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Journal of Environmental Biology

ISSN: 0254-8704 (Print)
ISSN: 2394-0379 (Online)
CODEN: JEBIDP

Comparison of synthetic dyes decolourisation by *Ganoderma* sp. using immobilized enzyme

Prathibha B. Iyer[1,2], B. Atchaya[1], K. Sujatha[1] and K. Rajmohan[2]

[1]Department of Chemical Engineering, Dr.M.G.R Educational and Research Institute University, Maduravoyal-600 095, India
[2]Department of Chemical Engineering, University of Petroleum and Energy Studies, Dehradun-248 007, India
*Corresponding Author E-mail: **baskaranpratibha@gmail.com**

Publication Info

Paper received:
16 October 2015

Revised received:
09 August 2016

Accepted:
28 August 2016

Abstract

The effluents from textile industries, which are disposed to the open environment and water sources, create major problem in polluting the environment. In Tamil Nadu districts like Tiruppur, people are facing problems due to disposal of untreated textile dyes. Pre-treatment of dyes help in reducing toxic chemicals present in the dye. Various species of white rot fungi are prominent producers of enzyme laccase. In the present study, *Ganoderma species* were screened isolated for laccase activity major laccase producing fungal strains was identified. In the second phase, the highest amount of laccase producing strain was compared with remaining strains, and the conditions for growth of *Ganoderma species* was standardized to maximize laccase production. Characterization of enzyme laccase synthesized by *Ganoderma species* was performed after optimization and partial purification. In the present study, application of characterized laccase in the degradation of textile dyes was investigated. Finally, pH and temperature optimum parameters were upgraded for dye decolorisation and compared to assess the maximum percentage of degradation. The percentage degradation of Direct Blue E was maximum than Acid Violet 4BY and Acid Brilliant Black due to maximum impact of laccase enzyme on that particular Direct Blue E dye.

Key words

Dye degradation, *Ganoderma sp.,* Immobilized enzyme, Laccase, White rot fungi

Introduction

Annual production of dyes is ever increasing due to its increased application mainly in textiles, cosmetics, food, leather industries. About 10 to 15% of the dye is released by the textile industry into waste water. Contaminated water, soil, and air by effluents from the industries are related to heavy disease burden (Murray and Lopez, 2002), and this could also be one of the reasons for the current shorter life expectancy in various countries (Evans and Beaglehole, 2003). Immense potential for microbiological decolourisation system development with almost complete colour removal within hours is possible, despite the challenges prevailing in textile as well as wastewater treatment industries (Halil *et al*., 2012). Azo, phthalocyanine and anthraquinone groups are commonly used dyes and possess toxic and carcinogenic properties (Arun Prasad *et al*., 2014). These toxic dyes are known to hinder light penetration in water, thereby damaging the hydrophytes which lead to decreased oxygen level in water. Also these dyes dissociate to form products which are harmful to aquatic life.

Biodegradation is one of the major natural process by which metals are removed from textile waste. Bioremediation efficiency to the textile waste were examined so that it may be important to combine the conventional methods of engineering task. Biotreatment are cost efficient and ecological alternative for the removal of textile waste and

metal (Subhathra *et al.,* 2013). In the recent past, microorganisms have shown remarkable ability to detoxify and decolorize dyes (Rajaguru *et al*., 2000; Kumar *et al*., 2006, 2007; Ramya *et al*., 2010; Adedayo *et al*., 2004). Since most of the dyes have complicate structure, they are hard to decolourize (Reddy Prasad Reddy *et al*., 2012). It has been reported that aerobic treatment by municipal sewage system has failed to decolorize the textile dye effluent (Willmott *et al*., 1998). Explicitly colored, acid dyes and water-soluble reactive are reportedly known to be the most difficult dyes to decolorize. Also, they easily pass through various conventional decolorizations ystems without undergoing any change (Murugesan *et al*., 2006).

