

# Biological removal of synthetic textile dye Reactive Red M5B by isolated white rot fungal culture AGYP-1 under optimized culture conditions

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## Abstract

The contamination of soil and water by dye containing effluents is the major and most important environmental problem. The removal of 10 different synthetic textile dyes using white rot fungal isolate AGYP-1 was investigated. The screening of decolorization using solid and liquid media demonstrated an effective removal of Reactive Red M5B by the isolate. Laccase and MnP were found to be major enzymes involved in the decolorization. The dye decolorization efficiency of the isolate was further improved by optimizing various physico-chemical parameters. The isolate was capable to decolorize 100 mg l<sup>-1</sup> dye at pH 5.0 and 30°C under shaking condition. The supplementation of maltose (20 g l<sup>-1</sup>) and gelatine (2.5 g l<sup>-1</sup>) improved the decolorization rate by 1.6 times along with 10.28 and 18.66 times higher production of laccase and MnP. A significant decolorization of 500 mg l<sup>-1</sup> of the dye was achieved by the isolate AGYP-1. The degradation of Reactive Red M5B was confirmed by Uv-visible spectrophotometric and HPTLC analysis. This suggests the potential application of the isolate AGYP-1 for the treatment of dye containing industrial effluents.

**Keywords:** Decolorization, Reactive Red M5B, Laccase, MnP, optimization

## Highlights:

- Isolation and Screening of different white rot fungi was performed using solid and liquid culture media. An isolate AGYP-1 decolorized 10 different textile dyes in liquid medium.
- The effect of various physico-chemical parameters was studied for the decolorization of model dye Reactive Red M5B by isolate AGYP-1 using single factorial design.
- The isolate AGYP-1 was able to decolorize 500 mg l<sup>-1</sup> Reactive Red M5B with an apparent maximum decolorization rate (RD<sub>max</sub>) and Michaelis constant (K<sub>m</sub>) 128.20 mg dye day<sup>-1</sup> and 166.66 mg l<sup>-1</sup> respectively.
- UV-Visible spectroscopy and HPTLC analysis revealed the formation of new metabolites or metabolic intermediates different from the parent dye Reactive Red M5B.

The global population expansion and an industrial based life style have created an anthropogenic impact on the biosphere. A variety of toxic compounds released from different industries adversely affect water source, soil fertility, aquatic organisms and ecosystem integrity (Khalid *et al.*, 2008). The textile and paper industries are considered as the largest water consumers and discharge

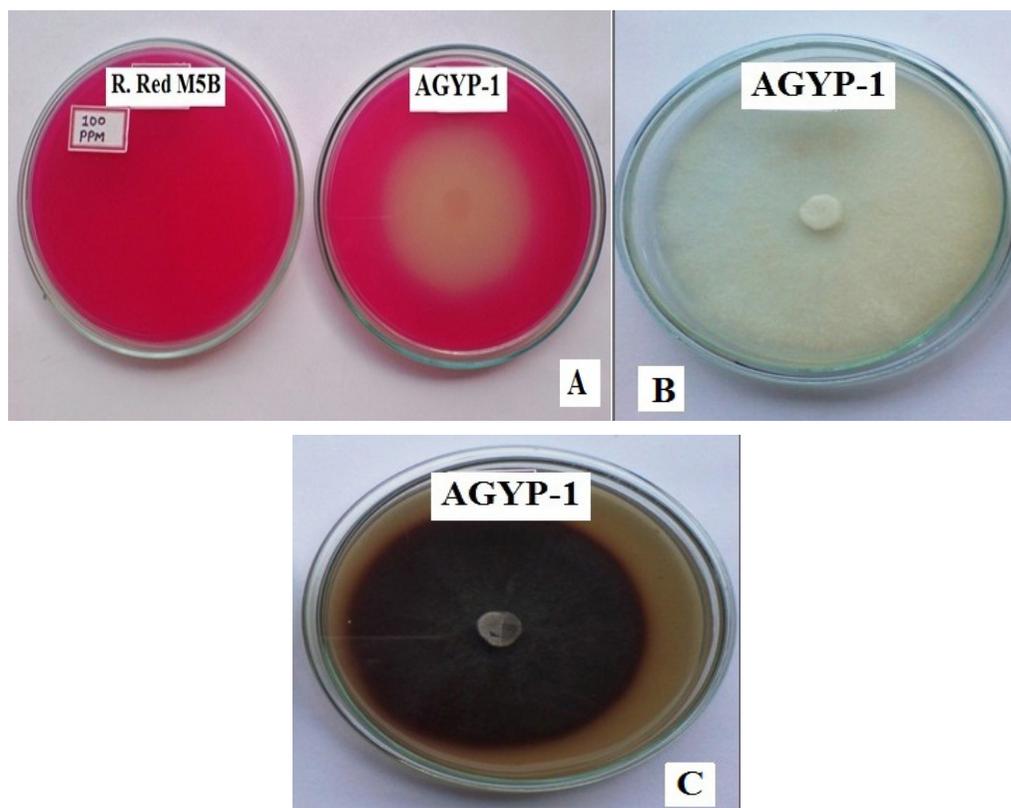
an enormous amount of dye containing wastewaters (Balaji *et al.*, 2012). Azo dyes with one or more (-N=N-) and sulfonic (-SO<sub>3</sub><sup>-</sup>) groups are the most important and widely used synthetic dyes having broad range of applications (Patel *et al.*, 2012). The discharge of poorly treated dye containing effluent alters the pH, increases the biochemical oxygen demand (BOD) and chemical

oxygen demand (COD) of the aquatic system as well as reduces sunlight penetration leading to negative impact on the water quality (Carias *et al.*, 2007). Many dyes and their transformation products are toxic, mutagenic and carcinogenic that can endanger human life (Bae and Freeman, 2007). Therefore, it is a great need to develop an economic and effective way for the treatment of textile dyeing wastewaters.

