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Degradation of textile dyes and characterization of ligninolytic enzymes of white rot fungi

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Abstract:
White rot fungus employ diverse ways to fully break down lignin and a broad range of environmental contaminants. Oxidative and reductive processes are essential for metabolising lignin and environmental contaminants. White rot fungi release several peroxidases to facilitate the direct and indirect oxidation of substances. *Trametes versicolor* degrades lignin efficiently through the use of a aggregation of ligninolytic enzymes of extracellular origin, organic acids, intermediary compounds, and auxiliary enzymes. The ability of test cultures to produce laccases and peroxidases was confirmed using Bavendamm's test. Bavendamm's test is effective in distinguishing between white and brown rot decomposition of wood and has shown that *L. betulina*, *T. versicolor*, UK4, TE, DSR-1, ISM, and UK2 are white rot basidiomycetes. These white rots degrades range of textile dyes. The *Trametes versicolor* culture on Kirk's medium agar plates decolorized textile dyes. Remazol Brilliant Blue R, Turquoise Blue H2GP, Reactive Magenta HB, Reactive Orange 3R, Reactive Red 6BX, V5R, BGR, Reactive Red HE3B, Fast Green, and Reactive Golden Yellow 145 (GY145). This study has focused the capacity of *Trametes versicolor* cultivated on Kirk's medium to remove colour from textile dyes. The correlativity between the growth of microorganisms and the decolorization of textile colours was examined by analysing their growth, decolorization, and the ratio between them throughout a 9-day cultivation period.These fungi exhibit ligninolytic activities, including manganese peroxidase (MnP), laccase (Lac)and manganese-independent peroxidase (MnIP), that decolorizes the textile dyes viz. Turquoise Blue GP, Remazol Brilliant Blue R, and Fast Green.

Keywords: *Trametes versicolor*, 3-Methyl 2- Benzothiazolinone Hydrazone(MBTH), 3-Dimethylamino Benzoic Acid (DMAB), Manganese Peroxidase, Manganese independent Peroxidase, Turquoise Blue 2GP, Fast Green, Remazol Brilliant Blue R, White Rot Fungi.

1. Introduction

In 1856, William Henry Perkin accidentally discovered the first synthetic dye successfully introduced to the market. By the end of the 1800s, ten thousand new synthetic dyes had been designed and manufactured. Prominent environmental agencies and organizations, like the US Environmental Protection Agency (US EPA), recognize the management and reduction of

extremely hazardous and toxic pollutants, such as dyes, as crucial tasks in their endeavors. Significant studies have been conducted to elucidate the impact of dye on human health and the environment, in both the immediate and prolonged periods[1], [2]. The issue of dye pollution has significantly increased in recent decades because to its widespread use in several textile industries in major. Industry utilises about 10 thousand types of dyes and pigments, with a production exceeding of 700,000 tonnes per annum[3].

Worries about the harmful effects and cancer-causing properties of textile dyes have been the primary reasons for investigating their ability to contribute to pollution. Many colours are made from substances such as benzidine and other aromatic chemicals, which are known to be carcinogenic and can undergo redox reactions due to microbial metabolism. Several studies have shown that dispersive dyes, which are organic compounds lacking ionising groups and have low water solubility, are the predominant dyes used for dyeing polyester yarn and fabricin the textile industry[4]. Dyes can create extremely dangerous aromatic compounds with mutagenic and carcinogenic properties when mixed with synthetic intermediates and their breakdown products[5].

Fungi have been proven to effectively degrade and break down resistant textile colours due to their strong physical structure, diverse metabolic abilities, and powerful extracellular ligninolytic enzyme system.[6], [7]. Currently, the most efficient lignin-degrading organisms are the white rot basidiomycetes. They are commonly utilised in industrial wastewater treatment due to their remarkable capacity to break down a variety of substances. [8]–[12]. The biological decolorization relies significantly on nonspecific ligninolytic enzymes such as laccase, manganese peroxidase (MnP), lignin peroxidase (LiP), and versatile peroxidase (VP) due to their capability to act on multiple substrates[9], [13]–[15]. Due to the high redox potentials of LiP, MnP, and VP, radical reactions require the addition of H_2O_2 to initiate. Several research have suggested using synthetic aromatic molecules to enhance the effectiveness of xenobiotic breakdown [16]–[20].However, the significance of lignin waste in dye degradation has not been thoroughly researched. Lignin is a complex biopolymer that usually accounts for 15–40% of the weight of lignocellulose in plant materials when dried. The structural unit is phenylpropane[21]–[24].The white-rot fungus is renowned for depolymerizing and converting lignin into aromatic compounds, which are considered waste by-products of the kraft-pulping process and lignocellulose biorefinery.

