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ANALYSIS OF LINGOLYTIC ENZYMES AND DECOLORIZATION OF DISPERSE VIOLET S3RL, YELLOW BROWN S2RFL, RED W4BS, YELLOW SRLP AND RED S3B BY BROWN ROT FUNGI

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Current study was designed to investigate the ability of *Coniophora puteana* IEBL-1, *Daedalea dickinsii* IEBL-2, *Piptoporus betulinus* IEBL-3 and *Fimitopsis pinicola* IEBL-4 to decolorized disperse textile dyes. Five disperse dyes, purchased from supplier were studied including; disperse violet S3RL, yellow brown S2RFL, red W4BS, yellow SRLP and red S3B. The decolorization process was observed for 10 consecutive days with the analysis of process on each day. The results showed that *D. dickinsii* IEBL-2 (70-80 %) has the more potential of biodegradation of disperse dyes while *P. betulinus* IEBL-3 (47-59 %) has the least. The study of lignolytic enzymes i.e. lignin peroxidase, manganese peroxidase and laccase showed that *C. puteana* IEBL-1 and *D. dickinsii* IEBL-2 produced most active enzymes. Higher enzymatic activities related with more degradation indicated that these are involved in this process. Enzymes showed maximum activities at 30 ^{0}C and pH 6.5 with good affinity towards their substrates as indicated by kinetic values. The result indicates that these BRF isolates are suitable for the treatment of textile wastewater.

Keywords: Brown rot fungi, lignin peroxidase, biodegradation, laccase, manganese peroxidase

INTRODUCTION

Textile industries used large amount of water and synthetic chemicals during the processing of their products at different levels (Andre *et al.,* 2007) and release large amount of these as effluent (Aksu, 2005). Dyes in effluent are mostly toxic in nature and is a major environmental problem and cause of diseases after entering into food chain (Pagga and Brown, 1986).

Many techniques (chemical and physical) have been used for the treatment of dyes containing effluent but these are not considered environmental friendly and are costly (Rauf *et al.,* 2007). Biological methods are relatively simple and cheap have been the focus of current studies for the dye biodegradation (Sirianuntapiboon and Srisornsak, 2007).

Wood as an organic and heterogeneous material, can be easily damaged by various microorganisms including fungi, bacteria and moulds. Microorganisms produce variety of enzymes which are responsible for this decay and due to non-specificity of most of these enzymes, can be used for the degradation of other similar compounds including dyes (Zabel and Korrell, 1992). Disperse dyes are water insoluble non-ionic dyes which are predominantly used for polyester fibers.

Brown-rot fungi mostly habitate and damaged coniferous and citrus plants with the presence of high moisture contents (Kirk *et al.,* 2008). These exhibit cellulolytic activity and damage timber, fibers, paints and drugs (Eggins and Allsopp, 1975; Berka *et al.,* 1992). Fungi secrete enzymes collectively termed as ligninases and classifies according to their reactions (Martinz *et al.,* 2005). Laccases enzyme uses molecular oxygen as a source of electron acceptor whereas peroxidases required hydrogen peroxide as a co-substrate (Mai *et al.,* 2004). These enzymes are responsible for the degradation of various organic, in-organic and aromatic compounds present in plant biomass as well as in dyes.

In present study five different disperse dyes were decolorized with four brown rot fungi. The lignolytic enzymes were also studied during decolorize process which gives insight of enzymes responsible for this process. This study would help to develop the process for the decolorization of industrial effluent and to protect the environment from adverse effect of effluent.

MATERIALS AND METHODS

***Microorganisms*:** Cultures of four brown rot fungi including *C. puteana* IEBL-1, *D. dickinsii* IEBL-2, *P. betulinus* IEBL-3 and *F. pinicola* IEBL-4 were obtained from industrial

environmental biotechnology laboratory, Department of Biochemistry PMAS-Arid Agriculture University Rawalpindi, Pakistan. These were cultured on malt extract agar media (malt extract 20g/L, dextrose 20g/L, agar 15g/L and peptone 2g/L in water) (Tein and Kirk, 1984). For further use in decolorization process cultures were maintained in liquid media (agar less MEA) and spore size was maintained from 10^7-10^8 spores/ml with biomass monitor (ABER 220) (Mahmood *et al.,* 2013).