Different physico-chemical methods are available for the remediation of dye wastewaters, but, their application is limited due to some technical and economical drawbacks (Ali, 2010). Bioremediation therefore offers a cheaper and environmentally friendlier alternative (Pathak *et al.* 2011). Many microorganisms have proven significant tools for the development of biological processes for the treatment of textile effluents (Yang *et al.*, 2009; Chen *et al.*, 2011; Gahlout *et al.*, 2013; Shah, 2014). The application of lignin-degrading white rot fungi (WRF) has attracted increasing scientific attention, as they are able to

degrade a wide range of recalcitrant organic compounds. They produce an array of lignin modifying enzymes (LME) such as Manganese peroxidase (MnP), lignin peroxidase (LiP) and laccases, which degrade not only lignin in their natural lignocellulosic substrates, but also various xenobiotic compounds including dyes (Kalpana *et al.*, 2012; Asgher *et al.*, 2013; Patel and Gupte, 2014). Various studies of ligninolytic enzymes mediated dye decolorization have been carried out using liquid culture conditions using homogenized mycelium or pellets of white rot fungi (Eichlerova *et al.*, 2005; Erkurt *et al.*, 2007, Singh *et al.* 2012).

The present work focuses on isolation and screening of synthetic textile dyes decolorizing white rot fungal culture AGYP-1. Various physico-chemical parameters like pH, temperature, dye concentration, carbon and nitrogen source were optimized for the enhanced decolorization of Reactive Red M5B. Furthermore, the relationship between dye decolorization and concomitant ligninolytic



**Figure 1: Decolorization of Reactive Red M5B by isolate AGYP-1 on solid medium (A), growth of AGYP-1 on malt extract agar (B), plate assay for ligninolytic enzyme on SDA plate containing 0.01% orth-dianisidine**



enzymes production by isolate AGYP-1 was evaluated. Decolorization and degradation of Reactive Red M5B was studied by UV-Visible spectrophotometric and HPTLC analysis.

## 2. Materials and methods

### 2.1 Dyes and chemicals

Commercially available synthetic textile dyes, Reactive Red M5B (C.I. Reactive Red 2), Acid Maroon V (C.I. Acid Red 119), Reactive Red BS (C.I. Reactive Red 111), Acid Red F2R (C.I. Acid Red 151), Acid Red 3BN (C.I. Acid Red 131), Reactive Blue 3R (C.I. Reactive Blue 28), Reactive Violet 5R (C.I. Reactive Violet 5), Acid Red BB (C.I. Acid Red 128), Acid Green GLW (C.I. Acid Green 27) and Reactive Red HE8B (C.I. Reactive Red 152) used for present study were procured from CAB Chemicals, Ankleshwar, Gujarat, India. ABTS (2, 2-Azino-bis (3-ethylbenzthiozoline-6-sulphonic acid) was purchased from Sigma (Sigma St. Louis, MO, USA), 2, 6-Dimethoxyphenol (DMP) and ortho-dianisidine were purchased from Lancaster (Lancs, UK) and CDH (Mumbai, India) respectively, Malt extract agar (MEA) was procured from Hi-Media Labs (Mumbai, India). All other chemicals used were of the highest purity and of analytical grade.

### 2.2 Nutrient media

Mineral Medium (MM) containing g l<sup>-1</sup>: glucose, 5.0, KH<sub>2</sub>PO<sub>4</sub>, 1.0, MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5, KCl, 0.5, yeast extract, 0.5, and pH 5.0 ± 0.5 was used for isolation and decolorization experiments. The fungal cultures were routinely transferred and maintained on malt extract agar (MEA) containing g l<sup>-1</sup>: malt extract, 20.0, agar-agar, 30.0, and pH 5.0 ± 0.5. The screening of ligninolytic enzymes was performed on Sabaroud's dextrose agar (SDA) containing g l<sup>-1</sup>: peptone 10.0, glucose 40.0, agar-agar 30.0, and pH 5.6 ± 0.5.

### 2.3 Isolation of dye decolorizing fungal cultures

The potential dye decolorizing fungal strains were isolated from contaminated soil samples obtained from different sites of common effluent treatment plant (CETP) - Nandesari - Vadodara, Gujarat, India. 250 ml Erlenmeyer flasks containing 100 ml MM and 100 mg l<sup>-1</sup> mixture of 3

dyes (Reactive Red M5B, Acid Green GLW and Reactive Violet 5R) were inoculated with 10 ml of soil suspension (10 % w/v) and incubated under shaking condition (120 rpm) at 30°C for 2 weeks. Repeated transfers in fresh dye containing media were performed till stable dye decolorizing fungal cultures were obtained. From the decolorized flasks, fungal mycelia were transferred to MEA for further purification.

### 2.4 Dye decolorization and enzyme screening on solid media

Plate assay was performed to detect the decolorization activity of fungal isolates. The MM agar plates containing individual dye (100 mg l<sup>-1</sup>) were inoculated with a mycelial disc (8 mm diameter) from previously grown fungal cultures and incubated at 30°C for 10 days. The plates were observed for the clearance of dye surrounding fungal growth. The production of ligninolytic enzymes was checked on SDA plate containing ortho-dianisidine (0.01% w/v). Uninoculated MM agar and SDA plates were served as control.

