International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2017; 5(6): 67-72

Received: 01-09-2017
Accepted: 04-10-2017

Raj Kumar Pandey
Department of Microbiology, College of Basic Sciences and Humanities, G. B. Pant University of Agriculture and Technology Pantnagar, U. S. Nagar, Uttarakhand, India

Lakshmi Tewari
Department of Microbiology, College of Basic Sciences and Humanities, G. B. Pant University of Agriculture and Technology Pantnagar, U. S. Nagar, Uttarakhand, India

Correspondence
Raj Kumar Pandey
Department of Microbiology, College of Basic Sciences and Humanities, G. B. Pant University of Agriculture and Technology Pantnagar, U. S. Nagar, Uttarakhand, India

Fungal decolourization study of chemically synthesised malachite green and brilliant green textile dyes

Raj Kumar Pandey and Lakshmi Tewari

Abstract
Chemical synthetic dyes are now widely used in many industries such as leather, paper printing, textiles, wool, cosmetics, and food. The major problems associated with the use of dye are that they are resistant to degradation, difficult to remove waste water, non eco-friendly and causing health hazards due to their carcinogenic nature. The use of extracellular enzyme systems from wood rotting fungi are now growing very fast as green technology to biologically remediate such aromatic coloured compounds. In view of above, present study was undertaken for decolorization malachite green and brilliant green using wood rot fungal cultures. Out of various fungal isolates eight fungal isolates and one standard culture of potent Lignolytic fungus *Phnerochaete chrysosporium* were used for dye decolorization study, both qualitatively and quantitatively. The cultures varied in their dye decolorizing potential, showing 47.31-97.36% and 14.18-93.63% decolorization of malachite green and brilliant green respectively in 24 d. Phase contrast microscopy clearly revealed the asorption of the dyes by fungal cultures (mycelia/spores) in the photomicrographs. Among various cultures tested, the isolate AFP5 showing maximum dye decolorizing/ bioabsorbing ability was found as the most potential isolate.

Keywords: Chemical dyes, Decolourization, Wood rot fungi, *Ganoderma lucidum*

1. Introduction
The dyes are synthetic compounds that are mutagenic/ carcinogenic and belong to the most dangerous pollutants [1]. Industrial dyes can be released into the environment as effluents from dye synthesizing or other synthesis plants and factories. It is estimated that approximately 15% of the total dye used in the dyeing process may be found in wastewater [2]. Several of these dyes are very stable to even at high temperature, as well as to microbial attack, making them recalcitrant and tough to degrade [3]. Dyes are also most commonly used in the textile industries. Use of these dyes constitutes about 30%–40% of the total consumption of dyes, and they are applied extensively on nylon, cotton, wool, and silk, for coloring food, oils, fats, waxes, varnishes, cosmetics, paper, leather, and plastics as well as for staining specimens in bacteriological and histopathological processes. The major drawback is also that these can be transformed to carcinogenic compounds under anaerobic conditions [4].
Various physicochemical methods, such as adsorption, electro-coagulation, precipitation, ozonation, and many other have been used for decolorization of, these harmful carcinogens, however, these methods possess inherent limitations such as high cost, formation of hazardous by-products, and intensive energy requirements [5]. But, the biological processes provide a low-cost, environmentally benign, and efficient alternative for the treatment of dye wastewater [6]. As a feasible alternative, dye decolorization using microorganisms has recently received much attention owing to their cost effectiveness [7]. Currently, a lot of studies have focused on white rot fungi or wood rot fungi that seem to be more prospective organisms because of their unique oxidoreductive enzyme systems. These fungi are efficient ligninolytic organisms capable of degrading many xenobiotic compounds including various types of dye such as azo, anthraquinone, reactive, and triphenylmethane dyes either by absorption into biomass or by degradation to some non colored compounds [9, 10]. However, due to operational ease and facile adaptability of microorganisms to a given set of conditions, the biodegradation mechanism is considered efficacious in comparison to biosorption for treatment of dye wastewater [10]. In view of above, present study was undertaken for decolorization of synthetic and toxic dyes such as malachite green and brilliant green using wood rot fungal cultures.

Various wood rot fungi were isolated and checked for their dye decolorization ability on the basis of the presence of dye decolorizing unique enzymes also.