Our study aimed to assess the ligninolytic enzyme production capability of White Rot Fungi. The found enzymes broke down various types of dye. We examined the impact of various physicochemical parameters on dye degradation. The dye degradation process was studied using spectrophotometry to understand its mechanism. Coloured wastewater can be treated with the fungus.

2.Methodology

2.1Maintenance of fungal strains

The basidiomycetes isolatenamely*Pleurotusostreatus*, *Lenzitesbetulina*, *Trametes versicolor (TRV)*, UK4, DSR-1, ISM, UK2 and TE were chosen for the study. Cultures were stored on malt extract agar (MEA) plates containing glucose – 10 g/L, malt extract powder- 10g/L, peptone - 2g/L, yeast extract powder -2g/L, L-asparagine -2g/L, KH_2PO_4 -2g/L, $MgSO_4.7H_2O$ -2g/L, thiamine HCl -0.001g/L and agar -30g/L. The pH of the medium was maintained 4.8 and the white rot fungal cultures were maintained at 4°C.

2.2Bavendamm's test

The Bavendamm's test was conducted using MEA plates with 0.1% tannic acid. An 8mm agar disc was used to inoculate the plates from a 7-day old fungal culture produced on MEA plates.The plates were kept at a temperature of 25°C for 7 days and monitored for colour changes around the agar disc to distinguish between White Rot Basidiomycetes and Brown Rot Basidiomycetes.

2.3Dye decolorization insolid state

From the previous test, the *Trametesversicolor* (TRV) was chosen for decolorization of ten different dyes namely Remazol Brilliant Blue R, Turqoise blue H2GP, Reactive magenta HB, Reactive orange 3R, Reactive red 6BX, ReactiveViolet 5,Rective Brown G, Reactive red HE3B, Fast Green and Reactive golden Yellow 145 (GY145). This assay was important for assessing the fungal strain's ability to decolorize variety of dyes.Dyedecolorization was carried out by inoculating 8 mm agar disc punched from old culture onKIRK'Smediumcontaining 0.1% of each textile dyes and incubates at 25°C temperature. The plates wereobserved at regularintervals until 6 days for evaluation of dye decolorization.

2.4Extraction of Ligninolytic enzyme and Enzyme activity assessment

The fungi TRV and UK4 were transferred from a preserved slant stored at 4°C to separate MEA plates and then cultured at 28°C in a humidity chamber for 7 days. Ten discs were extracted from the MEA plates and placed in a flask with 100 ml malt extract broth. They were then cultured under shaking conditions for 5 days at 28°C, referred to as an inoculum. The final inoculum was created by combining 20 ml of homogenised mycelia from the inoculum with 250 ml of 1.25x concentrated ME broth in an Erlenmeyer flask. The mixture was then placed in an incubator at 28°C for 5 days without agitation. The entire contents of these flasks were mixed well and used as the final inoculum. The production of ligninolytic enzymes utilised GVT (Glucose Veratryl alcohol Trace components) media. The supernatant from static cultures was filtered and centrifuged at 10,000 rpm for 15 minutes at 4°C. Subsequently, it was subjected to ammonium sulfate precipitation at 80% saturation. The precipitates were collected by centrifugation at 10,000 rpm for 15 minutes at 4°C and then dissolved in a small amount of acetate buffer (0.1 M, pH 4.2). MnP, MiP, and laccase activities were measured spectrophotometrically by monitoring the oxidation of 3-Dimethylamino Benzoic Acid (DMAB) and 3-Methyl 2-Benzothiazolinone Hydrazone (MBTH) as chromogen. One unit of enzyme activity is defined as the amount of MBTH-DMAB oxidised in the reaction mixture to produce 1µmol of indigo dye per minute[25].The isolated enzyme's ability to breakdown dyes was assessed. The TRV was selected for additional tests on dye degradation because to its superior performance in producing MnP and MiP enzymes. The studies were carried out by replacing MBTH-DMAB with 0.1% solutions of 50–250 g of Remazol Brilliant Blue R (RBBR), Turquoise Blue 2GP (TH2GP), and Fast Green (FG) in the reaction mixes for MnP. The reaction mixture comprised 1000 µl of succinate lactate buffer (100 mM, pH 4.5), 10 µl of H_2O_2 (10 mM), 100 µl of enzyme, and was adjusted to a total volume of 2 ml with distilled water. The pH was assessed within the range of 3 to 5.5. The degradation of the dye was measured using spectrophotometry within the wavelength range of 325 nm to 750 nm.