### 2.5 Decolorization studies in liquid medium

The dye decolorization experiments were carried out in 250 ml Erlenmeyer flasks containing 100 ml MM. The flasks were inoculated with 8 circular discs (8 mm diameter) cut from actively growing fungal cultures on MEA agar plate and incubated under shaking condition (120 rpm) at 30°C. Upon fifth day of incubation, an individual dye (100 mg l<sup>-1</sup>) was added and the flasks were further incubated at the same conditions for decolorization. In another set, the flasks were kept in static condition to check its effect on decolorization. Aliquots were withdrawn periodically from different flasks and were analyzed for decolorization and enzyme production.

### 2.6 Determination of enzyme activities

#### 2.6.1 Laccase assay

Laccase (E.C. 1.10.3.2) activity was determined by monitoring change in absorbance at 420 nm related to the rate of oxidation of 1 mM ABTS ( $\epsilon = 36,000 \text{ M}^{-1} \text{ cm}^{-1}$ ) following the method described by Niku-Paavola *et al.* (1990). The assay system contained 1 mM ABTS, 100 mM sodium acetate buffer (pH 5.0) and suitably diluted

sample. One unit of enzyme activity (U) was defined as the amount of enzyme required to oxidize 1 $\mu$ M ABTS per min.

### 2.6.2 Manganese Peroxidase (MnP) assay

Manganese peroxidase activity (E.C. 1.11.1.13) was measured by oxidation of DMP ( $\epsilon = 27,500 \text{ M}^{-1} \text{ cm}^{-1}$ ) in the presence of  $\text{H}_2\text{O}_2$  and  $\text{MnSO}_4$ . The reaction mixture contained 1 mM DMP, 0.1 mM  $\text{H}_2\text{O}_2$ , 1 mM  $\text{MnSO}_4$ , 100 mM Sodium tartarate buffer (pH 4.5) and suitably diluted sample. Increase in absorbance was measured at 469 nm and one unit of enzyme activity (U) was defined as the amount of enzyme catalyzing the oxidation of 1  $\mu$ M of DMP per min (Martinez *et al.* 1996). Presence of MnP activity was corrected for manganese-independent peroxidase (MnIP) activity by subtracting the activity obtained at pH 3.25 in the absence of  $\text{MnSO}_4$  at 469 nm.

### 2.7 Effect of Physico-chemical factors on decolorization

In order to study the effect of various physico-chemical parameters, the decolorization of Reactive Red M5B was assayed using different pH (3.0-8.0), temperature (15-50°C) and dye concentration (100-500 mg l<sup>-1</sup>). In the next step, the effect of various carbon and nitrogen sources was analyzed for the effective decolorization of the dye.

### 2.8 Analytical measurements

Two ml aliquot of decolorized medium was centrifuged at 10,000 rpm for 10 min and the clear supernatant was subjected for the measurement of absorbance at the maximum absorbance wavelength ( $\lambda_{\text{max}}$ ) of the dyes using UV-visible spectrophotometer (SHIMADZU UV-1800, Japan). The residual dye concentration in the sample was determined from the absorbance values using the calibration curve for absorbance versus dye concentration obtained by plotting the corresponding maximum absorbance in the UV-visible spectra at different concentrations of dye.

The percentage decolorization (% D) was calculated using the following formula:

$$\% D = \frac{C_o - C_f}{C_o} \times 100$$

Where,  $C_o$  and  $C_f$  are the initial and final concentrations respectively.

Fungal biomass was determined by weighing the dry mycelia after decolorization. The mycelial growth was squeezed to remove the medium, washed with distilled water, and dried completely at 60°C till constant dry weight. Biomass was calculated by subtracting the weight of initial fungal growth from the final weight.

HPTLC analysis was performed using precoated silica gel 60 F254 plate (Merck, Germany). A 5 $\mu$ l of the control and decolorized samples were spotted on TLC plate using micro syringe (HPTLC, Camag, Linomat 5). The solvent system used was isopropanol : ethyl methyl ketone : liquor ammonia in the ratio of 5:3:2. Developed plates were dried in air and the dye chromatogram was observed by exposing it to ultraviolet light (254 nm) and in visible light using Camag TLC scanner 3.

### 2.9 Data analysis

All the experiments and the analysis were carried out in triplicates and the data presented is the mean value of the triplicates. The standard deviation was calculated using the mean values and remained within the range of  $\pm 10\%$ .

## 3. Results and Discussion

### 3.1 Isolation and screening of dye decolorizing fungal cultures

The fungal cultures with an efficient decolorization potential of textile azo dyes were isolated from dye contaminated soil samples. It is expected that sites near these industries harbor several microorganisms which are capable to co-exist with higher toxic levels of pollution. Total four fungal cultures were isolated by the enrichment culture technique and were designated as AGYP-1, AGYP-2, AGYP-3 and AGYP-4 respectively. The screening in agar plates is considered as simple and efficient method to assess the dye decolorization potential of various microorganisms. All the four isolates were

able to grow and decolorize dyes in MM agar medium. However, the isolate AGYP-1 showed rapid and effective decolorization of Reactive Red M5B, Acid Green GLW, Reactive Violet 5R and Reactive Red HE8B on solid medium. The decolorization of Reactive Red M5B (100 mg l<sup>-1</sup>) started on 3<sup>rd</sup> day (Fig. 1A) and progressed rapidly along with the radial growth of AGYP-1 resulting into complete removal within 10 days. Furthermore, an adsorption of dye was not evident on fungal mycelia indicating decolorization of the dye was truly a microbial process. Other fungal isolates showed lesser degree of decolorization on the solid media. The initial identification by microscopy demonstrated that the mycelia of AGYP-1 were white and septate with abundant clamp connection.