***Disperse dyes*:** Disperse dyes used in current study includes disperse violet S3RL, yellow brown S2RFL, Red W4BS, yellow SRLP and Red S3B and these were purchased from supplier (Table 1).

Table 1. Details of disperse dyes used in study.

Sr.	Disperse dye	Formula	λ-max
1	Violet S3RL	$C_{19}H_{19}ClN_6O_3$	448 nm
2	Yellow brown S2RFL	$C_{19}H_{17}Cl_2N_5O_4$	612 nm
3	Red W4BS	$C_{19}H_{22}N_4O_6S$	647 nm
4	Yellow SRLP	$C_{21}H_{15}N_5O_2$	588 nm
5	Red S3B	$C_{20}H_{22}N_6O_2S$	662 nm

***Decolorization experiment*:** Decolorization process was carried out in 250 mL flasks by adding 90 ml of dye solution (0.02%) in acetonitrile:water (60:40), 7 mL inoculums media and 3 mL of fungal inoculum. Flasks were placed in shaking incubator at 28°C, 120 rpm for 1 to 10 days before measuring absorbance at ƛ max of each dye. Each experiment was performed in triplicate and after each day, the percet decolorization was measured by taking 2 ml of mixture, centrifuge at 10,000 rpm for 10 min and absorbance was noted at ƛmax. The percent decolorization was calculated with following formula;

$$\% \text{ decolorization} = 100 \times$$

Where; (A_{ini} = intial absorbance of mixture, A_{fin} = final absorbance)

***Lignolytic enzymes assays*:** With the observation of biodegradation, lignolytic enzymes like lignin peroxidase (LiP, manganese peroxidase (MnP) and laccase were also studied. The centrifuged mixture on each day was subjected to enzymatic activities as described by Wolfenden and Willson (1982) for laccase, Tien and Kirk (1984) for LiP and Wariishi *et al.* (1992) for MnP.

***Characterization of lignolytic enzymes*:** All the three lignolytic enzymes were characterized to find their optimum pH, temperature and kinetics parameters like K_m and V_{max}. For optimum temperature and pH, enzymes assays were performed at various temperature and pH values, respectively. To find out K_m and V_{max}, enzymes assays were performed at optimum pH and temperature with different concentrations of substrate of each enzyme. The data obtained was used to plot Line-Weaver burk plot, which was used to calculate K_m and V_{max} of each of the enzyme.

RESULTS AND DISCUSSION

Fungi have the ability to secrete different enzymes in the media depending upon the composition of surrounding media. If provided with certain nutrients, resultant enzymes can be used for various important processes. Keeping this in view, four brown rot fungi were tested to check their potential for the degradation of disperse textile dyes and production of lignolytic enzymes.

***Degradation of disperse violet S3RL*:** Solution of disperse violet S3RL dye (DD-1) in acetonitrile and water was kept for 10 days with the inoculums of all four brown rot fungi at controlled conditions. The results shows that maximum degradation (76%) was achieved with *D. dickinsii* IEBL-2 on the 7 day and after that there was a minimal change in the degradation (Figure 1). This reduction is possible due to accumulation of waste contents in mixture which reduces the growth of fungus and secretion of enzymes in media. There was 72% degradation achieved with *C. puteana* IEBL-1on same day followed by *P.betulines* IEBL-3 (59%) and *F. pinicola* IEBL-4 (45%) (Figure 1).

Effect of fungal extracellular enzymes on the days 7 of incubation at 28°C was also observed by Olfat *et al*. (2007). Similar kind of results was also reported by Abedin in 2008 during the decolorization of crystal violet and malachite green by *Fusarium solani*.

***Degradation of disperse yellow brown S2RFL*:** Disperse yellow brown S2RFL (DD-2) was degraded 75% by *C. puteana* IEBL-1 on day 7 followed by 72% by *D. dickinsii* IEBL-2 on the same day (Figure 2). The degradation became very low after day 7 to day 10, almost 1 to 2% in 3 days. *P. betulines* IEBL-3 showed 58% degradation and *F. pinicola* IEBL-4 showed 56% degradation of disperse yellow brown S3RFL (Figure 2).

