTION

Lignin breakdown is thought to occur by concomitant action of ligninolytic enzymes. The white rot fungi produce an array of extracellular oxidative enzymes that synergistically and efficiently degrade lignin. The major groups of ligninolytic enzymes include lignin peroxidases, Manganese Peroxidase, Versatile Peroxidase and Laccase (Dominic and Wong, 2009).The main extracellular enzymes participating in lignin degradation are heme-containing Lignin Peroxidase (Ligninase, LiP, EC 1.11.1.14), Manganese Peroxidase (MnP, EC 1.11.1.13) and Cu-containing Laccase (Benzenediol : Oxygenoxidoreductase, EC 1.10.3.2) (Abdul & Elisabeth, 2012). These lignin-degrading enzymes known so far are extracellular and nonspecific, participating in different oxidative reactions where the aromatic structure of lignin and bonds between the basic units are broken (Annele and Kenneth, 2010). The ability of the fungi to decolorize or degrade dyes is because of the presence of Lignin degrading enzymes (Couto 2009; Sanchez 2009; Patrick et al. 2010).). Because they are group specific enzymes they are thought to be responsible for decolourization or degradation of many synthetic dyes.

The production of the lignin degrading enzymes namely Laccase, Manganese Peroxidase (MnP) and Lignin Peroxidase (LiP) of selected Basidiomycetes white-rot fungus were examined on different media conditions. Production was studied using liquid medium. The effect of pH & different carbon sources namely lignocellulose-containing Rice Bran, Wheat Bran & Sugar Bagasse was examined on the production of these enzymes by the cultures. As it is these enzymes that help in decolorization/ degradation of dyes, (Adinarayana et al., 2007, Emrah, et al., 2007) the crude enzyme solution was also studied for its ability to decolorize selected dyes. Three fungal cultures were studied, two of which belong to Ganoderma sp. & one belongs to Schizophyllum sp.. Mycelia of Ganoderma sp. was designated as FU-1culture (ACCESSION JN049906). It was isolated by tissue culture method from local Ganoderma sp.. Mycelia of Schizophyllum sp. designated as FU-3 (ACCESSION JN049907) was procured from Dr. M. D. Shukla, Faculty, Microbiological Department M. G. Science Institute, Ahmedabad, India. The second Ganoderma sp.

culture designated as FU-2 (MTCC 1309) was procured from IMTECH, Chandigrah, India.

First the cultures were screened for the presence of the enzyme Laccase by Plate Assay techniques & once the presence was confirmed, all the three enzymes were quantitatively analyzed by Spectrophotometric method.

To study the effect of carbon source namely sugarcane bagasse, rice bran and wheat bran were used as different carbon sources. The pH of these media was kept at 5.0 as literature showed that production of Laccase in many fungal cultures is best at around 5.0 pH, (Adinarayana et al., 2007). The enzyme activity was assessed on different days of incubation. The enzyme samples were withdrawn in 2 days interval starting from 2nd day till 14th day of incubation. As production of Laccase in many fungal cultures was best at pH around 5.0, (Adinarayana et al., 2007) the pH of the media was kept at 4.0, 5.0, & 6.0pH.

Production of Lignin Degrading Enzymes in Different Liquid Media:

a) *Different Carbon Source:*

To study the effect of carbon source, sugarcane bagasse, rice bran and wheat bran were used as different carbon sources. Sterilized media for cultivation of fungi (FU-1, FU-2 & FU-3) contained 5 g / 100 ml dried carbon source (Sugarcane Bagasse / Rice Bran / Wheat Bran) and basal medium having 1.0 g yeast extract, 1.0 g KH2PO4, 0.5 g NH4NO3, 0.01 g CaCl2, 1.0 mg CuSO4. 5H2O, 1.0 mg FeSO4, 1.0 mg MnSO4 per litre. The pH of the media was kept at 5.0. Five plugs of fungal cultures from 7 days old cultures were transferred into flasks containing 100 ml of media under aseptic condition. Incubation was carried out till 14 days at 30°C on a rotary shaker at 125 rpm.

b) *Different pH & in presence of 2,5-Xylidine:*

The effect of pH on the production of enzyme too was studied. The media was varied in their pH along with their carbon sources namely wheat bran & rice bran. Different pH of 4, 5 and 6 was studied. Literature showed that Laccase production could be induced by 2,5-Xylidine (Bakkiyaraj et al., 2013) so to assess its effect on Laccase production in this culture 25 µl / 100 ml of 2 mM 2,5-Xylidine was added into the liquid medium. Control flasks without 2,5-Xylidine were also taken for media with 5.0 pH for comparison.