3. Results and Discussion:

Ligninolytic enzymes as synthesised by fungi can attach to a range of recalcitrant substances, including textile colours and insecticides. [26].It is essential to determine the efficiency of fungi in degrading specific persistent and foreign substances. The optimisation of dye-containing wastewater treatment has not been achieved, and no specific solutions have been reported to yet, despite substantial research in this area. The study demonstrates that ligninolytic enzymes can break down dyes.

3.1 Evaluation of fungal isolates by Bavendamm's test

The Bavendamm's test is generally utilized for the differentiation of white rot basidiomycetes from the brown rot basidiomycetes [27].It can also be used to observe the browning and bleaching of fungal isolates when grown on malt extract medium with tannic acid. The increased browning and bleaching effects indicate that these fungi produce ligninolytic enzymes as they grow on the media. [28]. The test cultures' capacity to generate laccases and peroxidases was verified using Bavendamm's test. Basidiomycetes, a

type of fungus, have the ability to decompose all parts of a plant's cell wall.

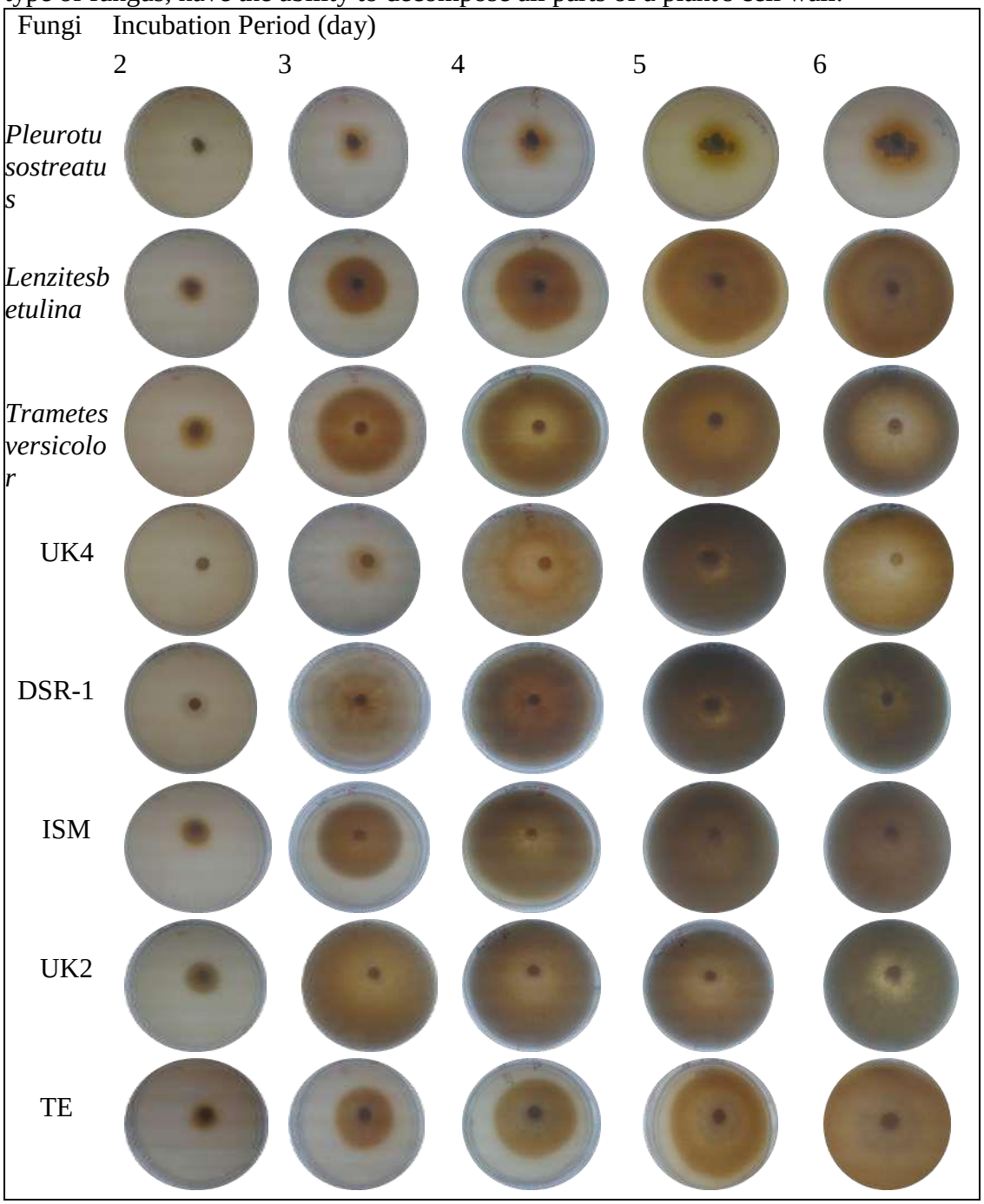


Fig.1 Development of Browning and bleaching effect by white rot basidiomycetes isolates(A) Pleurotusostreatus, (B) Lenzitesbetulina, (C) Trametes versicolor, (D) UK4, (E) DSR-1 (F) ISM, (G) UK2 and (H) TE

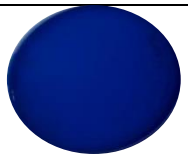
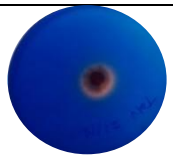
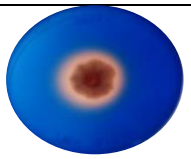
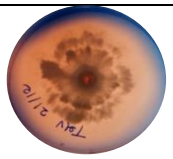
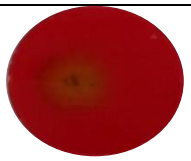
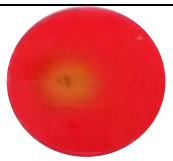
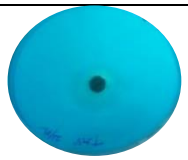
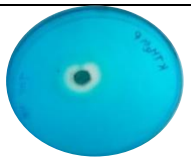
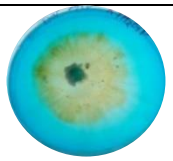
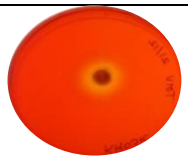
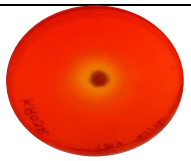
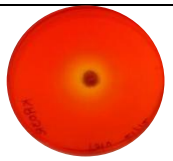
They can be categorized based on the kind of wood decay they induce. It can serve as the main assessment of enzymatic production. Fig.1clearly displaysthe gradual browning and bleaching effect of eight different fungal isolates. They were observed regularly from day 2 of fungal inoculation until day 6. The development of browning effect surrounding the fungal disc is clearly