Research on dye degradation using lignolytic enzyme present in *Ganoderma sp.* is meagre and Murugesan *et al.* (2006) reported the preliminary studies to explore the capability of extracellular enzymes of *Ganoderma sp.* for decolorization of reactive dyes. In contrary to basic methods, biological treatment for dye decolorization provides suitability to use for recalcitrant materials, ability to operate at high, as well as pH, temperature and salinity (Duran *et al*., 2000; Vasileva *et al*.,2009; Velu *et al*., 2011; Sekar *et al*.,2012).

It is essential to immobilize the enzymes to increase their stability and biological reuse in order to facilitate its application in wastewater treatment processes (Velu *et al*., 2011). Consequently, several studies have supported immobilization of laccase. High thermal stability and impedance to acute conditions and chemical testing agents are notable merits of immobilization of laccase enzyme (Georgieva *et al*., 2008). Immobilization of laccase can be carried out using adsorption, covalent attachment or entrapment on various supports mentioned in the literature (Metin *et al*., 2013). In view to the above, the present study was carried out to isolate *Ganoderma sp*., and assess its capacity to degrade synthetic dyes through laccase enzyme obtained from it (Murugesan *et al*., 2007).

Materials and Methods

Test organism : *Ganoderma sp.* was selected due to its major biotechnological importance, as well as remedial properties. The fungal culture *Ganoderma sp*. was obtained from the bark of oldest tree, at Thirukazukundram, Chengalpet. It was washed with mercuric chloride to remove the impurities. A small piece of fungi was isolated and preserved in Czepek Dox Broth and Potato Dextrose Agar medium (Velu *et al*., 2011).

Screening for laccase: For screening of laccase, Potato Dextrose Agar medium was prepared and after the medium was cooled, 1mM guaiacol was checked under sterile condition. The medium was poured onto petriplates, and the isolated fungal strain was inoculated on it. The plates were incubated at 37°C for 24 hrs and assayed.

Enzyme production : For enzyme production in solid-state fermentation (Hedge *et al*., 2006), ligno cellulosic substances such as rice bran, wheat bran and black gram were filled in different flask and damped (70%) with peptone water, and transferred to conical flask and sterilized. Innoculation was done by using five disc of five-day old mycelial culture and the flasks were incubated at 30°C. Each flask was kept still for 12 days. The fillings were extracted with 120 ml sodium acetate buffer (pH 5.8, 0.1M) and kept overnight at 4°C. The filtrate obtained was centrifuged at 5000 rpm for 20 min and the supernatant obtained was used as enzyme source to estimate laccase production.

Estimation of protein : Protein content in both crude, as well as salted out and partially purified enzyme, was estimated following the method of Bradford (1976). Extracellular laccase activity was estimated by Elico-192 UV visible spectrophotometer (Benny *et al.,* 1998) with syringaldazine as a substrate for both the crude, as well as salted out enzyme and partially purified enzyme. Bovine serum albumin was used as standard to estimate the protein content.

Purification of laccase: Using 75% ammonium sulphate saturation, precipitation of protein was carried out. All the subsequent purification steps were carried out at 4°C. Protein was dissolved in 0.1M CH_3COONa buffer, pH 5.8 and dialyzed against the same buffer.

Characterization of laccase: Inorder to perform SDS-polyacrylamide gel electrophoresis, slab gel with two dissimilar coagulating medium, namely separating and stacking (10% and 5% w/v) was performed following the method of Laemmli (1970). Also, Native PAGE was carried out following the procedure of Davis (1964). The purified enzyme were mixed with sample buffer 3:1 ratio for 3 min; boiled, cooled and then loaded to the wells. Power supply was connected such that cathode was connected to the upper tank and anode to the tank beneath. The process of electrophoresis was carried out at room temperature with consistent voltage and current supply was maintained at 20 mA till 0.5 cm of tracer dye reached the lower end. The gel was taken off and staining was carried out by silver nitrate (Blum *et al*., 1987) at the end of electrophoresis. The gels were preserved in 7 % (v/v) acetic acid. The pH was maintained at 8.8 in 10% polyacrylamide gel along with Tris/glycine buffer. After completion of Native-page, to examine the activity of laccase, gel was incubated at 35ºC for 30 min. The gels were then dipped for 10 min in sodium acetate buffer (0.2 M; pH 5.6) and at the same time rinsed with 0.1m Mguaiacol. (Reddy *et al*., 2012)