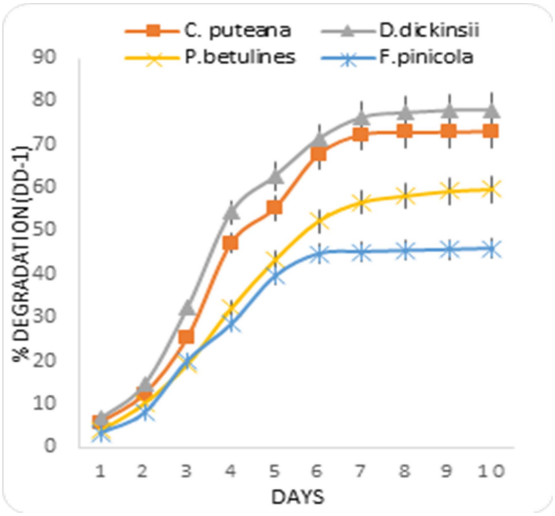


Figure 1. Degradation of disperse violet S3RL by brown rot fungi.

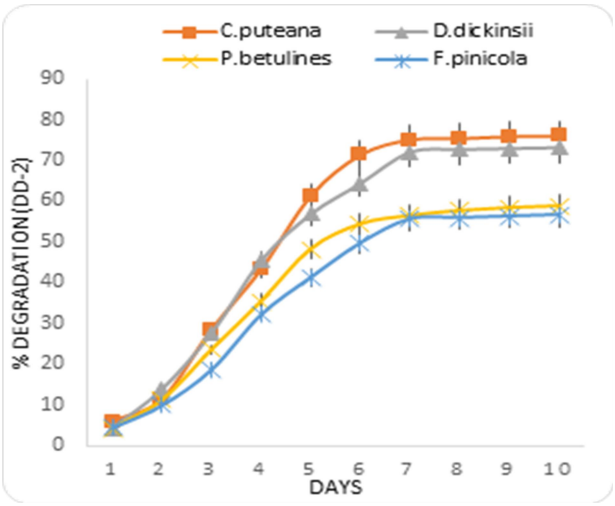


Figure 2. Degradation disperse yellowS2RFL by brown rot fungi.

These results are in line with the findings of Abedin in 2008, he achieved maximum decolorization of crystal violet and malachite green by *Fusarium solani* on day 7 at 28°C. Most of the fungus gave maximum decolorization of dyestuff around 30°C temperature (Jarsoz *et al.,* 2002).

***Degradation of disperse red W4BS*:** Disperse red W4BS (DD-3) solution (0.02%) was incubated with the inoculums of all four brown rot fungi at 28°C temperature and 5.5 pH for 10 days. It was maximum 79% degraded by *D. dickinsii* IEBL-2 followed by 64% (*C. puteana* IEBL-1), 58% (*F. pinicola* IEBL-4) and 50% (*P. betulines* IEBL-3) (Figure 3). There was good degradation up to day 7 after that the degradation process declined and gave less than 1% degradation per day. The degradation process is carried out by extracellular enzymes secrete by fungi in the media, as nutrients in the media decline and wastes accumulate fungal growth decreased and production of enzymes also decreased. Variable decolorization of dyes 75-100% by fungi using its active enzyme system was also observed by Jarsoz *et al.* (2002).

***Degradation of disperse yellow SRL*:** Disperse yellow SRLP (DD-4) was subjected to degradation/decolorization experiment as DD-3. The results showed that it was 75% degraded by *D. dickinsii* IEBL-2 on the day 7 and 69% by *C. puteana* IEBL-1 on day 8 (Figure 4). While, *P. betulines* IEBL-3 and *F. pinicola* IEBL-4 gave 47% and 54% degradation, respectively.

***Degradation of disperse red S3B*:** Disperse red S3B (DD-5) is one of the most commonly used dye for the dyeing process in textile industries of Pakistan and is source of environmental pollution in the form of effluent. In current study it was 80% degraded by *D. dickinsii* IEBL-1 and 59% degraded by *C. puteana* IEBL-1 on day 7 (Figure 5). There was 52% and 61% degradation achieved with *P. betulines* IEBL-3 and *F. pinicola* IEBL-4 on day 7 and 82, respectively. The accumulation of waste material in the mixture, decline the growth of fungi as well as secretion of enzymes, so degradation process decreased after day 7.