seen in the figure 1 and it varies according to fungal isolates. As the Bavendamm's test is useful in the differentiation of white and brown rot decay ofwood, it demonstrated that L. betulina, T. versicolor, UK4, TE, DSR-1,ISMand UK2belong towhiterot basidiomycetes.The maximum effect was observed in the *Trametes versicolor* and fungal isolate UK-4. The other fungal isolates showed slow or less bleaching effect.

Therefore, it was decided to use these two fungal isolates for carrying out further experiments.

According to Badalyan et al., the Bavendamm test was employed to detect the production of extracellular polyphenol oxidase activities and to evaluate growth on lignocellulosic substrates[29]. Malt agar medium supplemented with tannic acid has been used to detect the presence of extracellular oxidases which are generally characteristic of ligninolytic activity [30].

3.2 Dye decolorization by fungal isolate

Dyes are substances, either natural or synthetic, that impart color to various fibers used in industries such as textiles, pharmaceuticals, food, cosmetics, plastics, photography, and paper[31]. Colourants can either be pigments or dyes. Pigments are nearly insoluble, with particle sizes typically ranging from 1 to 2 µm. Dyes are readily soluble in water and typically have a particle size between 0.025 and 1.0 µm[32,33].The dyes are chemically stable and do not biodegrade in conventional sewage treatment plants. Physicochemical technologies are effective for dye degradation and addressing the issue of disposing industrial wastes with dyes, however they are costly and not environmentally friendly[34]. Various microorganisms have demonstrated the ability to break down and remove colour from textile dyes and waste. Basidiomycetes that degrade lignin have been researched for their potential to break down textile dyes using various oxidases and peroxidases that can degrade a variety of stubborn synthetic chemicals[35]. White rot basidiomycetes are known to act uponstructurallydiversexenobioticcompoundslikepolyaromatichydrocarbons,nitroaromatics and explosives, polychlorinated biphenyls and dyes[36]which aresomekeypollutantspresentineffluentsoftextileindustries.Mostofthereportedworkhasbeenfocusedon*Phanerochaetechrysosporium*and*Pleurotusostreatus*.