Immobilization of enzyme: The process of enzyme immobilization was taken up, so that calcium alginate puff containing the enzyme is formed (Velu *et al*., 2011).In 150 ml

of warm water 4 g of sodium-alginate powder was added pinch by pinch, stirring completely so that no lumps were formed. After total dissolution, 5-10 ml of enzyme was added and completely mixed. The sodium alginate dissolute solution was poured from 15-20 cm height into a beaker with 0.2M calcium chloride solution, so that calcium alginate puff containing the enzyme is formed.To allow hardening, calcium chloride solution was used where the puffs were added to the solution and maintained for 3-4 hrs at normal room temperature.

Application of laccase enzyme: Application of laccase enzyme was done on solid medium and liquid medium, where pH and temperature were optimized for each dye and compared. Textiles dyes Acid Violet 4BY, Acid Brilliant Black and Direct Blue E were obtained from ARMATS BIOTEK, Chennai. The Czapek Dox Basal broth (100 ml) was prepared in seven 250 ml Erlenmeyer flasks. Six azo dyes were held individually in the concentration of 10mg $100ml^{-1}$ (100ppm) and sterilized. Mycelial discs (5 No.) of 6mm from 3-day-old fungal culture was transferred to each flask and incubated at room temperature (30±2C). The culture was allowed to grow for 7days. A sterile control, without mycelial discs, were maintained for each dye and medium with cultures were also maintained under similar conditions. Culture filtrates were harvested every 24 hr interval and monitored for decolourization.

pH stability: Laccase that was partially purified from *Ganoderma sp.*, was used to determine the optimum pH. The enzyme was incubated with different buffers of pH, ranging from 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0. After 60 min incubation, the samples were assayed for enzyme activity and a stable pH was verified (Velu *et al*., 2011).

Temperature stability : A optimum temperature was stabilized for laccase enzyme that was partially purified from *Ganoderma sp*., and incubated enzyme with buffer was maintained at pH 5 with varying temperature ranging from 50°C, 60°C, 70°C, 80°C, 90°C and 100°C was carried out. After 2 hours of incubation, the samples were assayed for enzyme activity and the stable temperature was verified.

GC/MS analysis: The extracts of methanol were examined by Thermo Scientific GC-MS [SHIMADZU QP2010] Gas chromatograph Software: GCMS solution ver.2.53. Split split-less injector were used to fit the extracts and MS PolarisQ-Quadrupole Ion Trap (Thermo Electron) merged silica column VB5 (95% methylpolyxiloxane, 5% phenyl, 30 m with 0.25 mm ID (Inner diameter) film thickness 0.25 µm) was connected. The injector and interface were operated at 250°C and 300°C, respectively. The oven temperature was maintained as follows : 50°C increased to 250°C ($4°C\ min^{-1}$) and enabled for 3 min. Helium of selected as carrier gas was taken at 1 ml min^{-1}. The sample (1 µl) was fed (1:20). The condition for Mass Spectroscopy was: mass range 10-350, amuionization voltage EI of -70 eV.

Results and Discussion

This strain of *Ganoderma sp.* fungal culture acquired from the bark of oldest tree, shown in Fig 1, was able to decolourise all the tested dyes, but at different individual operating conditions. Mycelial disc (6 mm) from 5-day-old pure culture of the fungus was grown on PDA plates (Fig. 2) and were used as inoculums for further experiment**.**

During screening of enzyme activity (Fig. 3), the presence of reddish brown colour zone confirmed that laccase enzyme was positive in the fungal strain.

The culture was isolated by solid state fermentation using wheat bran, rice husk and black gram. These substrates were prioritised as they are proven raw materials for bulk enzyme production with less investment. Among the 3 substrates (Fig. 4), wheat bran showed highest protein concentration (1.25 mg ml^{-1}) for crude enzyme and increase in protein concentration (1.34 mg ml^{-1}) after dialysis. Wheat bran was witnessed to contain more enzyme activity (1.128 Unit ml^{-1}) for crude enzyme and dialysis (1.250 Unit ml^{-1}), after 24 hrs of incubation.