II. EXTRACTION OF ENZYME FROM LIQUID MEDIA

As Laccase, MnP and LiP are extra cellular enzymes secreted by the fungi in the growth media, the broth in which the fungi was grown was withdrawn aseptically from flask & was used as crude enzyme extract. For the study of different carbon source, the broth was withdrawn from flasks after 2, 4, 6, 8, 10, 12 and 14 days of incubation. The broth was filtered with the help of Whatman filter paper number.1. The filtrate thus obtained was used as crude enzyme solution for Laccase, MnP and LiP enzyme assays. For the study of different pH and 2,5-Xylidine media, the broth was withdrawn from flasks after 8 days of incubation, filtered & used as crude enzymes. Crude extracts were kept at 4oC before use.

Lignin Degrading Enzyme Assays:

The cultures were first screened for the presence of Laccase enzyme by Plate Assay techniques (C. Srinivasan, 1995; P. Cassland and L. J. Jönsson, 1999).

Plate Assay for Laccase by ABTS Method:

The Laccase plate assay allows rapid determination of the presence of Laccase in the extracellular fluid. For this 100 ml of PDA medium containing 0.5 ml of 2 mM ABTS (2,2'azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) in sodium acetate buffer (pH 4.5, 0.1 M) & 4 ml of CuSO4 100 mM (25 mg/ml) was prepared & sterilized. Twenty milliliter of this media is poured in a 90 mm petriplate. Once the media had solidified, wells were bored with the help of a borer & 50 µl of crude enzyme extracts were injected into these wells & the plates were incubated at 300C for 24-48 hours. The development of bluish-green halos around the wells was considered as a positive test for laccase activity (C. Srinivasan, 1995).

Plate Assay for Laccase by Guaiacol Method:

Guaiacol (0.01%) was added to 100 ml PDA medium for this assay. Twenty millilitre of this sterilised media was poured in a 90 mm petriplate. Once the media had solidified, wells were bored with the help of a borer & 25-50 µl of crude enzyme extracts were injected into these wells & the plates were incubated at 30°C for 24-48 hours. The development of orange/brown halos around the wells was considered as a positive test for Laccase activity. (P. Cassland, and L. J. Jönsson, 1999)

Once the presence of Laccase was confirmed quantitative analysis for all the three enzymes Lacase, MnP and LiP was carried out spectrophotometrically.

Enzyme Assays for Media with Different Carbon Source:

This study was carried out with FU-1 & FU-2 fungal cultures both of Ganoderma Sp.

Laccase Enzyme Assay:

Laccase activity was determined by the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS method (R. Bourbonnais, 1998). The method was based on the oxidation of ABTS at 420 nm with an absorbance coefficient value 36,000 M-1 cm-1. The non-phenolic dye ABTS is oxidized by Laccase to the more stable and preferred state of the cation radical. The concentration of the cation radical responsible for the intense blue-green color can be correlated to enzyme activity and is read at 420 nm (A. Majcherczyk et al., 1998). Oxidation of ABTS was monitored by determining the increase in A420 (ε420, $3.6 \times 104\ M{-}1cm{-}1$). The reaction mixture contained 0.5 mM substrate (ABTS) (100 µl of 5 mM/1ml ABTS), 2.8 mL of 0.1 M sodium acetate buffer of pH 4.5 and 100 µl of crude enzyme solution and incubated for 10 minutes. Absorbance was read at 420 nm in a spectrophotometer against a suitable blank (Sunil et al., 2011). The enzyme Unit was calculated using molar extinction coefficient value of 36,000 M-1 cm-1 for ABTS (at 420 nm.). One unit was defined as the amount of the Laccase that oxidizes 1 micromole of ABTS substrate under assay conditions (Childs and Bardsley, 1975).

Manganese Peroxides (MnP) Assay:

MnP activity was monitored with Phenol red as substrate at 30±2°C (Glenn and Gold, 1983).The reaction mixture contained 200 µl of 25 mM lactic acid, 200 µl of 0.1 mM $MnSO_4$, 200 µl of 1mg/ml BSA, 200 µl of 1mg/ml phenol red and 1000 µl of enzyme culture filtrate in 200 µl of 20 mM sodium acetate buffer (pH 4.5) in a total volume of 2 ml. The reaction was started with the addition of 100 µl of 0.1 M H_2O_2 and stopped after one minute with 100 µl of 10% NaOH. The absorption at 610 nm was measured against a blank without any manganese in the reaction mixture. The enzyme Unit was calculated using molar extinction coefficient value of 22,000 M^{-1} cm^{-1} for oxidized phenol red (at 610 nm). One unit of Manganese Peroxidase was defined as the amount of enzyme which oxidized 1 micromole of phenol red under the conditions specified in the assay system.