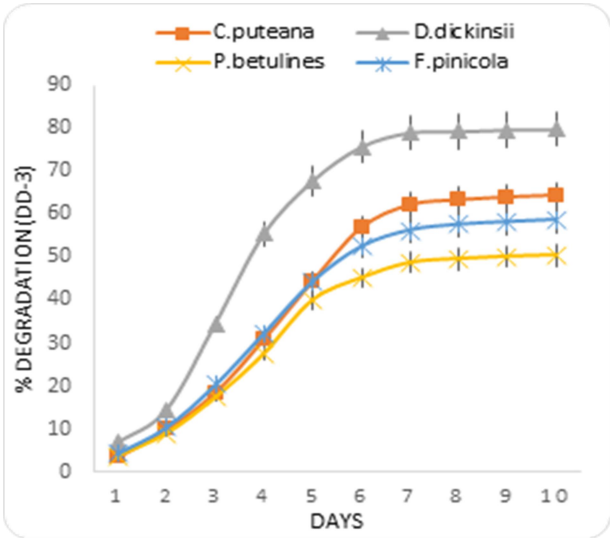


Figure 3. Degradation of disperse red W4BS by brown rot fungi.

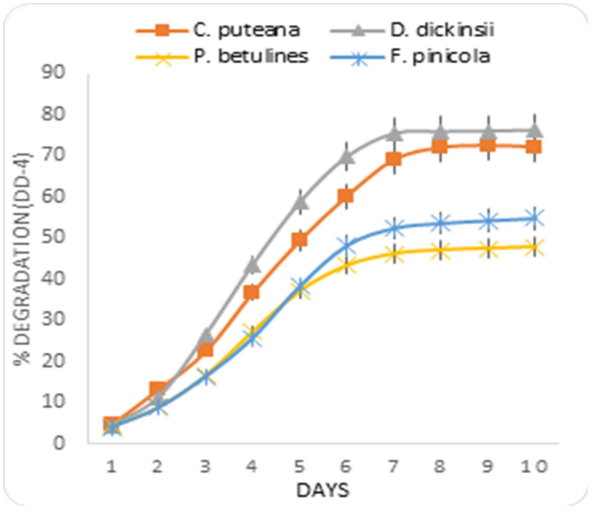


Figure 4. Degradation of disperse yellow SRLP by brown rot fungi.

There are many previous studies similar to current study for the decolorization of various toxic dyes. Jarsoz *et al.* (2002) reported that cultures of *Bjerkandera fumosa* and *Kuehneromyces mutabilis* gave 100% decolorization of Acid red 183 and 75–100% of Basic Blue 22. The decolorization of various textile dyes by fungal cultures depends upon the production of lignolytic enzymes which gave very good decolorization results at acidic pH range from pH 4 to pH 6

for different fungi (Mazmanci and Ali, 2010; Shazia and Safia, 2011; Ali and Mohamedy, 2012).

These results indicated that fungi are most active around physiological conditions. This is very important to carry out these beneficial processes at normal conditions without much maintenance of process. The current results also support the previous results which can be utilize very effectively for the biodegradation of textile waste water at local environmental conditions.

***Extracellular lignolytic enzymes*:** Extracellular enzymes released by brown rot fungi plays important role in the decolorization/removal of dyes. In current study, it was noted that enzymatic activity increased with increasing decolorization process denoting direct relation between these two parameters.

***Production of lignin peroxidase (LiP)*:** Lignin peroxidase produce by fungi plays role in the degradation of lignin and other aromatic compounds by oxidation process. In current study lignin peroxidase production of four brown rot fungi was observed during the decolorization of disperse dyes. It was observed that lignin peroxidase produced by *D. dickinsii* IEBL-2 gave maximum activity of all four fungi (865.55 U/ml) followed by *C. puteana* IEBL-1 (842.12 U/ml), *F. pinicola* IEBL-4 (745.34 U/ml) and *P. betulines* IEBL-3 (729.38 U/ml) (Figure 6). *D. dickinsii* IEBL-2 gave maximum degradation of disperse dyes as well as maximum production of lignin peroxidase. These findings indicated that lignin peroxidase involved in the degradation of disperse dyes.

There are many other studies elucidating the role of microbial ligninolytic enzymes involved in removal of various dyes from effluent (Selvam *et al.,* 2006; Asgher *et al.,* 2009; Shazia and Safia, 2011; Asgher *et al.,* 2012). These enzymes are usually non-specific and have the ability to act on similar compounds from various origins (Asgher *et al.,* 2012).