At different pH range (3.0-9.0), (Fig 6), maximum enzyme activity (14.129 IU ml^{-1}) was seen at pH 5.0.The maximum enzyme activity (10.160 Units ml^{-1}) was observed at 70°C.

Saturation of 75% ammonium sulphate was used to precipitate the protein present in the supernatant. Protein sample of laccase enzyme from *Ganoderma sp.*, were examined using SDS-PAGE and silver nitrate staining was observed. Fig. 8 shows multiple bands indicating that the dialyzed samples were electrophorotically not homogenous. Purified laccase was tested for its activity with crude positive control on native PAGE using a base source (guaiacol) Dark brown band in Fig. 9 shows the presence of oxidized region.

As shown in Fig. 10, decolourization of Direct Blue E was observed on Potato Dextrose Agar plate with dye concentration of 100ppm, whereas other dyes were found to be impotent in undergoing degradation on solid media. Dye decolourisation was also observed on Potato Dextrose basal broth for Acid brilliant black with dye concentration of 100ppm (Fig 10).The pH was optimized for the textile dyes is as follows: Acid brilliant Black-pH 5.0, Direct Blue E-pH 5.0 and Acid Violet 4BY-pH 5.0, (Fig. 11, 12 and 13). The optimum temperature (Fig 14, 15 and 16) estimated for the textile dyes as follows: Acid Violet 4BY-90ºC, Acid brilliant Black-80ºC and Direct Blue E-90ºC.

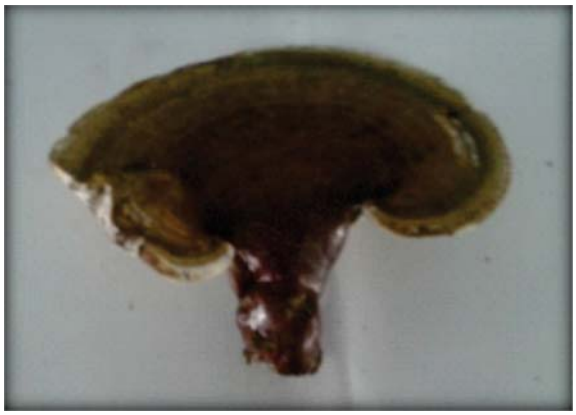

Fig. 1 : *Ganoderma* fungal sp.