Lignin Peroxidase (LiP) Assay:

Lignin Peroxidase assay was done by oxidation of Veratryl alcohol (3, 4-dimethoxy benzyl alcohol) to Veratryl aldehyde (Tien and Kirk 1984).
The reaction mixture contained 750 µl Sodium tartarate buffer (0.33 M, pH 3.0), 250 µl Veratryl alcohol (4 mM), 100 µl H_2O_2 (10 mM), 900 µl distilled water and 500 µl culture medium. Absorbance was measured at 310 nm.
The enzyme unit was calculated using molar extinction coefficient value of 9300 M^{-1} cm^{-1} for Veratryl aldehyde (at 310 nm.) The unit of Lignin Peroxidase was defined as the amount of enzyme which converted 1 micromole of veratryl alcohol to veratryl aldehyde under the conditions specified in the assay system.

Enzyme Assays for Media with Different pH:

This study was carried out with FU-1, FU-2 and FU-3 fungal cultures.

While studying the effect of pH, for the assays of all the enzymes, except for the volume all the other conditions were as mentioned in the above assay systems. But the volume of enzymes was halved as the activity was high due to the addition of 25 µl/ 100ml of 2 mM 2,5-xylidine an inducer of Laccase activity. So the volume was 50, 250 & 500 µl for Laccase, LiP & MnP respectively. This was also done for control tubes.

Use of Enzymes for Decolorization of Selected Dyes:

Crude enzyme extract was used to check the decolorization of different dyes. The ability of the fungi to decolorize or degrade dyes is because of the presence of Lignin degrading enzymes (Couto 2009; Sanchez 2009; Patrick et al. 2010).). So the crude enzyme extract which was rich in these enzymes was studied for its ability to degrade selected dyes. The dyes & their λ max are listed in table number 1.

The extract that showed maximum Laccase activity was selected for this purpose. 1ml of enzyme extract was added to 9ml of different dye solution of different concentrations (10 ppm, 25 ppm, 100 ppm). These were incubated at 30°C temperature. The colour of the dye solution before (0 day) & after the enzyme treatment (after filtration & centrifugation to remove the turbidity) was measured

spectrophotometrically at their respective λ max as mentioned in the table 1 after a time interval of 2 days. The experiments were carried out in triplicates and the mean values were taken. Decolorization was observed & measured in terms of percentage of decolorization with control taken as 100 percentage, using the following formula:

% Degradation = 100 - (Ae/Ac × 100); Where, Ae is OD of the fungal treated sample & Ac is OD of the control at 0 day.

Sr. No	Name of dye	λmax (nm)
1	Blue 221	610
2	Black B	588
3	Red ME4BL	518
4	0.2 R	468
5	T Blue	614
6	Red HE7B	520
7	Golden Yellow Merl	418
8	Blue 222	598

Table 1: Dyes and their λ max

III. RESULTS & DISCUSSIONS

For Plate Assay:

The enzyme extract withdrawn on the 6th day of incubation of all the three cultures grown on different media showed huge halos by both the plate methods, indicating that the fungi were positive for the presence of the Laccase enzyme. Studies have shown the presence of Laccase enzyme in basidiomycetes fungi (Sasidhara and Thirunalasundari, 2014,). A number of screening studies of white rot basidiomycetes (WRB) and other classes of fungi were conducted to discover promising producers of ligninolytic enzymes (Elisashvili *et al.,* 2001; Elisashvili *et al.,* 2009; Myasoedova *et al.,* 2008).

Figure 1**:** Plate assay for Laccase by Guaiacol method. Brown halos around the wells containing culture shows presence of Laccase, C=Control, 1=FU-3 (Rice Bran), 2= FU-2 (Rice Bran), 3=FU-1(Rice Bran), 4=FU-2 (Sugarcane Bagasse)

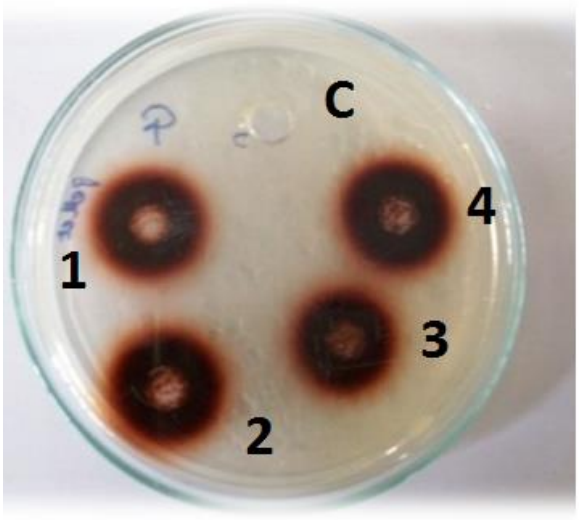

Fig 1. Plate assay for Laccase by Guaiacol method.