The isolate grew well on malt extract agar plate covering the entire petri plate within 10 days of incubation. The aerial mycelia were found to be dense and white in color (Fig. 1B). Based on above observations, the isolate AGYP-1 was identified as white rot fungus belonging to class basidiomycete.

The use of chromogenic substrates like ortho-dianisidine, ABTS, guaiacol etc. is considered as important aspect for the detection of ligninolytic enzymes. In the present study, all isolates were screened on the SDA plates amended with 0.01% ortho-dianisidine. The oxidation of substrate was observed as brown colored zone surrounding the mycelia of the culture indicating Bevandamm's reaction. Out of four isolates, AGYP-1 and AGYP-4 showed

**Table 1: Decolorization of different textile dyes by fungal isolate AGYP-1. Concentration of each dye was 100 mg l<sup>-1</sup>**

Dye	$\lambda_{max}$	Molecular weight	CAS No.	% Decolorization
Acid Maroon V	523	628.27	12220-20-1	51.22±3.6
Reactive Red BS	517	NA	88232-20-6	45.97±3.5
Acid Red F2R	510	454.43	6406-56-0	25.28±1.8
Reactive red M5B	538	615.34	12226-03-8	91.36±4.8
Acid Red 3BN	551	NA	12234-99-0	10.69±2.2
Reactive Blue 3R	577	NA	12225-45-5	88.58±5.9
Reactive Violet 5R	560	735.59	12226-38-9	86.54±4.8
Acid Red BB	532	862.28	6548-30-7	50.78±4.1
Acid Green GLW	617	706.73	6408-57-7	65.0±5.3
Reactive Red HE8B	548	1752.11	71870-80-5	85.49±6.1

NA-Not available

**Table 2: Effect of co-substrate on decolorization of Reactive Red M5B and ligninolytic enzymes production by fungal isolate AGYP-1**

Carbon source	% Decolorization	Laccase activity (U ml <sup>-1</sup> )	MnP activity (U ml <sup>-1</sup> )
Glucose	91.35±3.5	235.8±9.5	88.4±5.5
Sucrose	74.32±5.4	156.9±13.3	34.36±2.6
Xylose	89.55±2.8	219.4±6.9	62.85±4.5
Maltose	96.11±2.7	572.34±24.6	171±7.2
Lactose	78.88±3.4	145.26±12.9	36.25±8.5
Fructose	87.44±1.9	89.4±7.6	38.07±2.5
Starch	81.07±6.2	179.4±9.8	70.01±5.3
Sodium acetate	64.32±3.3	21.01±3.3	10.16±4.8

Bevandamm's reaction for positive ligninolytic enzyme activity (Fig. 1C). However, the intensity of the color produced by AGYP-1 was much higher as compared to AGYP-4, suggesting greater production of ligninolytic enzymes.

### 3.2 Decolorization of Reactive Red M5B by fungal isolates in liquid medium

Since long, the use of fungi has attracted increasing scientific attention, as they are able to degrade a wide range of recalcitrant organic compounds due to production of an array of highly efficient nonspecific ligninolytic enzymes (Christian *et al.*, 2005; Nozaki *et al.*, 2008). The color removal ability of the fungal isolates was checked in MM amended with 100 mg l<sup>-1</sup> Reactive Red M5B. As depicted in Figure 2, maximum decolorization of the dye (91.65±3.4%) was obtained with AGYP-1 on 12<sup>th</sup> day of incubation. The color removal performances of AGYP-3 and AGYP-4 were 71.16±4.3% and 74.32±2.2% respectively. The lowest decolorization of the dye (35.66±1.3%) was achieved by AGYP-2 indicating poor color removal ability of the isolate. The superior decolorization activity of AGYP-1 may be correlated with maximum biomass production along with concomitant production of ligninolytic enzymes. An effectiveness of white rot fungi for the decolorization of different synthetic dyes has been well documented (Erkurt *et al.*, 2007; Yang *et al.*, 2009).

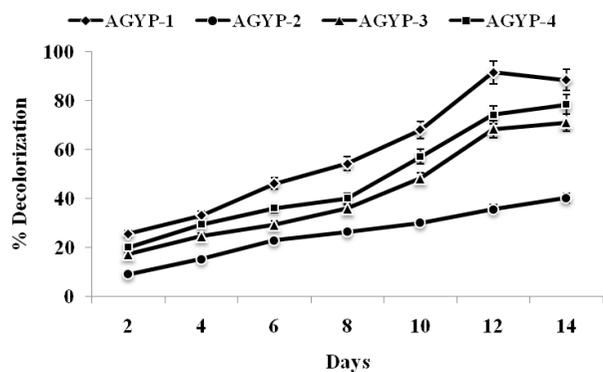


Figure 2: Time course study of decolorization of Reactive Red M5B (100 mg l<sup>-1</sup>) by isolated fungal cultures in liquid medium

### 3.3 Screening of decolorization of different dyes by fungal isolate AGYP-1

Industrial effluent contains varieties of dyes with different structural and functional properties. Therefore, it is important to assess the capability of microorganisms to decolorize various dyes. A varying degree of decolorization of different dyes was obtained by the isolate AGYP-1 (Table 1). The isolate displayed maximum decolorization of Reactive Red M5B (91.36±4.8) followed by Reactive Blue 3R (88.58±5.9%). The decolorization of Acid Green GLW, Reactive Red HE8B and Reactive Violet 5R was in the range of 65.0±5.3 to 86.54±4.8%. The dyes Reactive Red BS, Acid Red BB and Acid Maroon V were decolorized moderately with decolorization between 45.97±3.5 and 51.22±3.6%, whereas, the decolorization of Acid Red 3BN was 10.69±2.2%. The observed variation in decolorization may be due to structural diversity of dyes. It is observed that dyes with simple structures and low molecular weight are decolorized faster. On the other hand, highly substituted, high molecular weight dyes are prone to decolorize slowly. Moreover, the number of azo bonds and substitution of electron withdrawing groups (-SO<sub>3</sub>H, -SO<sub>2</sub>NH<sub>2</sub>) in dye molecule also confer the extent of color removal (Sani and Banerjee, 1999). The results thus illustrate the efficiency of the isolate AGYP-1 for decolorization of different dyes and hence, its potential application for the treatment of multiple dyes containing industrial effluent.