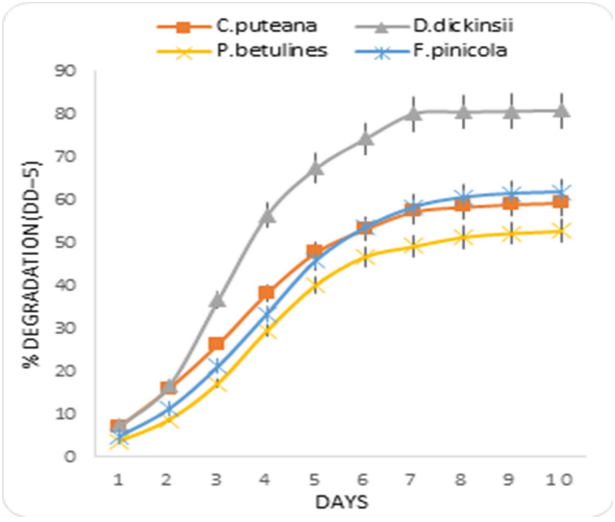


Figure 5. Degradation of disperse red S3B by peroxidase brown rot fungi.

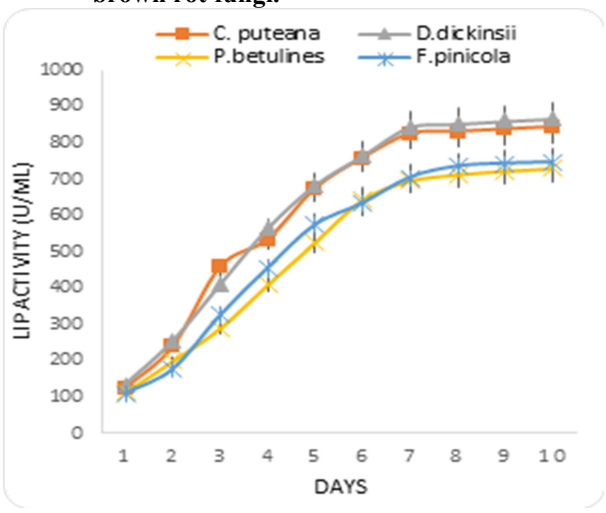


Figure 6. Comparison of lignin activity produce by brown rot fungi.

***Production of manganese peroxidase*:** Manganese peroxidase is Mn dependent oxidase that cause the oxidation and degradation of lignin in plant cell wall. In current study its secretion by four brown rot fungi was reported during the biodegradation of five disperse dyes. Higher production and activity indicate that this enzyme play some role in the decolorization of disperse dyes. It was observed that *D. dickinsii* IEBL-2 produce manganese peroxidase with maximum activity (612.31 U/ml) and it has the ability to degrade 80% of dye contents from the solution (Figure 7). While *C. puteana* IEBL-1 produces manganese peroxidase with activity 603.16 U/ml followed by *F. pinocola* IEBL-4 (486.65 U/ml) and *P. betulines* IEBL-3 (453.18 U/ml). The activity increased with increasing degradation and remains static after achieving maximum degradation (Figure 7).

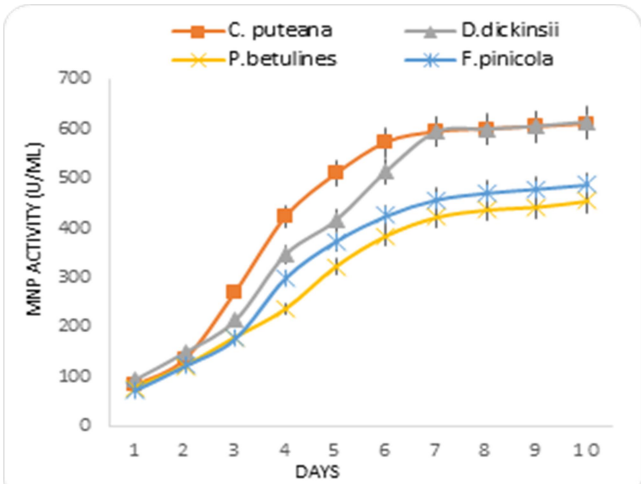


Figure 7. Comparison of manganese peroxidase activity, produce by brown rot fungi during the degradation of disperse dyes.