Dyes	Incubation period (days)			
	Control	2	4	6
V5R				
Remazol Brilliant Blue R				
RRHE3B				
Reactive orange 3R				
Turquoise Blue 2GP				

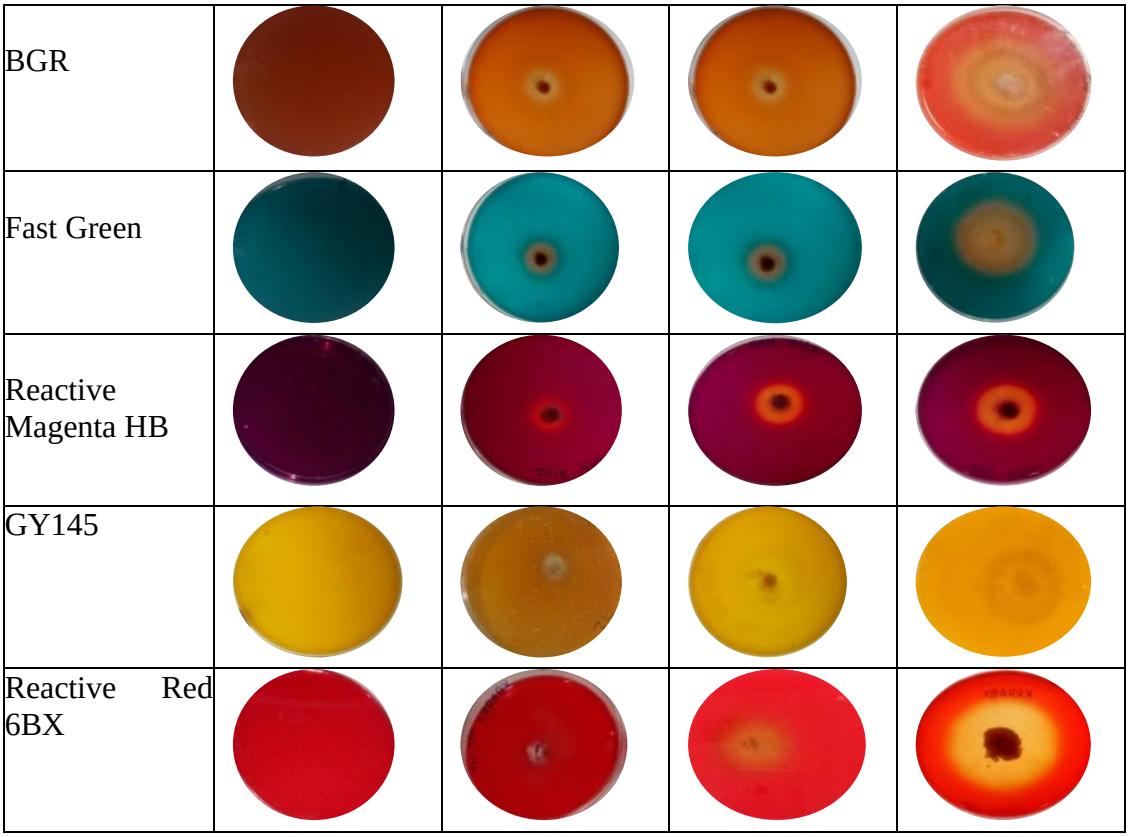


Fig. 2 Decolorisation of textile dyes-V5R, Remazol Brilliant Blue R, RRHE3B, Reactive orange 3R, Turquoise Blue 2GP, BGR, Fast Green, Reactive Magenta HB, GY145, Reactive Red 6BX by *Trametesversicolor*isolate