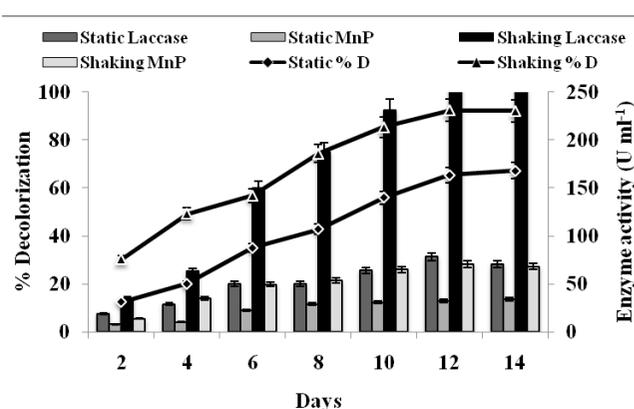


Figure 3: Decolorization of Reactive Red M5B by isolate AGYP-1 under static and shaking conditions

**Table 3: Effect of nitrogen source on decolorization of Reactive Red M5B and ligninolytic enzymes production by fungal isolate AGYP-1**

Nitrogen source	% Decolorization	Laccase activity (U ml <sup>-1</sup> )	MnP activity (U ml <sup>-1</sup> )
Yeast extract	93.73±4.4	935.3±12.3	468.88±12.2
Peptone	87.34±3.7	837.8±23.7	377.93±8.9
Gelatine	96.74±1.7	1537.1±14.6	791.64±12.5
Urea	92.41±3.5	1170.4±12.8	518.47±11.2
Ammonium sulfate	74.33±4.1	143.46±10.9	45.40±7.7
Ammonium nitrate	60.34±2.7	111.52±6.3	54.37±6.3

### 3.4 Effect of Physico-chemical parameters on decolorization of Reactive Red M5B

#### 3.4.1 Effect of static and shaking conditions

The isolate AGYP-1 exhibited maximum decolorization of Reactive Red M5B (92.33±2.4%) under shaking condition, whereas, 65.34±4.2% color removal was achieved in static condition on 12<sup>th</sup> day incubation (Fig. 3). Moreover, AGYP-1 displayed higher activities of laccase (253.8±14.5 U ml<sup>-1</sup>) and MnP (70.36±8.6 U ml<sup>-1</sup>) under shaking condition. The estimated biomass of the isolate was 0.257±0.04 and 0.164±0.03 g under shaking and static conditions respectively. Conversely, the isolate showed very low level of laccase and MnP activities (78.25±4.2 and 32.4±2.2 U ml<sup>-1</sup> respectively) in static condition. Moreover, it was found that laccase activity in all the experimental flasks was much higher as compared to MnP activity, suggesting its possible role in decolorization process. Above results demonstrate that shaking condition favored the growth of the isolate and resulted into 1.41 times higher decolorization of the dye. This may be attributed to better oxygen transfer and nutrient distribution as compared to static condition. Miranda *et al.* (2012) reported an effective decolorization of textile dyes under shaking condition using *Phanerochaete chrysosporum* CCT1999, *Lentinula edodes* CCT4519 and *Curvularia lunata* UFPEDA885. However, Kirby *et al.* (2002) observed better dye decolorization by *Phlebia tremellosa* under static condition. Many reports have described a relationship between ligninolytic enzyme activities and decolorization of different dyes by white rot fungi (Erkurt *et al.* 2007; Moreira-Neto *et al.* 2013).

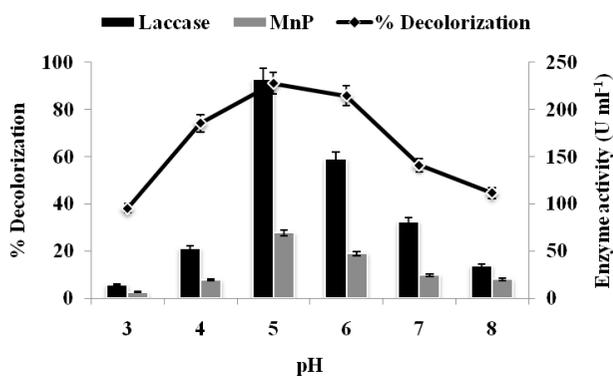
#### 3.4.2 Effect of pH

The hydrogen ion concentration greatly affects various biological activities of organisms. The decolorization of Reactive Red M5B was investigated in the range of pH 3.0 to 8.0. As shown in figure 4, that the decolorization efficiency of the isolate AGYP-1 was found to be increase with an increase in pH, reaching maximum at pH 5.0 (91.17±3.2%). Further increase in pH resulted into decrease in decolorization efficiency. However, at pH 4.0 and 6.0, the color removal was 74.18±4.4% and 85.86±3.8% respectively. Moreover, the maximum fungal biomass (0.253±0.03 g) along with highest activities of laccase (232.38±10.9 U ml<sup>-1</sup>) and MnP (69.27±3.6 U ml<sup>-1</sup>) were obtained at pH 5.0, suggesting that acidic pH favoured the decolorization of the dye. Vaithanomsat *et al.* (2010) demonstrated maximum decolorization of Reactive Black 5 along with highest laccase activity at pH 5.0 using white-rot fungus *Dratronia* sp. KAPI0039. Based on the study carried out by Young and Yu (1997), the azo-based dye was more effectively degraded by white-rot fungi under an acidic condition. Many other reports suggest that the fungi can grow best at low pH normally ranging from 4.0 to 6.0. Furthermore, fungal ligninolytic enzymes show maximum activity at low pH; hence, an efficient decolorization is observed at low pH (Saranthima *et al.*, 2009; Patel and Gupte 2014).