Fig 2: Plate assay for ABTS method.

Figure 2: Plate assay for ABTS method. Green halos around the wells containing culture shows presence of laccase, C=Control, 1=FU-3 (Rice Bran), 2=FU-2(Rice Bran), 3=FU-1 Rice Bran), 4=FU-2 (Sugarcane Bagasse), 5= FU-1(Sugarcane Bagasse)

Once the cultures were confirmed for the presence of Laccase activity, the qualitative measurements of Laccase, MnP & Lip enzyme activity was done spectrophotometrically.

Enzyme Activity for Crude Extract obtained from Liquid Media with Different Carbon Source on Different Days:

The effect of the different carbon source on the production of the enzymes in the cultures can be seen from Tables 2, 3 & 4.

Laccase Enzyme Assay:

As seen in table 2, crude enzyme extract of FU-1 grown at 5.0 pH from rice bran containing liquid media showed highest Laccase activity and peak value for Laccase production on the 8th and 10th day of incubation while in wheat bran containing media peak value for Laccase production was observed on 12th day of incubation. In sugarcane bagasse containing media peak value for Laccase production was observed on the 10th day of incubation which was almost half as compared to the other two media.

Crude enzyme extract of FU-2 grown in wheat bran and sugarcane bagasse liquid media at 5.0 pH showed the highest Laccase activity on the 8th day of incubation while in rice bran containing media peak value for Laccase production was observed at 12th day of incubation.

The Laccase activity in FU-1 varied from as low as 4.33 units to as high as 66.97 units while in FU-2 from 6.81 to 88.42 units.

Table 2: Laccase Activity for crude extract obtained from liquid media on different days

FU-1 (Activity of Laccase)			
Days	**Wheat Bran Enzyme Units**	**Rice Bran Enzyme Units**	**Sugarcane Bagasse Enzyme Units**
2	5.67	4.33	3.03
4	20.58	27.81	16.36
6	37.19	66.81	28.33
8	49.42	**66.97**	28.44
10	49.42	**66.97**	**30.08**
12	**62.19**	37.53	27.83
14	61.31	36.14	27.00

FU-2 (Activity of Laccase)			
Days	**Wheat Bran Enzyme Units**	**Rice Bran Enzyme Units**	**Sugarcane Bagasse Enzyme Units**
2	12.00	6.81	10.06
4	28.11	17.33	25.03
6	78.33	28.31	55.19
8	**88.42**	28.75	**55.94**
10	67.39	40.19	51.86
12	59.03	**62.25**	49.78
14	55.61	61.92	48.94

Manganese peroxidase (MnP) assay:

As seen in table 3, crude enzyme extract of FU-1 at 5.0 pH from rice bran and sugarcane bagasse containing liquid media showed highest MnP activity and peak value for MnP production on the 10th day of incubation while in wheat bran containing media peak value for MnP production was observed on 8th day of incubation.

Crude enzyme extract of FU-2 at 5.0 pH from Wheat bran containing liquid media showed highest MnP activity and peak value for MnP production on the 10th day of incubation while in rice bran and sugarcane bagasse containing media peak value for MnP production was observed on 8th day of incubation. The MnP activity in FU-1 ranged from 9.18 units to 62.00 and in FU-2 from 8.95 to 53.73 units. .

Table 3: MnP Activity for crude extract obtained from liquid media on different days

FU-1(Activity of MnP)			
Days	**Wheat Bran Enzyme Units**	**Rice Bran Enzyme Units**	**Sugarcane Bagasse Enzyme Units**
2	12.23	9.18	13.45
4	14.77	13.05	18.95
6	18.91	15.45	27.09
8	**31.32**	15.82	30.32
10	25.55	**62.00**	**38.73**
12	20.09	22.59	38.00
14	18.23	19.45	35.77

FU-2 (Activity of MnP)			
Days	**Wheat Bran Enzyme Units**	**Rice Bran Enzyme Units**	**Sugarcane Bagasse Enzyme Units**
2	12.36	11.55	11.55
4	14.27	13.27	13.27
6	15.55	14.50	14.50
8	17.32	**18.09**	**18.09**
10	**53.73**	10.91	10.91
12	16.50	10.91	10.91
14	15.95	8.95	8.95

Lignin peroxidase (LiP) assay:

As seen in table 4, crude enzyme extract of FU-1 at 5.0 pH from rice bran containing liquid media showed highest LiP activity and peak value for LiP production on the 10th day of incubation while in wheat bran containing media peak value for LiP production was observed on 12th day of incubation. In sugarcane bagasse containing media peak value for LiP production was observed on the 8th day of incubation.