Thepresentstudyinvolveddecolorizationofvarious textiledyessuch as V5R, Remazol Brilliant Blue R (RBBR), RRHE3B, Reactive orange 3R (RO3R),Turquoise Blue 2GP (TH2GP),BGR, Fast Green(FG),Reactive Magenta HB(RMHB), GY145 and Reactive Red 6BX (RR6BX) bywhiterotfungi *Trametes versicolor*.Figure 2 displayed the decolorization of dyes on KIRK's medium upto 6 days with comparison to control dye plates. TRV showed highest potential for decolorization of RBBR dyewhich is an anthraquinone dye. [36] also confirmed the potential of *Trametes versicolor* CB8 stain for degradation of dye belonging to azo, triphenylmethane and anthraquinone dyes.Moreover, TRV was successful in decolorizing additional dyes, including Turquoise Blue 2GP, Fast Green, Reactive Red 6BX, V5R and Reactive orange 3R. It showed the potential of TRV for dye decolorization belonging to different classes.Binding of dyes to the fungal hyphae, physical adsorption and enzymatic degradation by extracellular enzymes as reasons for the colour removal. The dye-saturated mycelium can be regenerated and used for repeated dye adsorption. They havefurther stated that dyes were not decolourized by manganese dependent peroxidase (MnP) while above 80% colour was removed by ligninase-catalyzed oxidation. Dyes with different structures are decolourized at different intrinsic enzymatic rates and high dye concentration results in slower decolourization rate. The dye was adsorbed to the mycelia pellets in both ligninolytic and non-ligninolytic cultures [37]. Other white-rot fungi (WRF) studied include Trametes versicolor, Pleurotus ostreatus, Bjerkandera adusta, Clitocybe ladusenii, and Phlebia species. These fungi have been reported to decolorize six structurally diverse dyes: Amaranth, Remazol Black B, Remazol Orange, Remazol Brilliant Blue, Reactive Blue, and Tropaeolin O. Among them, Bjerkandera sp. BOS55, Phanerochaete chrysosporium, and Trametes versicolor showed extensive decolorization of these dyes on agar plates[38].

3.3 Assessment of Enzymatic activity

White rot fungus naturally produce ligninolytic enzymes with poorer specificity. These enzymes have been utilised to degrade many xenobiotics, which are resistant compounds present in the environment. Two white rot basidiomycetes, *Trametes versicolor* and UK4 fungal isolates, were used for this study to generate ligninolytic enzymes such laccase, MnP, and MiP. Enzyme activity have been graphically depicted in Figure 3 from day 4 to day 9 of the incubation period. The MnP enzyme activities were detected on the 4th day of incubation in both strains at a level of around 600 U/ml. Activity of MnP significantly rose on the 5th day in TRV, surpassing that of the UK4 strain. MiP activity exhibited a progressive increase from the 4th to the 7th day, followed by a modest reduction thereafter. The peak MiP activity of approximately 1000 U/ml was recorded on the 7th day in the TRV strain. It is highly effective for breaking down contaminants. Laccase activity ranged from 120-200 U/ml and remained somewhat stable throughout time, showing lower levels compared to MnP and MiP activities.