2. Experimental

2.1 Chemicals and Cultures Used

Veratryl alcohol, and 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and synthetic dyes were purchased from HiMedia Mumbai, India. The chemical structures and characteristics of the dyes used are depicted in Table 1. All other chemicals used were of analytical grade. Various lignocellulolytic fungal cultures used during present investigation, were isolated from diverse sources (decaying door and other woods, infected wood and infected trees) collected Pantnagar, Uttarakhand. The standard cultures of *Phanerochaete chrysosporium*, *Fusarium oxysporium*, *Rhizoctonia solani*, *Helminthosporium maydis*, and *Alternaria* spp. were obtained from the department.

2.2 Isolation and Conservation of Microbial Gene Pool

All the fungal cultures were isolated and maintained on Potato Dextrose Agar (PDA) medium (gL^{-1}; potato peeled 200.0, dextrose 20.0 g, and agar 15.0, pH 5.6±0.2). The fruiting bodies from decaying wood samples used for isolation of fungi. Small piece from the fruiting body surface sterilized using 70% ethanol, then washed with sterile distilled water and inoculated in triplicates at centre of the potato dextrose agar plates. The plates were incubated at 28±1 ^{0}C for 5-10 d for fungal growth. Pure mycelia and bacterial growth appeared on the plates were further purified. The purified plates were routinely subcultured at an interval of 30 d on same medium and preserved at 4^{0}C in refrigerator as slants and at -20^{0}C as glycerol stocks in deep freeze for short and long term storage, respectively. All the microscopic analyses were done based on LPCB (Lacto phenol cotton blue) staining.

2.3 Qualitative Screening for Production of Lignin Degrading Enzymes

2.3.1 Qualitative Screening for Overall Lignin Modifying Activity

Screening of the cultures for overall lignin modifying activity (ligninases production) was done using Lignin Modifying Enzyme Basal Medium (LBM) contained (g/L) KH_2PO_4, 1.0; $C_4H_{12}N_2O_6$, 0.5; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.01; Yeast Extract, 0.01; $CuSO_4.5H_2O$, 0.001; $Fe_2(SO_4)_3$, 0.001; $MnSO_4.H_2O$, 0.001; Agar, 16.0. LBM was supplemented with 1.6% w/v agar and autoclaved. To this added 1 ml of separately sterilized 20% glucose solution and 1 ml of aqueous tannic acid solution to each 100 ml of growth medium prepared. The medium was pour plated in sterilized petri plates. Active fungal culture disc was inoculated on LBM agar medium in triplicates. Un-inoculated plate served as control. The inoculated plates were incubated at 28±1^{0}C for 5-10 d. The qualitative measure of extracellular lignin modifying activity is the presence of brown oxidation zone around the fungal colony. It is reported as the index of relative enzyme activity (I_{LIG}). The following formula was used for calculating the I_{LIG} index.

$$I_{LIG} = \frac{Zone\ \ diameter}{Colony\ \ diameter}$$

2.3.2 Qualitative Screening for Extracellular Laccase Activity

Screening of the cultures for extracellular laccase activity (laccase production) was done using assay plates contained 15 ml of Potato Dextrose Agar (PDA) media, amended with 0.01% guaiacol. Active fungal culture disc was inoculated on agar medium in triplicates. Un-inoculated plate served as control. The inoculated plates were incubated at 28±1^{0}C for 5 d. The qualitative measure of extracellular laccase activity observed as presence of brick red zone of oxidized guaiacol around the fungal colony. It is reported as the index of relative enzyme activity (I_{LAC}) and calculated as the section 2.3.1.

2.3.3 Qualitative Screening for Overall peroxidase Activity

The fungal mycelial disc glucose malt extract salt agar medium contained glucose 2% (w/v); malt extract 2% (w/v); $NaNO_3$ 0.2% (w/v); KH_2PO_4 0.2% (w/v); KCl 0.2% (w/v); $MgSO_4.7H_2O$ 0.1% (w/v); $FeSO_4.7H_2O$ 0.002% (w/v); pH 6.5. Plate was incubated at 28 °C for 3 days and thereafter, 3ml of 1.7mM and 2.5 mM of ABTS and hydrogen peroxide respectively were overlapped on the plate and were kept in dark at 25 °C for 5 minutes. Appearance of clear bluish green zone around the fungus gave an indication of peroxidase production by the fungus. It is reported as the index of relative enzyme activity (I_{PER}) and calculated as the section 2.3.1.