Crude enzyme extract of FU-2 at 5.0 pH from sugarcane bagasse containing liquid media showed highest LiP activity and peak value for LiP production on the 8th day of incubation while in rice bran and wheat bran containing media peak value for LiP production was observed on the 10th day of incubation. The LiP activity in FU-1 ranged from 22.9 units to 96.56 and in FU-2 from 20.22 to 94.30 units. .

Table 4: LiP Assay for crude extract obtained from liquid media at different days

FU-1 (Activity of LiP)			
Days	**Wheat Bran Enzyme**	**Rice Bran Enzyme Units**	**Sugarcane Bagasse Enzyme Units**
2	22.90	33.76	27.10
4	44.30	42.26	34.62
6	73.01	65.05	53.44
8	78.28	72.58	**64.52**
10	83.01	**96.56**	43.98
12	**91.08**	54.52	39.57
14	88.92	53.55	38.39

FU-2 (Activity of LiP)			
Days	**Wheat Bran Enzyme Units**	**Rice Bran Enzyme Units**	**Sugarcane Bagasse Enzyme Units**
2	46.99	20.22	33.44
4	55.27	27.74	43.33
6	64.30	43.66	56.34
8	65.48	44.41	**94.30**
10	**69.89**	**57.10**	71.72
12	60.54	26.24	65.16
14	54.52	21.94	64.52

Various studies have shown that the production of enzymes vary with media conditions (Elshafei *et al*., 2012; R. Sivakumar *et al*., 2010). This study too shows the change in the production of enzymes with time of incubation & media composition for all the three cultures studied and for all three enzymes produced. There is an initial increase in the enzyme concentration & then decreasing later after certain period. This type of peaking of enzyme production is seen with most organisms, where there is a gradual increase & then after there is a decrease in the production of enzymes. This may be due to depletion of nutrients in the media with time, thus decrease or inactivation of protein synthesising machinery in the cell, (Elshafei *et al*., 2012).

Laccase and Manganese peroxidase yield by white-rot *Basidiomycetes* is found to be species-dependent and strain-dependent, (Cilerdzic *et al*., 2016; Elisashvili *et al*., 2009). The carbon source and lignocellulosic substrate also play a crucial role in enzyme production. (Elisashvili & Kachlishvili, 2009). Thus the difference seen in this study could be because of different strain and different carbon source and lignocellulosic substrates used in the present study in the media.

Enzyme activity at different initial pH:

The activity of the enzymes in different carbon source media at different initial pH on the 8th day of incubation is shown in Table 5a, 5b & 5c.

Laccase Production:

FU-2 culture grown in liquid media at pH 4.0 with wheat bran had highest Laccase production while FU-

1& FU-3 from wheat liquid media at pH 6.0 and 5.0 respectively had highest Laccase production.

Table 5a: The **Laccase** activity at different pH:

		Laccase		
Carbon source	pH	FU-1	FU-2	FU-3
Wheat bran	4	52.11	**64.03**	11.44
Rice bran	4	62.61	45.92	01.19
Wheat bran	5	53.94	63.42	**17.31**
Rice bran	5	36.69	31.44	11.83
Wheat bran	6	**62.78**	63.28	09.89
Rice bran	6	59.94	60.56	10.31

LiP Production:
FU-2 and FU-3 cultures grown in wheat bran liquid media at 5.0 pH showed highest Lip production, while FU-1 culture grown in rice bran liquid media at 5.0 pH showed highest Lip production.

Table 5b: The **LiP** activity at different pH:

		LiP		
Carbon source	pH	FU-1	FU -2	FU -3
Wheat bran	4	53.55	117.85	69. 78
Rice bran	4	41.18	43.44	55.91
Wheat bran	5	101.29	**145.16**	**112.47**
Rice bran	5	**116.45**	81.72	111.61
Wheat bran	6	86.88	80.32	77.74
Rice bran	6	101.18	97.53	108.39

MnP Production:
All the three cultures FU-2, FU-1&FU-3 cultures grown in wheat bran liquid media at 5.0 pH showed highest MnP production.