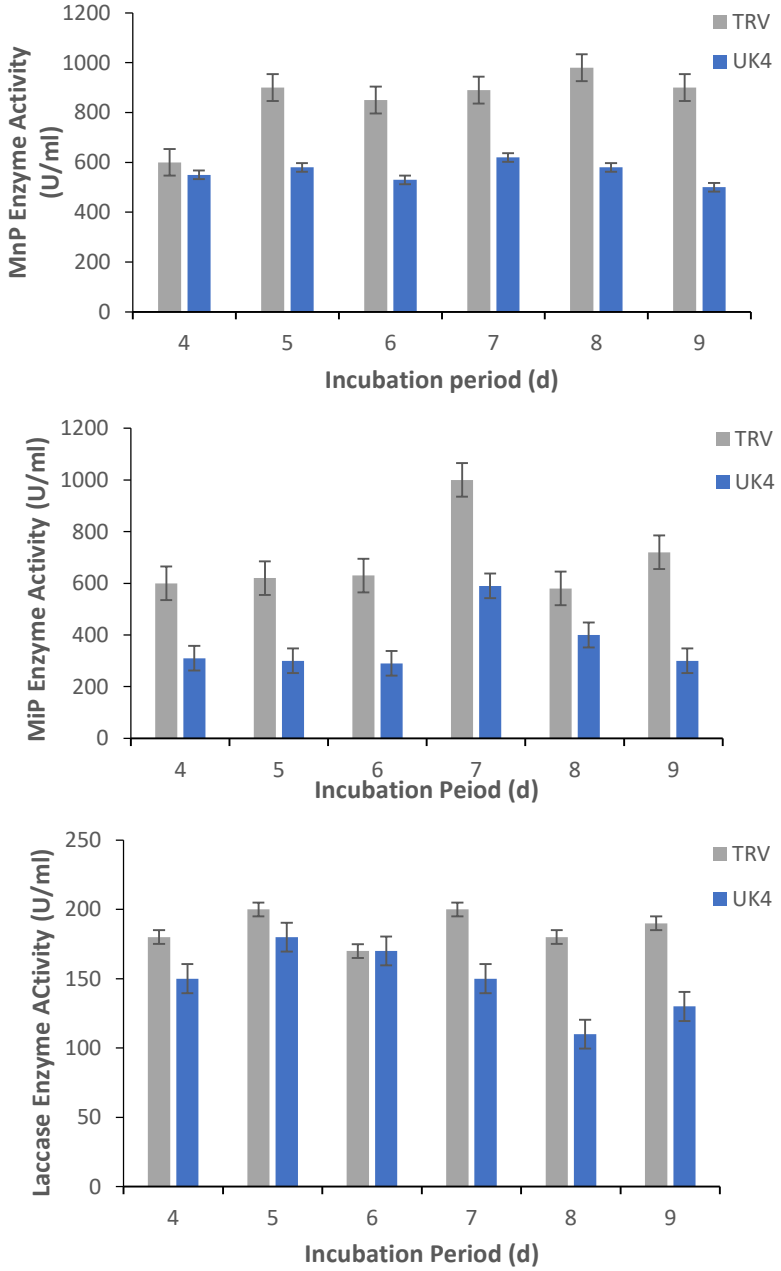


Fig.3 Production of ligninolytic activities (a) MnP, (b) MiP and (C) Lac by staticcultures of *Trametes versicolor and UK4 isolate*grown on GVT medium at 28ºC.

3.4 Dyedegradation

The extracellular ligninolytic enzymes of T. versicolor cultures were incubated for 7 days in GVT at 20ºC under static conditions to breakdown Remazol Brilliant Blue R, Turquoise Blue GP, and Fast Green. MnP enzyme decolorized RBBR, TH2GP, FG3-3.5, and FG4-4.5 with pH optima of 4, 3.5, 4.5, and 4.0, respectively.At pH 4, spectral changes during RBBR decolorization indicated a decrease in absorbance between 375-415 nm and 525-700 nm, whereas absorbance increased between 325-375 nm and 415-520 nm. The alterations indicated that the dye had deteriorated, resulting in decolorization. MnP enzyme did not remove colour from TH2GP at pH 4. Spectral changes were observed in the wavelength range of 325-420 nm and 525-750 nm, showing a decrease in absorbance during TH2GP decolorization by MnP. The spectral changes observed during the decolorization of FG by MnP activity at the tested pH were dissimilar. Decrease in absorbance was noted between 610-665 nm and 460-575 nm wavelengths when FG was decolorized at pH 3 and 3.5. Changes in spectral properties were observed during the decolorization of FG by MnP at pH 4 and 5. At pH 4, absorbance decreased, whereas at pH 5, absorbance rose in the 350-400 nm range when FG was decolorized. MnP decolorized FG differentially at various pH levels as depicted in Figures 4.1, 4.2, and 4.3.

MiP activity effectively decolorized the dyes Remazol Brilliant Blue R, Turquoise Blue GP, and Fast Green. The ideal pH for decolorization was found to be between 4 and 4.5. Specifically, decolorization was somewhat more effective at pH 4.5 for RBBR and TH2GP, and at pH 4 for FG. When RBBR was decolorized, MiP activity led to an increase in absorbance between 325-365nm and 410-500nm, whereas absorbance decreased between 370-410nm and 510-700nm. The decolorization of TH2GP by MiP activity resulted in an increase in absorbance within the 300-425 nm and 500-750 nm ranges. FG decolorization by MiP activity resulted in increased absorbance in the ranges of 275-300 nm, 320-410 nm, and 440-580 nm, whereas absorbance decreased in the range of 590-680 nm (Figures 4.4, 4.5, and 4.6).

The fungi's ligninolytic enzymes, laccase (Lac), manganese peroxidase (MnP), and manganese-independent peroxidase (MiP), are capable of decolorizing textile dyes such as Remazol Brilliant Blue R, RO3R, Turquoise Blue GP, Fast Green, ROH2, and RMHB. T. versicolor's laccase activity can decolorize RBBR across a wide pH range, making it valuable for treating effluent with textile dyes. Laccase degrades TH2GP and RBBR textile dyes, leading to their decolorization as shown by spectrum changes during the process. Laccase, an oxidase enzyme containing copper, is produced by several sources including bacteria, fungus, and plants. Laccase facilitates the breakdown of contaminants such synthetic colours, aromatic chemicals, phenolic compounds, and aliphatic amines using four copper atoms located at three distinct locations (Type I, Type II, Type III).[39]. The three copper sites' significance in catalysing pollutants and activating the laccase enzyme is significant because to their specific habitat and spectroscopic characteristics[40].