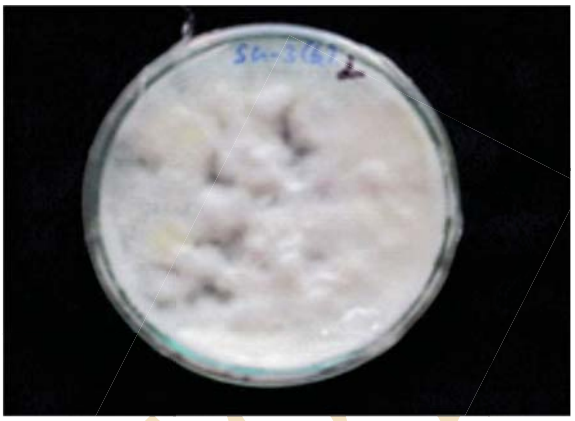

Fig. 2 : *Ganoderma* sp. pure culture

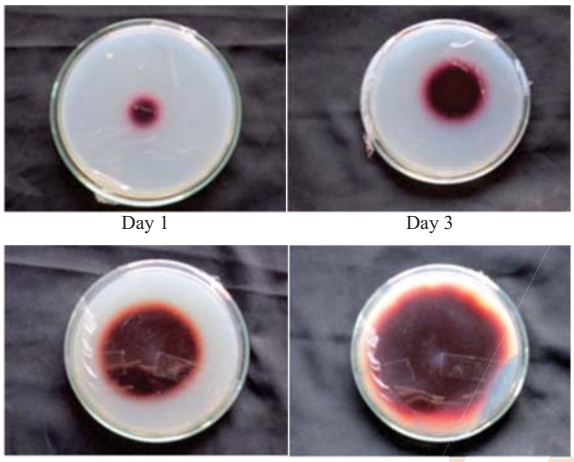

Fig. 3 : Screening for laccase

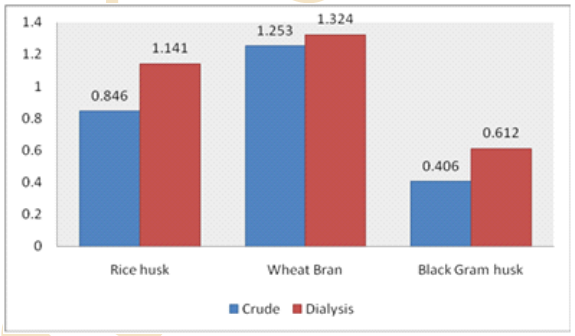

Fig. 4 : Protein estimation for laccase

Fig. 17 shows the percentage comparison of degradation of dyes- Acid Violet 4BY, Acid Brilliant Black and Direct Blue. The results revealed that the most degrading dye was Direct Blue.

Diverse form of oxidative enzymes has been found in white rot fungi so that lignocellulosic substrates can be utilized for nutrition (Martin *et al*., 2008; Rogalski *et al*., 1999). Solid state fermentation is considered as a resourceful technique for enzyme production in biotechnological process due to its potential advantage and high yield. Agro waste such as wheat bran, rice husk and black gram are chosen as substrates for optimizing the superlative solid support in order to show maximum laccase activity. Hedge *et al*. (2006) reported that wheat bran and rice bran shows maximum laccase activity due to the presence of ferulic acid.

Similar observations have been reported by Sathiyamoorthi *et al*. (2007). Enzyme activity of the culture filtrate was found to be 0.125 U ml^{-1} using *P. florida* and *T. hirsuta sp.* The decolourization effect of three dyes on solid state were resulted to be: Blue CA dye- 96.4% for *P.florida* and 91.1% for *T. hirusita*, Black B133 dye- 95.2% for *P. florida* and 65.5% for *T. hirusita* and Corazol violet dye: 99.5% for *P. florida* and 56.98% for *T. hirusita* respectively. Also, in aqueous state at 25mg l^{-1} dye concentration, Blue CA showed maximum decolourisation of 93.54% by *P. florida* and 92.17% by *T. hirusita* at 10^{th} day of assessment. Black B133 dye reflected low decolourization capacity of 64.47% by *P. florida* and 57.21% by *T. hirusita* and 83.70% Corazol violet SR dye was dicolourized by *P. florida* and 62.02% by *T.hirusita*. The author through their results proved that with moderate rate of time and with greater capability of enzyme activity, the species chosen in the current study provided enhanced and efficient results than the species compared on this note.

The results of Dayaram *et al*. (2007), confirmed that the laccase activity was about 17.1 U ml^{-1} under 2,6-dimethoxyphenol method and this activity was produced by them at 48^{th} hour. The dye concentration chosen here were 100 mg l^{-1} and 1000 mg l^{-1}, that gave a major shoot up of results due to the immense time rate consideration of about 5 days of incubation. Also, optimization of pH and