Table 5c: The **MnP** activity at different pH:

		MnP		
Carbon source	pH	FU-1	FU-2	FU-3
Wheat bran	4	30.91	47.95	81.32
Rice bran	4	43.45	34.14	45.00
Wheat bran	5	**87.45**	**74.18**	**86.64**
Rice bran	5	57.32	45.45	56.45
Wheat bran	6	22.91	27.41	54.27
Rice bran	6	55.23	33.86	63.23

The pH optima for enzymes in this study matches with some of the studies while it contradicts other studies done previously by other investigators.
Laccase activity was found maximum for *Schizophyllum sp*. at pH 6.5 by Ruchika Mahajan *et al.*, 2015, while this study shows that FU-3 *Schizophyllum sp* culture shows maximum activity at 5 pH in wheat bran and then the activity decreases at 6.0 pH.

As for *Ganoderma* sp., Zill-e-Huma Aftab *et al*., 2015, while studying the effect of different pH on laccase production by *Ganoderma Lucidum* found pH 5.5 as the best for Laccase synthesis. The activity of the enzyme decreased above and below of the optimum pH of 5.5. One of the three isoenzymes of Laccase produced by *G. lucidum* 77002 when using wheat bran and peanut powder as energy sources in liquid culture medium showed an optimum pH of 4.5 to 5.0 (Zemin Fang *et al.*, 2015) which matches with this study of *Ganoderma* cultures. The results in this study also matches with study conducted by Hariharan & Nambisan, 2013, where they found that the optimum pH was 5 for all the three enzymes ie. Laccase, LiP, MnP.
The variation in the pH optima seen in other studies when compared to this study could be due to the difference in the fungal strains & / or due to the differences in the growth media conditions used in these studies which could have lead to the production of enzymes having different kinetic properties.

Effect of 2,5 Xylidine on Enzyme production:

The effect of 2,5 Xylidine on enzyme production is shown in Tables 6, 7, 8 & Figures 3, 4 ,5.

Table 6 & Figure 3 indicates that in FU-1culture grown in different media at different pH and having 2,5 Xylidine, maximum amount of Laccase was produced in wheat bran medium at pH 6.0 followed by rice bran medium at 4.0 pH. Minimum amount of LiP was produced in rice bran medium at 5.0 pH. Maximum amount of LiP was produced in rice bran at pH 5.0 followed by wheat bran at 5.0 pH and then by Rice bran at 6.0 pH. Minimum amount of LiP was produced in rice bran medium at 4.0 pH. Maximum amount of enzyme MnP was produced in wheat bran medium at 5.0 pH. Minimum amount of MnP was produced in wheat bran medium at 6.0 pH.

Table 6: Enzyme activity of FU-1 at different pH & carbon source with 2, 5 Xylidine

		FU -1		
Carbon	pH	Laccase	LiP	MnP
Wheat bran	4	52.11	53.55	30.91
Rice bran	4	62.61	41.18	43.45
Wheat bran	5	53.94	101.29	**87.45**
Rice bran	5	36.69	**116.45**	57.32
Wheat bran	6	**62.78**	86.88	22.91
Rice bran	6	59.94	101.18	55.23

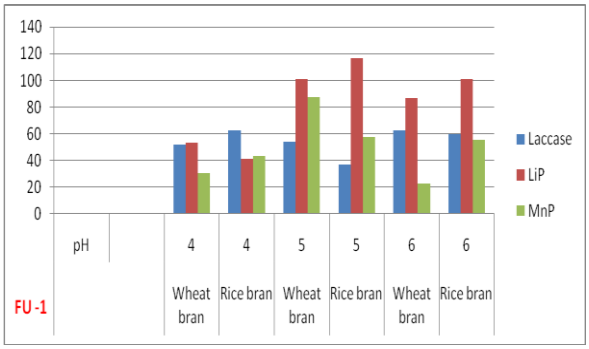

Figure 3**:** Enzyme activity of FU-1 at different pH & carbon source

Table 7 & Figure 4 indicates that in FU-2 culture grown in different media at different pH and having 2,5 Xylidine, maximum amount of Laccase was produced in wheat bran medium at pH 4.0 followed by wheat bran medium at 5.0 pH. Minimum amount of Laccase was produced in rice bran medium at 5.0 pH. There was a lot of variation in the production of LiP in different media conditions. Minimum amount of LiP was produced in rice bran medium at 4.0 pH. Maximum amount of LiP was produced in wheat bran at pH 5.0 followed by wheat bran at 4.0 pH and then by Rice bran at 6.0 pH. Minimum amount of LiP was produced in rice bran medium at 4.0 pH. Maximum amount of enzyme MnP was produced in wheat bran medium at pH 5.0 Minimum amount of MnP was produced in wheat bran medium at 6.0 pH.