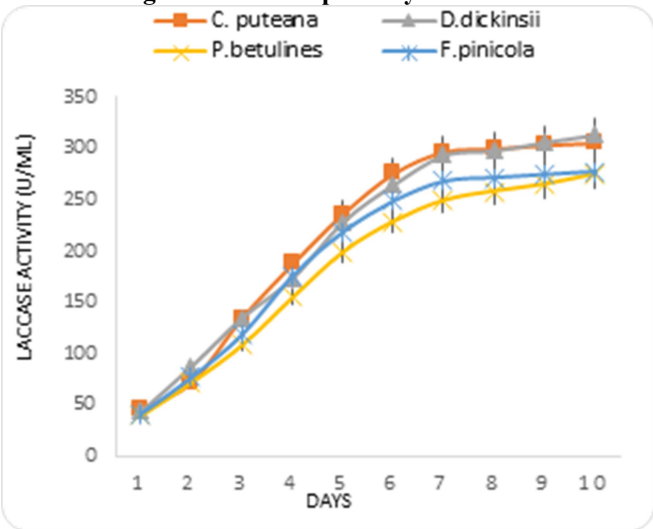


Figure 8. Comparison of laccase activity, produce by brown rot fungi during disperse dyes biodegradation.

***Production of laccase*:** Laccase is a Cu containing oxidase that catalyzed the oxidative cleavage of aromatic compounds located in the cell wall and dyes. Active laccase were produced by four brown rot fungi during the decolorization of disperse dyes indicating their involvement in disperse dyes biodegradation and decolorization. The most active laccase was produced by *D. dickinsii* IEBL-2 (312. 71 U/ml) followed by *C. puteana* IEBL-1 (305.27 U/ml), *F. pinicola* IEBL-4 (277.12 U/ml) and *P. betulines* IEBL-*3* (274.34 U/ml) (Figure 8). These values of activities are directly related with the % degradation of the most degraded dye by that fungus.

Various studies have been reported for the decolorization of dyes by lignolytic enzymes and effect of various factors on the production of these enzymes. The amount and type of enzymes secreted by fungi depends up on the concentration and type of dye in the solution (Vaithanomast *et al.,* 2010). The secretion of enzyme and decolorization of dye directly related with the size of inoculum and other factors that affect the growth of fungi. Kashif *et al.* (2011) reported 78% decolorization of solar golden yellow R dye by *P. ostreatus* with high activity of laccase, indicated the role of laccase in decolorization.

***Characterization of lignolytic enzymes*:** The most active lignolytic enzymes produced by brown rot fungi were characterized for their optimum temperature, pH and values of kinetics parameters. Finding of these values of an enzyme increase its feasibility and utilization in different industrial processes. The results revealed that all three enzymes show same optimum temperature (30°C) and pH (6.5) (Figure 9-10). Enzymatic activities remain very good from pH 5.25 to pH 6.75, wide range of activity made these enzymes attract scientists for their industrial use. These enzymes are mesophillic and are active in acidic conditions near to neutral pH value. Most of the industrial effluents are basic in nature, so in order to get better results there is need of addition of acid to reduce pH. Various concentrations of substrates of these enzymes were used to calculate the values of K_m and V_{max}. The enzymes activities obtained from various concentrations were used to plot double reciprocal or Line Weaver-Burk plots (Figure 11). The values of K_m obtained from these were 0.751 mM, 0.700 mM and 0.571 mM for Lip, MnP and laccase, respectively. While, the values of V_{max} were 1250 µM/ml/min, 1000 µM/ml/min and 1428.57 µM/ml/min for LiP, MnP and laccase, respectively. These results indicated the efficiency and specificity of these enzymes. Due to good affinity of these enzymes towards their substrates with high speed these can be used for various other biotechnological processes.

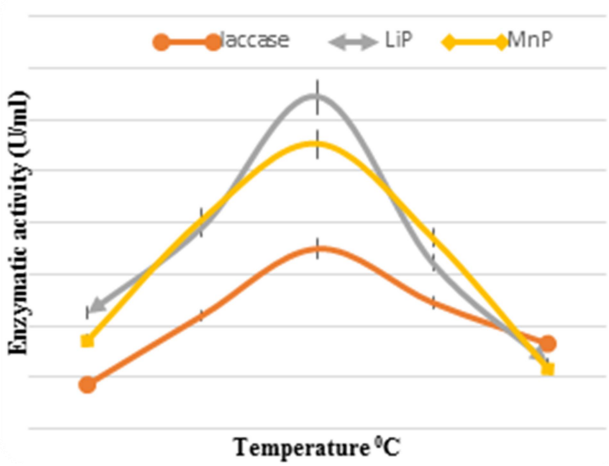


Figure 9. Activity of lignolytic enzymes at different temperature.