2.4 Dye Decolorization Study

2.4.1 Dye decolorization on agar plate

Dye degradation ability of selected fungal cultures was assayed in low nitrogen basal medium containing (g/L) glucose, 1.0; $CaCl_2$, 1.5; $MgSO_4$, 2.0; KH_2PO_4, 1.5; NH_4Cl, 0.15, and 1.6%.The media were supplemented with various textile dyes at the concentration of 100 mg/l. The above medium was poured on petri-dishes and inoculated with mycelial disc and incubated at 30 ^{0}C under dark. Plates were regularly monitored at every 24 h for growth and decolorization activities.

2.4.2 Quantitative Study of Dye Decolorization

The cultures screened out from the above experiments were used to quantify dye decolorizing potential *in vitro*. The five active fungal discs were grown in the broth medium containing (g/L) glucose, 1.0; $CaCl_2$, 1.5; $MgSO_4$, 2.0; KH_2PO_4, 1.5; NH_4Cl, 0.15, supplemented with different dyes at the concentration of 100 mg/l. Samples were withdrawn periodically at an interval of 72 h and observed for colour change by measuring optical densities, spectrophotometrically. The cultures showing the bio-sorption of the dye were also checked visually and by microscopically. The percent decolorization (%) was calculated using the following formula:

$$D\,(\%) = \frac{Initial\ \ OD - Final\ \ OD}{Initial\ \ OD} \times 100$$

2.5 Dye Decolorizing Enzyme Production from Selected Cultures

The selected isolates were further screened for extracellular enzymes- lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase- in carbon limited liquid medium which contained (g %): glucose, 0.3; KH_2P0_4, 0.5; NH_4N0_3, 12.5 mM; $MgS0_4.7H_20$, 0.1; tween 20, 0.02; veratryl a1cohol, 1mM at pH 5.0. For the determination of MnP activity, the basal medium was supplemented with $MnS0_4$ (0.05%).

Growth medium (100 ml) was taken in 500 ml Erlenmeyer flasks and inoculated with the 5 mycellial discs. Samples were removed at regular intervals and crude enzyme collected after centrifugation at 10,000 rpm for 10 min, at 4 °C. This cell free supernatant was used as the source for crude enzymes.

2.6 Enzyme assays

Culture supernatants were used for the assay of the various lignolytic ernzymes enzymes. Lignin peroxidase (LiP) activity was estimated by measuring the rate of H_20_2-dependent oxidation of veratryl alcohol to veratraldehyde, spectrophotometrically[11]. The standard reaction mixture (2.05 ml) contained 0.8 mM veratryl alcohol in 0.1 M citrate buffer (pH 3.0) and 1 ml of culture supematant. The reaction was started by the addition of 150 mM H_20_2 and the linear increase in absorbance at 310 nm was monitored for one minute at 30°C. One unit of LiP was defined as 1 umol of veratraldehyde formed per minute and was expressed as U/ml. MnP activity was measured by monitoring the oxidation of Mn^{2+} toMn^{3+}. The assay solution (3.06 ml) contained 0.1 mM guaiacol and 0.1 mM $MnS0_4$ in 0.1 M citrate buffer (pH 5.0) with 1ml of culture filtrate. The reaction was started by 0.1 mM H_20_2 addition. One unit of enzyme activity was defined as the increase in absorbance at 465 nm per minute[12]. The laccase activity was determined by monitoring the oxidation of 500 µM ABTS (2,2' - azino - di - [3 - ethyl benzothiazoline - 6 - sulphonic acid]) buffered with 50 mM citrate buffer (pH 4.5) at 436 nm[13]. The reaction mixture (3 ml) contained 1 ml of culture filtrate. One unit was defined as 1 ~M ofABTS oxidized per minute.

3. Results and Discussion

White rot fungi have been demonstrated for decolourization of synthetic dyes mediated by their lignolytic enzymes such as lignin peroxidase, manganese peroxidase and laccases [14, 15]. To achieve the goal, a total of forty fungal strains (including both Mushrooms and other fungi) were isolated from various samples from the different geographical areas of Uttarakhand. Standard culture of *Phanerochaete chrysosporium* was produced from MTCC Chandigarh, India.