Table 7: Enzyme activity of FU-2 at different pH & carbon source with 2, 5 Xylidine

		FU-2		
Carbon source	pH	Laccase	LiP	MnP
Wheat bran	4	**64.03**	117.85	47.95
Rice bran	4	45.92	43.44	34.14
Wheat bran	5	**63.42**	**145.16**	**74.18**
Rice bran	5	31.44	81.72	45.45
Wheat bran	6	63.28	80.32	27.41
Rice bran	6	60.56	97.53	33.86

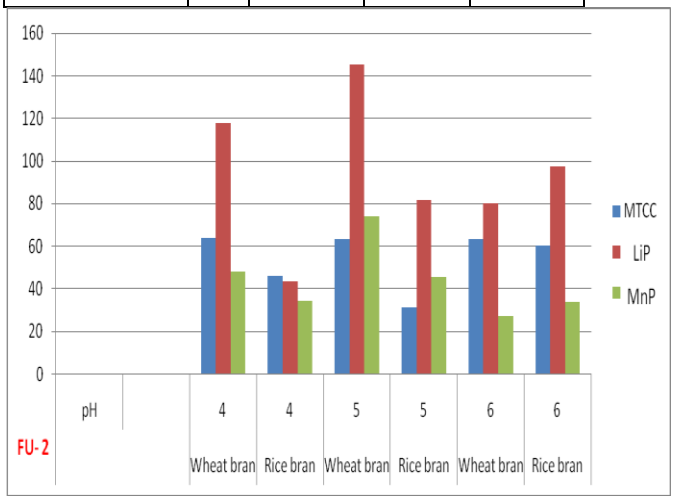

Figure 4: Enzyme activity of FU-2 at different pH & carbon source

Table 8 & Figure 5 indicate that in FU-3culture grown in different media at different pH and having 2, 5 Xylidine, produced very little Laccase in comparison of other two cultures. Maximum amount of Laccase was produced in wheat bran medium at pH 5.0. The culture excepting rice bran medium at 4.0 produced more or less the same amount of Laccase. Rice bran medium at pH 4.0 barely produced any Laccase. Minimum amount of LiP was produced in rice bran medium at 4.0 pH. Wheat & rice bran at pH 5.0 produced almost same LiP, closely followed by rice bran at 6.0 pH. Maximum amount of enzyme MnP was produced in wheat bran medium at pH 5.0. Minimum amount of MnP was produced in rice bran medium at 4.0 pH.

Table 8: Enzyme activity of FU-3 at different pH & carbon source with 2,5 Xylidine

		FU- 3		
Carbon source	pH	Laccase	LiP	MnP
Wheat bran	4	11.44	69.78	81.32
Rice bran	4	1.19	55.91	45.00
Wheat bran	5	**17.31**	**112.47**	**86.64**
Rice bran	5	11.83	111.61	56.45
Wheat bran	6	9.89	77.74	54.27
Rice bran	6	10.31	108.39	63.23

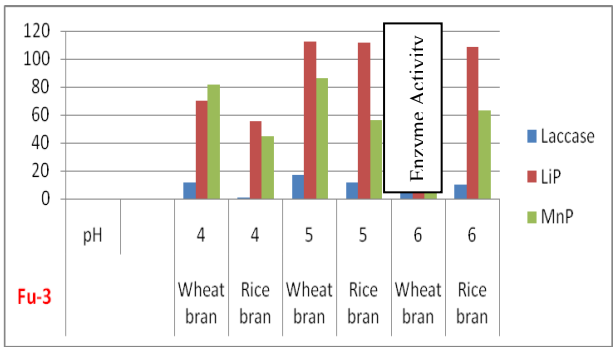

Figure 5: Enzyme activity of FU-3 at different pH & carbon source

Effect of 2,5 Xylidine on the production of the enzymes:

As can be seen from table 9 there is a clear effect of 2,5 Xylidine on the production of the all the three enzymes.

Table 9: Effect of 2,5 Xylidine on the production of the enzymes:

		Laccase			**LiP**			**MnP**		
Carbon source	pH	FU-1	FU-2	FU-3	FU-1	FU -2	FU -3	FU-1	FU-2	FU-3
Wheat bran#	5	25.71	44.23	@	41.12	31.48	@	15.23	9.05	@
Wheat bran*	5	**53.94**	**63.42**	17.31	**101.29**	**145.16**	**112.47**	**87.45**	**74.18**	**86.64**
Rice bran#	5	32.55	14.37	@	38.29	24.20	@	08.49	9.95	@
Rice bran *	5	36.69	**31.44**	11.83	**116.45**	**81.72**	111.61	**57.32**	**45.45**	56.45

- Media without 2,5 Xylidine, *-Media with 2,5 Xylidine @- Contaminated

2,5 Xylidine seemed to be able to induce the production of all the three enzymes studied in both the wheat bran & rice bran media. In most cases the production of all three enzymes increased in both the FU-1 & FU-2 cultures. In case of FU-3 cultures results could not be obtained due to contamination. Laccase production seemed to have increased in wheat bran media by more than two times. LiP production seemed to have increased by three to four times in FU-1 culture in both wheat bran & rice bran media. The effect on MnP production was remarkable in both the FU-1 & FU-2 cultures. There was an increase of production of MnP in both the wheat & rice bran by almost five to eight times in presence of 2,5 Xylidine. Except for LiP production by FU-1 culture, the presence of 2,5 Xylidine in wheat bran media has induced a high production of enzymes.