#### 3.4.3 Effect of temperature

Temperature is an important environmental factor; change in temperature affects the biodegradation activities of microorganisms. The effect of temperature on decolorization of Reactive Red M5B by isolate AGYP-1

is depicted in figure 5. The linear relationship between incubation temperature and color removal was observed in the temperature range of 15 to 30°C. The isolate exhibited maximum decolorization of the dye (91.34±4.5%) along with 237.17±11.3 U ml<sup>-1</sup> and 68.13±5.4 U ml<sup>-1</sup> activities of laccase and MnP respectively at 30°C. The dye was decolorized 84.32±3.9% at 37°C, and the color removal activity of the isolate was severely reduced at 50°C. The fungal growth was highest (0.258±0.04 g) at 30°C indicating an optimum temperature for the growth, enzyme production and decolorization activity of the isolate AGYP-1. Different fungi grow at different optimum temperatures, with most of them growing at 25-35°C (Fu and Vararaghavan 2001). Similar results were reported by Gahlout *et al.* (2013), stating that the decolorization of Reactive Violet 1 was maximum at 30°C and lesser decolorization was observed at 45°C using *Ganoderma cupreum* AG-1. Beyond the optimum temperature, the slower growth and deactivation of enzymes responsible for degradation are main reasons for decreased degradation activities of the microorganisms (Ali, 2010).

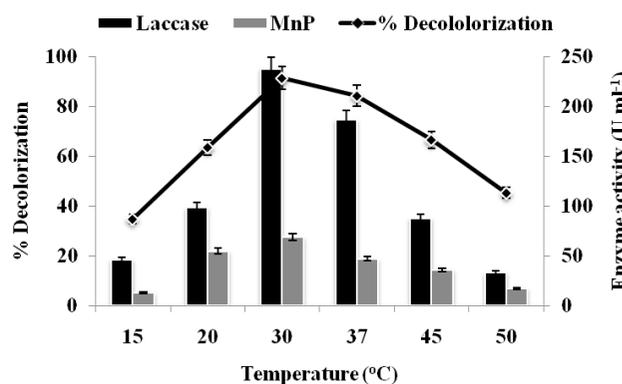


**Figure 4: Effect of pH on decolorization of Reactive Red M5B by isolate AGYP-1**

#### 3.4.4 Effect of initial dye concentration

The decolorization capability of the isolate AGYP-1 was studied by varying initial dye concentration (100-500 mg l<sup>-1</sup>) under shaking condition. Maximum decolorization of Reactive Red M5B (92.18±2.2%) was observed at 100 mg l<sup>-1</sup> concentration (Fig. 6). A marginal reduction in decolorization efficiency of the isolate was observed at 200 mg l<sup>-1</sup> concentration (86.45±4.5%). The decolorization of the dye was decreased with increase in initial dye

concentration thereafter. However, the isolate AGYP-1 was quite stable and exhibited 74.34% decolorization at 500 mg l<sup>-1</sup> Reactive Red M5B concentration. Moreover, the decolorization rate increased from 7.68 to 30.97 mg dye day<sup>-1</sup> with an increase in initial dye concentration from 100 to 500 mg l<sup>-1</sup> explaining a typical Monod-type profile. Under experimental conditions, the correlation between decolorization rate ( $R_d$ ) and initial dye concentration ([RRM5B]) was obtained by Michaelis-Menten double reciprocal model ( $R_{Dmax} [RRM5B] / (Km + [RRM5B])$ ). The values of an apparent maximum decolorization rate ( $R_{Dmax}$ ) and Michaelis constant ( $Km$ ) were 128.20 mg dye day<sup>-1</sup> and 166.66 mg l<sup>-1</sup> respectively. Above results thus demonstrate a good capability of the isolate AGYP-1 for the decolorization of higher concentration of the dye. The effect of Malachite Green concentration on decolorization capacity of white rot fungus *Dichomitus squalens* has been studied; wherein the dye at 50 mg l<sup>-1</sup> concentration inhibited the fungal growth (Eichlerova *et al.*, 2006).