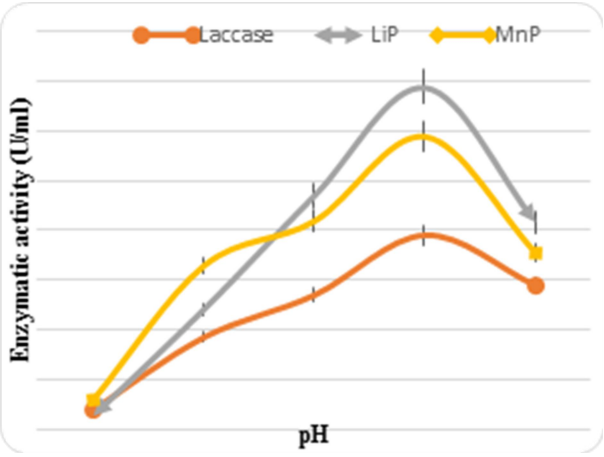
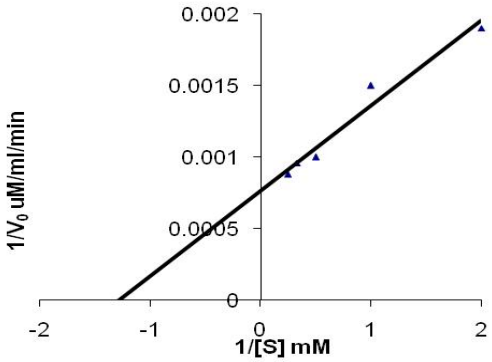


Figure 10. Activity of lignolytic enzymes at different pH values.

Optimum temperature for various other mushrooms has been reported between 25 ^{0}C to 37 ^{0}C for the decolorization of industrial dyestuff (Agher *et al.*, 2009). Further increase in temperature results into decrease in growth of fungi and less decolorization of dyestuff due to no or less production of lignolytic enzymes.

A



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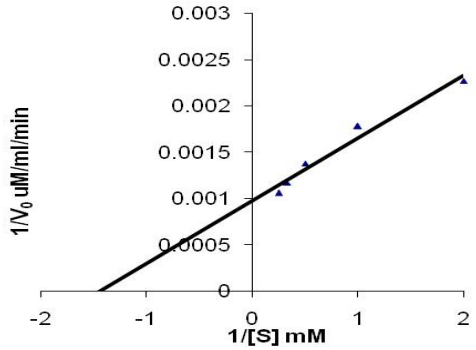

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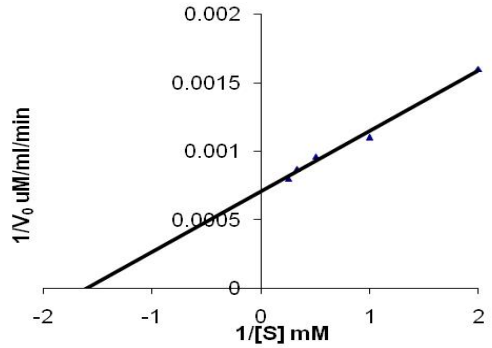

Figure 11. Line-Weaver Burk plot against reciprocal of substrate concentration on X-axis and rate of reaction on Y-axis for the calculation of Km and V_{max} of lignolytic enzymes produces by brown rot fungi during the decolorization of disperse dyes (A)-Lip, (B)- MnP and (C)-Laccase.

The temperature plays vital role in the growth as well as secretion of important enzymes (Asgher *et al.,* 2008; Assadi *et al.,* 2001). Change in pH of decolorization mixture effect the decolorization process has also been reported by various other researchers (Sawhney and Kumar, 2011; Kumar *et al.,* 2012). Fungi produce different lignolytic enzymes during the decolorization of different dyes which are active in acidic range of pH (Asgher *et al.*, 2008; Mahmood *et al.,* 2015).

***Conclusion*:** In this study, it was observed that *D. dickinsii* IEBL-2 and *C. puteana* IEBL-1 are the efficient brown rot fungi for the decolorization of disperse dyes. These isolates of Pakistan can be used for the decolorization of industrial wastewater. Further, these fungi can be used for the commercial production of lignolytic enzymes which can be used for various industrial processes.

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