MnP and MiP efficiently and quickly remove colour from dyes over a wide pH range, highlighting the significance of these enzymatic activities in treating effluents. MnP and MiP destroy RBBR and TH2GP in many phases, resulting in the breakdown of the dye and its byproducts. The decolorization of FG by MnP, but not MiP, is significant, as indicated by the spectrum alterations associated with FG decolorization. MnP activity is more effective in decolorizing textile colours compared to MiP and Lac activities.

White rot basidiomycetes have advantages in treating industrial effluents containing dyes because their ligninolytic enzymes can both decolorize and degrade various types of resistant organic pollutants, such as PAH, PCB, nitroaromatics, chloroaromatics, organophosphates, anthranilic diamide, and neonicotinoids.It is therefore crucial to showcase the decolorization of textile dyes in industrial effluents, which are complicated mixtures of many contaminants that change in composition over time.

Fig 4.1 Spectral changes observed during decolorization of Remazol BrilliantBlue Rat pH (**a**) 3, (**b**) 3.5, (**c**) 4,(**d**) 4.5, (**e**) 5 and (**f**) 5.5 by MnP activityproducedbythestaticcultureof*Trametesversicolor*grownonGVTmediumfor7daysat 28ºC.

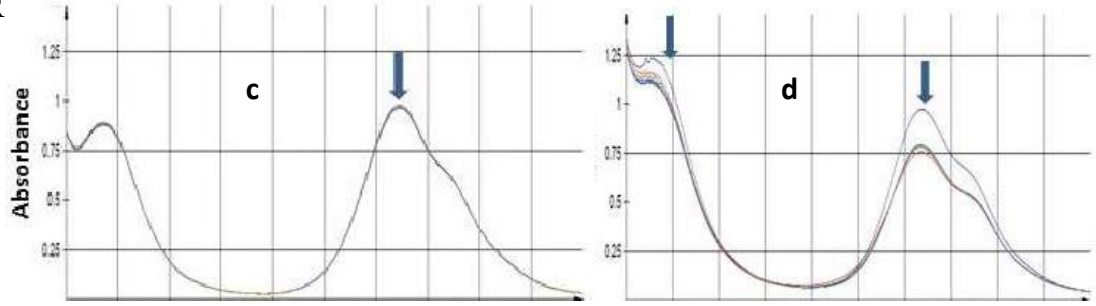


Fig 4.2. Spectral changes observed during decolorization of TurquoiseBlue GP atpH (**a**) 3, (**b**) 3.5, (**c**) 4,(**d**) 4.5, (**e**) 5 and (**f**) 5.5 by MnP activity produced by thestaticcultureof *Trametes versicolor*grown onGVT mediumfor7 daysat 28ºC

Fig 4.3. Spectralchangesobservedduringdecolorizationof FastGreenatpH(**a**)3,(b) 3.5,(**c**)4,(**d**)4.5,(**e**)5and(**f**)5.5byMnPactivityproducedby thestaticcultureof*Trametes versicolor*grownon GVTmediumfor7 daysat 28ºC.

Fig.4.4. Spectral changes observed during decolorization of Remazol Brilliant Blue Rat pH (**a**) 3, (**b**) 3.5, (**c**) 4,(**d**) 4.5, (**e**) 5 and (**f**) 5.5 by MiP activity produced by thestaticcultureof *Trametes versicolor*grownon GVTmedium for7days at28ºC

Fig.4.5. Spectralchangesobservedduringdecolorizationof TurquoiseBlueGPatpH(a) 3,(**b**)3.5,(**c**)4,(**d**)4.5,(**e**)5and(**f**)5.5byMiPactivityproducedbythestaticcultureof*Trametesversico lor*grown on GVTmediumfor7 days at28ºC.

Fig.4.6. SpectralchangesobservedduringdecolorizationofFastGreenatpH(**a**)3,(b) 3.5,(**c**)4,(**d**)4.5,(**e**)5and(**f**)5.5byMiPactivityproducedbythestaticcultureof*Trametes versicolor*grownonGVTmediumfor7 daysat 28ºC

Conclusion

Whiterotbasidiomycetestrains*Trametesversicolor,Lenzitesbetulina*andUK2 isolate were decolorized the textile dyes during growth on MEA 3 to 10days of incubation. While White rot basidiomycetes strain *Trametes versicolor* andUK4 isolate were effectively producing ligninolytic enzyme during growth onGVT 3 to 9 days of incubation. Their enzymes *viz.* manganese peroxidase, manganeseindependent peroxidase and laccase decolorize and simultaneously alter the structureand chemical composition of the dyes Remazol Brilliant Blue R, Turquoise Blue GPand Fast Green.

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