**Figure 5: Effect of incubation temperature on decolorization of Reactive Red M5B by isolate AGYP-1**

#### 3.4.5 Effect of co-substrate

In general, dyes are deficient in carbon content and their degradation without any extra carbon and nitrogen source is found to be very difficult (Stolz, 2001). In order to evaluate the effect of co-substrate, the decolorization of Reactive Red M5B by isolate AGYP-1 was monitored in the presence of various carbon sources. Variable results of decolorization of the dye were obtained with different co-substrates tested (Table 2). The decolorization performance of the isolate AGYP-1 was better in the presence of maltose (96.11±2.7%) with maximum laccase



( $572.34 \pm 24.6$  U ml<sup>-1</sup>) and MnP ( $171 \pm 7.2$  U ml<sup>-1</sup>) activities followed by glucose ( $91.35 \pm 3.5\%$ ). Sodium acetate was found to be poor carbon sources allowing  $64.32 \pm 3.3\%$  decolorization, whereas sucrose, lactose, starch, fructose and xylose supported the decolorization in the range between  $74.32 \pm 5.4$  and  $89.55 \pm 2.8\%$  by AGYP-1. Glucose is known as the most readily usable carbon source for most of the fungi. However, it is a costly carbon source and is generally not used in wastewater treatment (Erdal and Taskin, 2010). With this context, the preference of maltose as a co-substrate by isolate AGYP-1 offers an additional advantage for the removal of dye containing wastewaters. Similar results were reported by Asgher *et al* (2013), in which they obtained 91% decolorization of Solar Brilliant Red 80 by *Schizophyllum commune* IBL-06 in the presence of maltose.

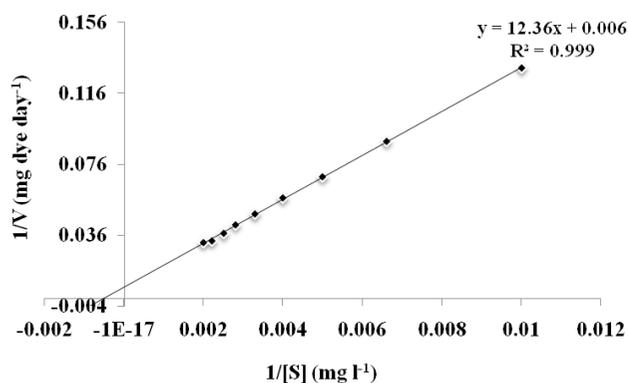


Figure 6: Double reciprocal plot of Reactive Red M5B biodecolorization by fungal isolate AGYP-1

The concentration of maltose in the medium was varied in the range of 1.0 to 50 g l<sup>-1</sup> (w/v) for maximal removal of Reactive red M5B by isolate AGYP-1. The decolorization efficiency of the isolate AGYP-1 increased with an increase in maltose concentration from 1.0 to 20 g l<sup>-1</sup>; with maximum color removal ( $96.33 \pm 1.2\%$ ) and laccase ( $963.38 \pm 28.34$  U ml<sup>-1</sup>) and MnP ( $441.63 \pm 20.13$  U ml<sup>-1</sup>) activities at 20 g l<sup>-1</sup> (Fig. 7). The obtained fungal growth was  $0.277 \pm 0.03$  g at 20 g l<sup>-1</sup> maltose concentration. Any further increase in maltose concentration did not influence an overall decolorization potential suggesting 20 g l<sup>-1</sup> maltose concentration as an optimum for growth and decolorization of the dye. The decolorization of the dye was  $73.48 \pm 2.2\%$  at 50 g l<sup>-1</sup> concentration of maltose. The reduced decolorization along with lower ligninolytic

enzyme activities may be owing to highly acidic condition or catabolic repression at higher maltose concentration.

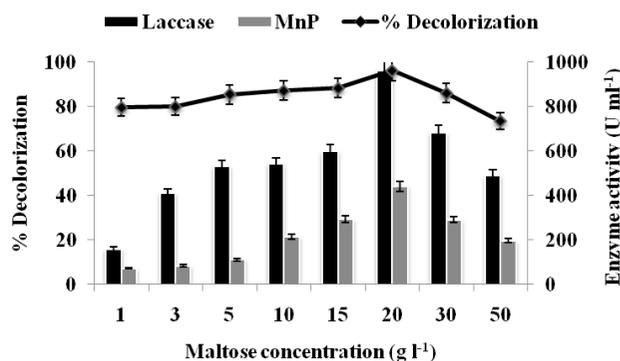


Figure 7: Effect of maltose concentration on decolorization of Reactive Red M5B by fungal isolate AGYP-1

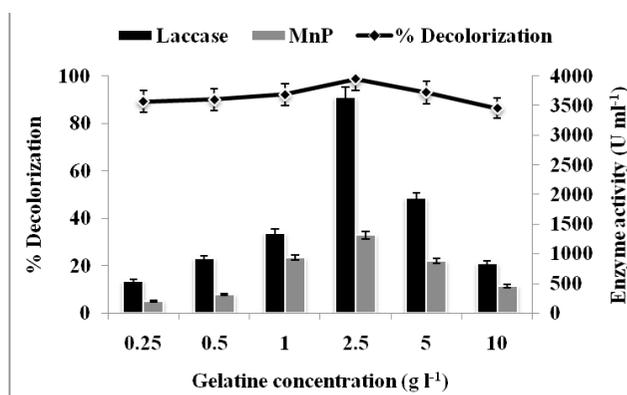
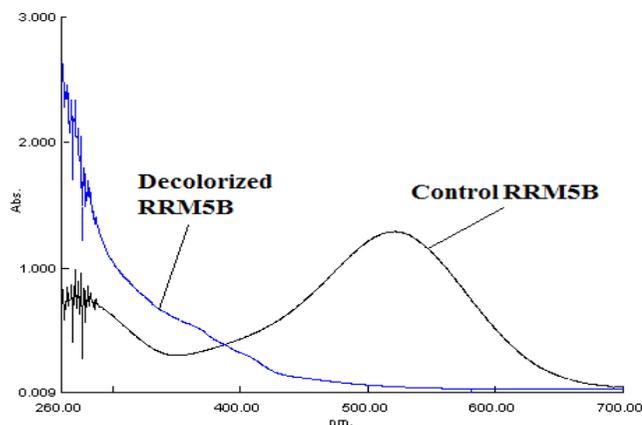