3.1 Selection of dye decolourizing fungal strains

Out of forty one isolates nine isolates were showed the overall lignin modifying activity, whereas the laccase and peroxidase activity was observed in seven and two isolates respectively (Table 2). Nine isolates were selected on the basis of zone formation (Fig 1) and relative enzyme activity indices (Table 3). Maximum overall lignin modifying activity was found in fungal culture AFP7 (2.0) and AFP5 (1.818), followed by AFP4 (1.567); whereas maximum laccase activity was found in fungal culture AFP5 (3.0) and AFP4 (2.777), followed by PN1 (2.5). Only two fungal cultures PN1 (2.5) and WDP2 (1.6) were found positive for peroxidase production.

3.2 Dye Decolorization Study

To confirm that various strains of the fungi tested were capable of decolorization capacity, the five common dyes were incubated with the fungal strains for 10-20 days on solid medium and up to 1 month in liquid medium at 28 °C and 150 rpm. The percent decolorization of malachite green and brilliant green dyes are presented in Table 4. On the solid cultures, decolorization began with formation of very light decolorized zones, and the decolorization capacity of the fungus was determined by measuring the diameters of the discolored rings (Fig 2). On the basis of spectrophotometric quantification results, all the cultures showed very high percent decolourization except the cultures AFP7 and *P. chrysosporium* (Table 4). During the liquid cultivation experiments, the batch cultures turned from an initial deep coloration to a lighter color, eventually becoming colorless, indicating the dye adsorption and dye decolorization (Fig 3). The cultures varied in their dye decolorizing potential, showing 47.31-97.36% and 14.18-93.63% decolorization of malachite green and brilliant green respectively in 24 d. Maximum decolourization of brilliant green (97.36%) was showed by fungal isolate AFP5, followed by PN1 with 96.59% decolorization. Malachite green was efficiently decolourised by fungal isolate AFP1 with a maximum of 93.63 % decolourization.

3.3 Enzyme Assay

The selected fungal cultures were grown in enzyme production medium with the varatryl alcohol as inducer for enzyme production. The cell free culture filtrate was used as crude enzyme for calculating the crude enzyme activity. The induction of enzymes was correlated with their involvement in dye decolorization. Various oxidative and reductive enzymes were synthesized by various cultures in enzyme production medium. The enzymes were synthesized with peak laccase activity (171.83 U mL^{-1} by AFP5) on 18th day (**Fig 4**), peak LiP and MnP activity on the twenty one day (0.34 U mL−1 by AFP4) and fifth day (0.0021 U mL−1 by AFP5) respectively (data not shown). These results reveal that laccase, LiP, MnP, and other enzymes play vital roles in the bioremediation of used dyes during present investigation dyes. The laccase enzyme was maximally produced in positive strains thus used for further study.

Earlier studies have also reported differential dye removing capabilities of diverse microbial cultures but wood rot fungi specifically have been reported to be more efficient for dye removal through its enzymatic/ non enzymatic mechanisms. Previously only 40% decolourization of anthraquinone dye, Remazol Brilliant Blue R (RBBR) by *Ganoderma* sp. was reported which could be increased up to 92.4% upon addition of HBT as redox mediator[16]. However, contrary to previous findings, much higher decolourization rate for the for brilliant green by our indigenous fungal isolates could be achieved during present study indicating higher bioremedial potential of the cultures. All the five test fungal isolates decolouried all three dyes efficiently in comparision to the standard culture of *Phanerochaete chrysosporium*. It is evident from the results that the cultures showed better biosorption efficacy towards brilliant green but only wood rot fungal isolate AFP5 could show better efficacy for removal of the dye. As per many earlier reports, the bioremediation of dye by the ligninolytic fungi and their ligninases enzymes (lignin peroxidase, manganese dependent peroxidase and laccase) is more environmentally friendly strategy to degrade synthetic textile dyes [17, 18]. The decolourization of dyes with the help of extracellular lignin degrading/modifying enzymes is also supportable because of the broad spectrum substrate range that is very low substrate specificities of these enzymes from the fungal system. The low substrate specificities of ligninases enzymes from wood rot fungi make them more capable to degrade a wide range of xenobiotic compounds including the synthetic industrial dyes [19, 20]. The present investigation deals with removal of synthetic dyes by biological means using white-rot fungal cultures. All the cultures showed the good dye decolorizing and bio-absorption ability in comparison to the standard culture of *P.*

chrysosporium, thus having a good potential. Lignolytic enzyme producing fungi have many biotechnological applications and the isolated fungi might be used for further research projects.