Aromatic compounds regulate the ligninolytic enzyme synthesis although their effect is very specific depending on fungi (Eva Kachlishvili *et al*., 2014; Elisashvili *et al*., 2009; M S Revankar and S S Lele, 2006).Studies have shown increased production of Laccase, MnP, in response to the addition of aromatic compounds in several white rot causing fungi, (Piscitelli *et al*., 2011, Adinarayana, 2007, Robson *et al*., 2015). Laccase induction with veratryl alcohol was reported for another strain of *G. lucidum* while ferulic acid had no effect (D'Souza *et al*., 1999). The same study shows that high concentrations of nitrogen increased up to five times the production of the enzyme.

The inductive role of the 2,5-xylidine in the production of Laccase by white rot fungi has been demonstrated by number of studies (Bakkiyaraj Selvaraj et al., 2015; Viviane *et al.*, 2009; M. S. Revankar, S. S. Lele,2006; C Eggert *et al*., 1996,)

The results about the effect of 2,5-xylidine from this study are comparable to those described by other authors. There was an increase of production of Laccase, LiP & MnP in both the wheat & rice bran by almost twice and maximum 8 times in presence of 2, 5 Xylidine. Except for LiP production by FU1 Culture, the presence of wheat bran along with 2,5 Xylidine has induced a high production of enzymes in all the other cases. The variation in the degree of induction in this study shows that the physiological responses of Laccase, LiP & MnP production could be different depending on the fungal strain used and the media conditions. It is possible that the inducer 2,5 Xylidine may be inducing the production of some isoenzymes the way it has seen in a study where

different inducers are able to induce the production of isoenzymes, (Kuhar & Papinuti, 2014).

Thus the presence of 2,5 Xylidine and media conditions influenced the production of Laccase.

Decolorization of dye (in %) by crude enzyme:

Crude extract from eight days old FU-2 culture grown in liquid media with wheat extract at 5. 0 pH showed highest production of Laccase. This extract was used for dye decolourization study. Table 10 shows that all dyes at different concentrations (10 ppm, 25 ppm, 100 ppm) showed varied percentage of decolourization after three days of incubation. Dyes Black B, Blue 221, Red ME4BL show maximum decolourization followed by Red HE7B, Golden Yellow Merl and 0.2 R., followed by T Blue while Blue 222 showed minimum decolourization.

Table10: Decolorization of dye (in %) by crude enzyme

Dyes	Black B	0.2 R	Red HE7B	Blue 222	Blue 221	Red ME4BL	T Blue	Golden Yellow Merl
Concentration								
10 ppm	85	71	70	61	82	76	67	60
25 ppm	89	72	72	63	88	83	74	52
100 ppm	93	82	85	71	92	91	72	84

A number of investigations show that *Ganoderma* sp. has been successfully employed for decolorization of different synthetic dyes. (Lingan *et al.*., 2014; Gahlout, *et al*.; 2012**;** ***et al.,*** 2010; Asgher *et al*.., 2010; Chang-en Tian, 2013; M. Mohammadian, 2010). The study here too, show that all the dyes that were studies were decolorized in varying percentages by crude extract from eight days old FU-2 culture grown in liquid media with wheat extract at 5.0 pH.

IV. CONCLUSION

The present study clearly indicated that all three fungal cultures FU-1, FU-2 & FU-3 were found to have the ability to produce ligninolytic enzymes Laccase, Manganese Peroxide and Lignin Peroxidase. By varying the media conditions of growth all the three fungal cultures produced different amounts of all these enzymes. Addition of 2, 5 Xylidine could induce the production of all the three enzymes. Thus this study demonstrated that media manipulation can change the enzyme production in the fungal cultures. By optimizing the media condition & adding 2, 5 Xylidine to the media it was possible to increase the production of enzymes Laccase, LiP & MnP.

The crude enzyme of the culture studied was efficient in degrading most of the dyes studied here. Thus this study also showed that rather than using fungi it is possible to directly use crude enzymes for decolourization of dyes & probably for effluent treatment.

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