Figure 8: Effect of gelatine concentration on decolorization of Reactive Red M5B by fungal isolate AGYP-1

#### 3.4.6 Effect of nitrogen source

The decolorization of Reactive Red M5B was performed in the presence of different organic and inorganic nitrogen sources; maximum decolorization of the dye was obtained with gelatine ( $96.74 \pm 1.7\%$ ) after 10 days (Table 3). The growth of the isolate AGYP-1 was  $0.297 \pm 0.02$  g along with laccase and MnP activities of  $1537.1 \pm 14.6$  and  $791.64 \pm 12.5$  U ml<sup>-1</sup> respectively. Following gelatine, yeast extract and urea exhibited  $93.73 \pm 4.4$  and  $92.41 \pm 3.5\%$  decolorization of Reactive Red M5B. The color removal efficiency of the isolate was much lower in the presence of inorganic nitrogen sources. Many researchers have reported enhanced decolorization activity of fungal cultures in the presence

of various organic nitrogen sources (Saratale *et al.* 2009; Ponraj *et al.* 2011). In contrast to this, various inorganic nitrogen sources like ammonium nitrate and ammonium sulfate have been reported to enhance decolorization of different dyes by white rot fungi (Asgher *et al.*, 2009; and Singh *et al.* 2012).



**Figure 9: UV-Visible spectral analysis of Reactive Red M5B during decolorization by fungal isolate AGYP-1**

The decolorization of Reactive Red M5B by isolate AGYP-1 was monitored in the presence of gelatine in the range of 0.25 to 10.0 g l<sup>-1</sup> (Fig. 8). At 2.5 g l<sup>-1</sup> concentration of gelatine, the isolate exhibited maximum decolorization of the dye (98.78±2.5%) within 8 days of incubation. No significant decolorization of the dye was obtained with an increase in concentration of gelatine. The isolate AGYP-1 could grow best (0.308±0.04 g) and produced high amount of ligninolytic enzymes at 2.5 g l<sup>-1</sup> concentration of gelatine. Under optimized culture condition, the laccase and MnP activities were 10.28 and 18.66 times higher, which resulted into 1.6 times faster decolorization of reactive Red M5B by isolate AGYP-1. The improvement in the production of ligninolytic enzymes as well as dye removal by different fungal strains has also been previously reported with optimum carbon and nitrogen sources (Asgher *et al.*, 2012).

### 3.5 Decolorization and degradation analysis of Reactive Red M5B

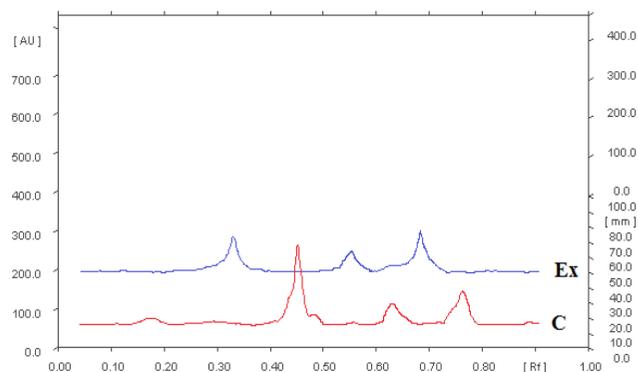
#### 3.5.1 UV-visible spectrophotometric analysis

Degradation of dyes can be revealed by examination of light absorbance spectra during decolorization. The

control and decolorized samples of Reactive Red M5B were analysed at 538 nm for assessment of biodegradation. The absorption peak for treated dye samples gradually decreased with the time and disappeared completely, which correlate with an apparent decolorization of the dye. In addition, extra peaks appeared in Uv region may be due to biotransformation or degradation of the dye and generation of different metabolites (Fig. 9). Moreover, the pH of the decolorized medium was around 5.5, indicating that the color removal was due to biological activity of the isolate AGYP-1, not because of the change in pH. The observed results suggested the biodegradation capacity of the isolate AGYP-1.

#### 3.5.2 HPTLC analysis

The assessment of degraded metabolites was further performed by HPTLC. Figure 10 demonstrates the HPTLC analysis of parent dye Reactive Red M5B and metabolites formed after degradation. The chromatogram when observed under UV light (254 nm), the control sample showed presence of 3 bands with R<sub>f</sub> values of 0.45, 0.62 and 0.76. After decolorization, the control bands disappeared and new bands with R<sub>f</sub> values of 0.31, 0.55 and 0.71 appeared. The results obtained in decolorized dye sample indicated the formation of new metabolites or metabolic intermediates that are different from the parent dye. This signifies the effectiveness of the isolate AGYP-1 for the degradation of Reactive Red M5B.



**Figure 10: Densitometric scan of HPTLC chromatogram of control Reactive Red M5B (C) and its decolorized samples (Ex)**

#### 4. Conclusion

The present study displays the efficiency of the white rot fungal isolate AGYP-1 for the removal of different textile dyes. The isolate showed the decolorization of Reactive Red M5B on both solid and liquid media with significant rate. Maximum removal of the dye and ligninolytic enzymes production was achieved at pH 5.0 and 30°C under shaking condition. Maltose and gelatine were found to be an optimum carbon and nitrogen sources for enhanced decolorization of Reactive Red M5B. The isolate AGYP-1 was able to decolorize higher concentrations of the dye. The UV-visible spectrophotometric and HPTLC analysis revealed the degradation of Reactive Red M5B. Therefore, it is concluded that the fungal isolate AGYP-1 could be promising for bioremediation of synthetic dyes from industrial waste water.

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