Table 1: The structural formulae, industrial uses and hazardous effects of the synthetic Dyes decolourized during present study*

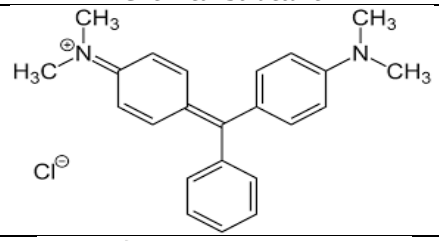
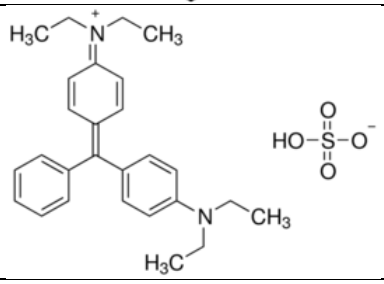
S. No	Dye	Chemical Structure	Use and Hazards
1	Malachite Green		Used as a dye, frequently been used to catch thieves and pilferers, active against the oomycete Saprolegnia, which infects fish eggs, cause carcinogenic symptoms, The LD50 (oral, mouse) is 80 mg/kg, Extreme irritant.
3	Brilliant Green		Related to malachite green, genotoxic and carcinogenic properties induces vomiting when swallowed and is toxic when ingested

Table 2: Diversification of Fungal Isolates On the Basis of Qualitative Enzyme Assay

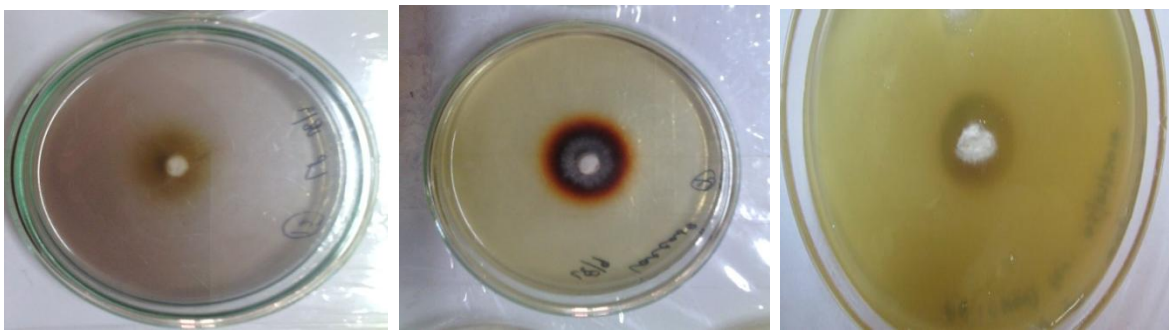
S. No	Enzyme Activity	Total Isolates	Isolates showing positive enzyme activity	Isolates do not showing enzyme activity	Selected Isolates
1	Overall lignin modifying activity	41	9	32	WDP1, PN1, AFP7, AFP5, AFP3, AFP1, WDP2, AFP4, and *P. chrysosporium*
2	Laccase activity	41	7	34	WDP1, PN1, AFP7, AFP5, AFP3, WDP2, AFP4
3	Overall peroxidase activity	41	2	39	PN1, and WDP2

Table 3: Relative lignin degrading enzyme activity indices of selected fungal cultures

S. No	Culture	Relative index (I_{LIG})	Relative index (I_{LAC})	Relative index (I_{PER})
1	WDP1	1.166	2.3	-
2	PN1	1.408	2.444	2.5
3	AFP7	2.0	2.3	-
4	AFP5	1.818	3.0	-
5	AFP3	1.4	1.363	-
6	AFP1	1.466	-	-
7	WDP2	1.076	1.83	1.6
8	AFP4	1.562	2.777	-
9	*P. chrysosporium*	1.0	-	-
		cd at 5% = 0.135 sem = 0.0416	sem = 0.029 cd at 5% = 0.1	sem =0.01 cd at 5% = 0.15