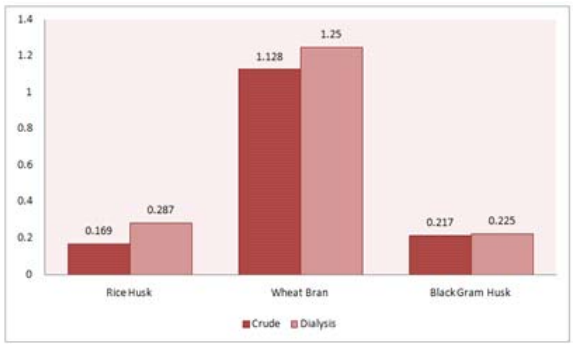

Fig. 5 : Laccase enzyme assay using Syrangaldazine

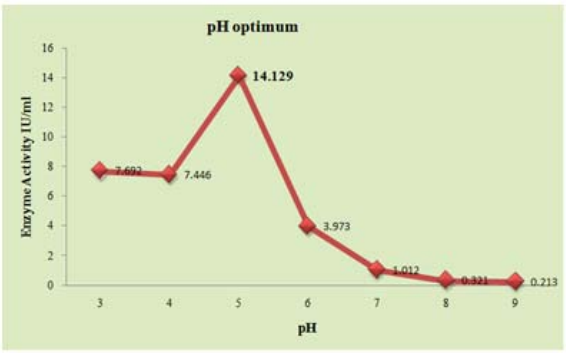

Fig. 6 : pH optimum for laccase enzyme

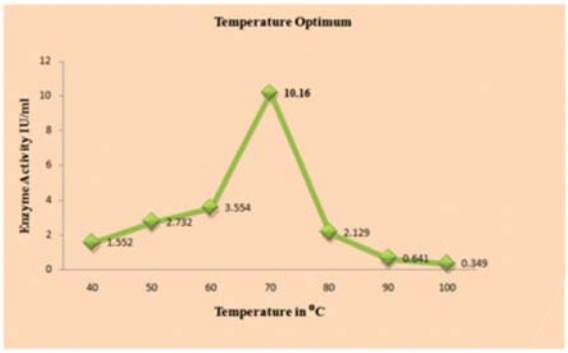

Fig. 7 : Temperature optimum for laccase enzyme

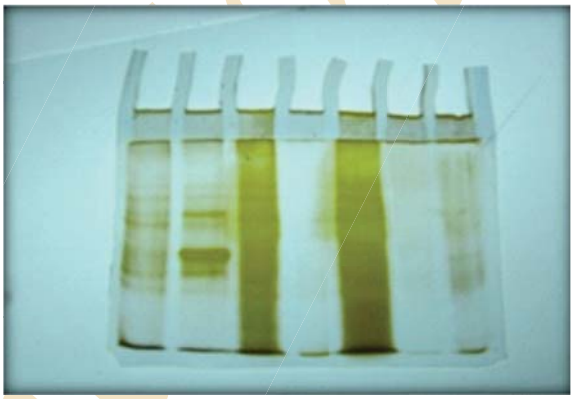

Fig. 8 : Extracellular polypeptide profile on SDS-PAGE

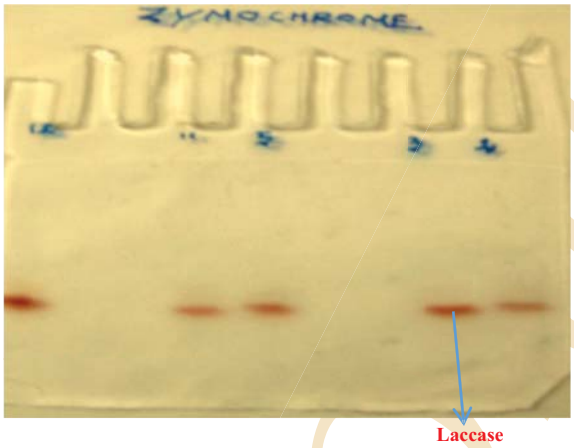

Fig. 9 : Purified laccase

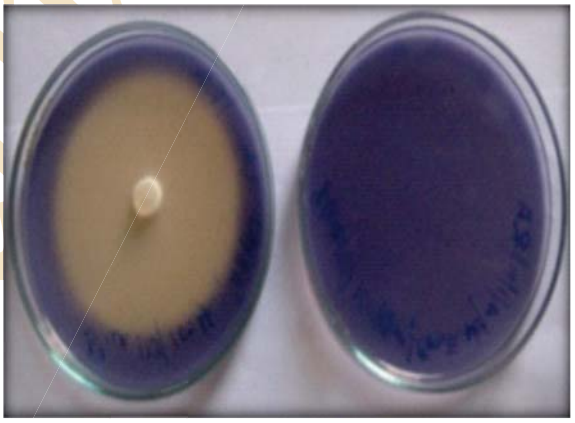

Fig. 10 : Direct Blue dye decolourisation on solid medium

temperature, that are the key parameters for effective dye decolourization, were not carried out and focussed by the authors.