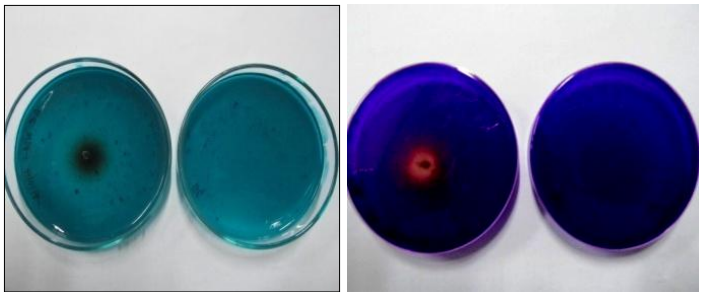
Table 4: Percent decolorization of malachite green and brilliant green using lignolytic fungal cultures

S. No	Fungal Culture	Percent Decolourization (%)	
		Brilliant Green Decolourization	Malachite Green Decolourization
1	WDP1	92.66	58.6
2	PN1	96.59	14.18
3	AFP7	47.31	14.9
4	AFP5	97.36	88.85
5	AFP3	96.33	43.84
6	AFP1	89.56	93.63
7	WDP2	92.76	21.27
8	AFP4	95.19	92.32
9	*P. chrysosporium*	58.62	32.99
		sem =0.15 cd at 5% =0.505	sem =0.142 cd at 5% =0.464



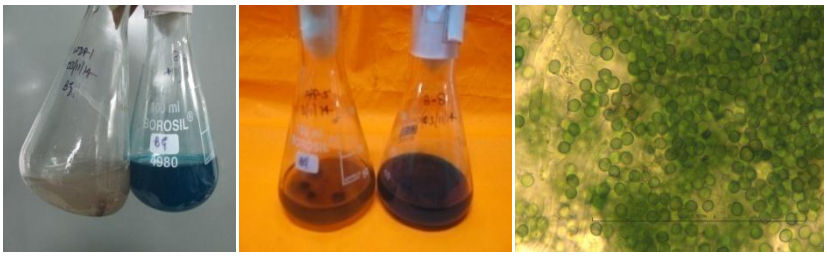
(a) (b) (c)

Fig 1: Zone formation by fungus isolate AFP5. Overall activity (a), laccase activity (b) and peroxidase acitivity (c).



(a) (b)

Fig 2: Qualitative detection of the dye decolorizing potential of the fungal isolates. Malachite green decolorization (a) and brilliant green decolourization (b) by fungal isolate AFP5



(a) (b) (c)

Fig 3: Removal of malachite green from broth medium (a) and brilliant green (b) by fungal isolate AFP5, phase contrast microscopic view of the fungal mycelia and spores showing the accumulation of the dye brilliant green by fungal isolate AFP5 (c).

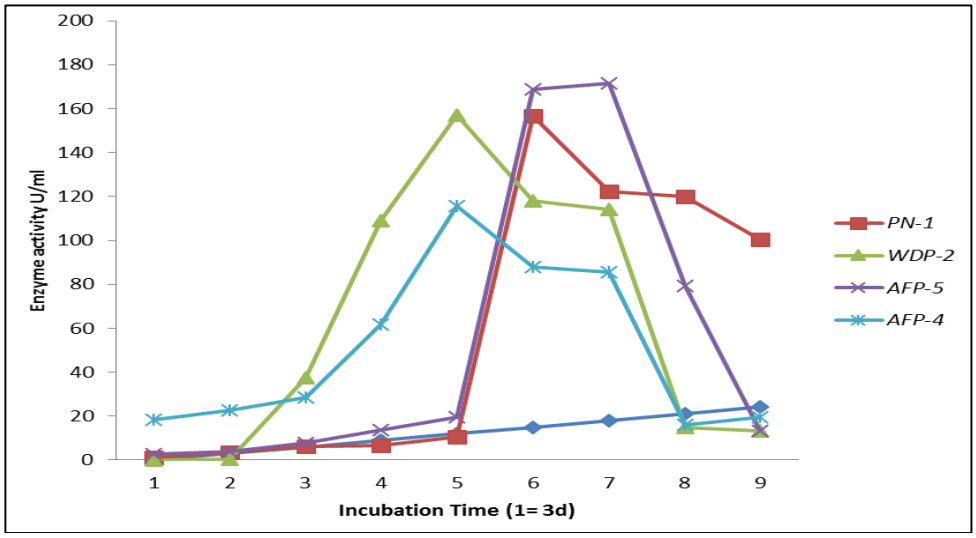


Fig 4: Laccase production during growth of fungi. Fungal cultures grown in standard conditions in defined medium at pH 6, and 30 °C. Maximum enzyme activity was observed in culture filtrate of fungal culture AFP5.

4. References

1. Mohan SV, Rao NC, Srinivas S, Prasad KK, Karthikeyan J. Treatment of simulated Reactive Yellow 22 (azo) dye effluents using Spirogyra species. Waste Manag. 2002; 22:575-582.
2. Elizabeth Rodrı´guez, Michael A. Pickard, Rafael Vazquez-Duhalt.1998. Industrial Dye Decolorization by Laccases from Ligninolytic Fungi. Current Microbiology 1999; 38:27-32

3. Pagga U, Brown DH. The degradation of dyestuffs, part II. Behavior of the dyestuffs in aerobic biodegradation test. Chemosphere. 1986; 15:479-491.
4. Bumpus JA, Brock BJ. Biodegradation of crystal violet by white rot fungus Phanerochaete chrysosporium. Appl Environ Microbiol. 1988; 54:1143-1150
5. Daneshvar N, Ayazloo M, Khataee AR, Pourhassan M. Biological decolorization of dye solution containing Malachite Green by microalgae *Cosmarium* sp, Bioresource Technology. 2007; 98(6):1176-1182.
6. Ali H, Ahmad W, Haq T. Decolorization and degradation of malachite green by Aspergillus flavusand Alternaria solani, African Journal of Biotechnology. 2009; 8(8):1574-1576,
7. Chivukula M, Renganathan V. Phenolic azo dye oxidation by laccase from Pyricularia oryzae. Appl Environ Microbiol. 1995; 61:4374-4377.
8. Dey S, Maiti TK, Bhattacharyya BC. Production of some extracellular enzymes by a lignin peroxidase-producing brown rot fungus, Pleurotus ostreiformis, and its comparative abilities for lignin degradation and dye decolorization. Appl Environ Microbiol. 1994; 60:4216-4218.
9. Zhou W, Zimmermann W. Decolorization of industrial effluents containing reactive dyes by actinomycetes, FEMS Microbiology Letters. 1993; 107(2-3):157-161.
10. Tien M, Kirk K. Lignin peroxidase of Phanerochaete chrysosporium. Method. Enzymol. 1988; 161:238-249.
11. Paszczynski A, Crawford RL, Huynh V. Manganese peroxidase of phanerochaete chrysosporium: purification. Method. Enzymol. 1988; 161:264-270.
12. Niku-Paavola ML, Karhunen E, Kantelinen A, Viikari L, Lundell T, Hatakka A. The effect of culture conditions on the production of lignin modifying enzymes by the white-rot fungus Phlebia radiata. Journal of Biotechnology. 1990; 13:211-221.
13. Heinfling A, Martinez MJ, Martinez AT, Bergbauer M, Szewzyk U. Transformation of industrial dyes by Manganese peroxidase from *Bjerkandera adusta* and *Pleurotus erynjii* in a manganese-independent reaction. Appl Envrion Microbiol. 1998; 64:2788-93.
14. Abadulla E, Tzanov T, Costa S, Robra KH, Cavaco-Paulo A, Gubitz GM. Decolorization and detoxification of textile dyes with a laccase from *Trametes hirsute*. Appl Environ Microbiol. 2000; 66:3357-62.
15. Murugesan K, Arulmani M, Nam IH, Kim YM, Chang YS, Kalaichelvan PT. Purification and characterization of laccase produced by a white rot fungus *Pleurotus sajorcaju* under submerged culture condition and its potential in decolorization of azo dyes. Applied Microbiology and Biotechnology. 2006; 72(5):939-946.
16. Raghukumar C, D'Souza-Ticlo D, Verma A. Treatment of colored effluents with lignin-degrading enzymes: an emerging role of marine-derived fungi. Critical Reviews in Microbiology. 2008; 34(3-4):189-206.
17. Plácido J, Chanagá X, Ortiz-Monsalve S, Yepes M, Mora A. Degradation and detoxification of synthetic dyes and textile industry effluents by newly isolated *Leptosphaerulina* sp. from Colombia. Bioresources and Bioprocessing. 2016; 3(1):1-14.
18. Spadaro JT, Gold MH, Renganathan V. Degradation of azo dyes by the lignin-degrading fungus *Phanerochaete chrysosporium*. Applied Environmental Microbiology. 1992; 58:2397-2401.
19. Kaushik P, Malik A. Fungal dye decolourization: Recent advances and future potential. Environment International. 2009; 35:127-141.