Karnika *et al*. (2014) reported 145 U l^{-1} laccase activity (in the presence of copper) on the 12th day and 103 U l^{-1} (in the absence of copper) on the 14th day of assessment in *L. polychrous* sp. Highest decolourisation of 85% of acid Blue dye in 180 min at pH 5.0 and temperature 30-60^{o}C was reported. They also confirmed that *Ganoderma sp.* revealed greater activity at acidic condition than *L.polychrous sp.* Also, the method of enzyme production carried out by the author was in the presence of copper that proved this research to be undemanding with massive productivity and low production cost.

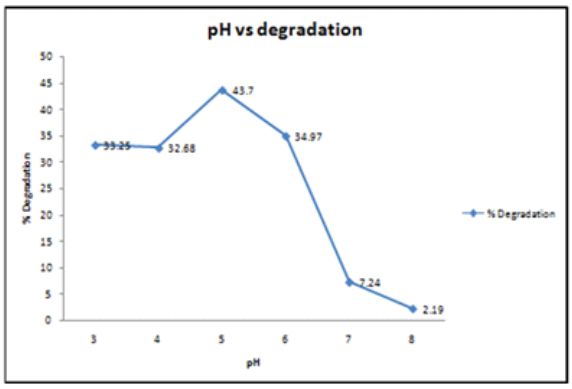

Fig. 11 : pH optimization for Acid Violet 4BY dye degradation using laccase enzyme

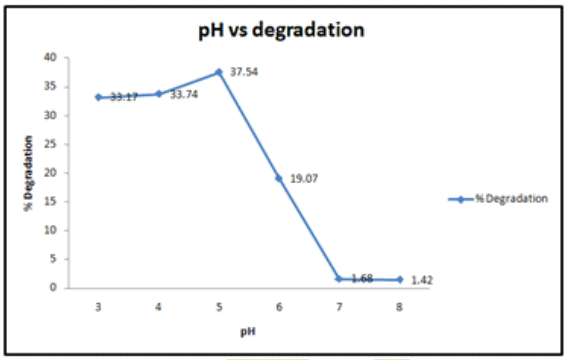

Fig. 12 : pH optimization for Acid Brilliant Black dye degradation using laccase enzyme

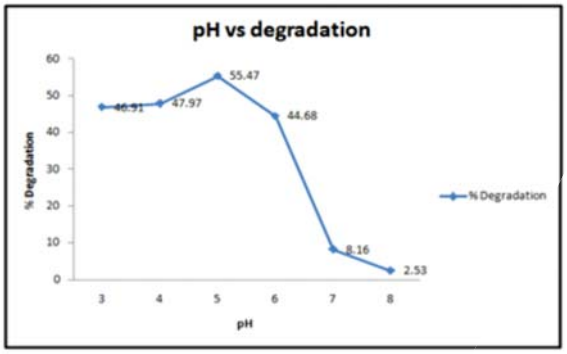

Fig. 13 : pH optimization for Direct Blue E dye degradation using laccase enzyme

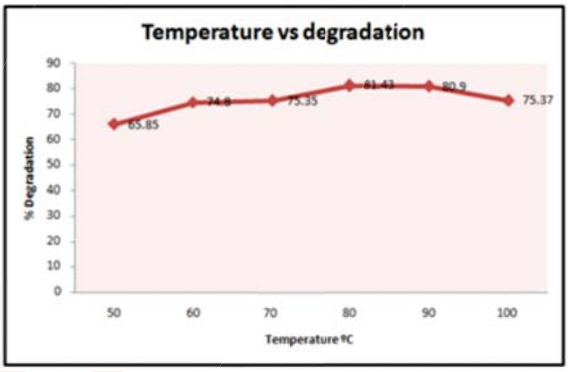

Fig. 14 : Temperature optimization for Acid Violet 4BY dye degradation using laccase enzyme

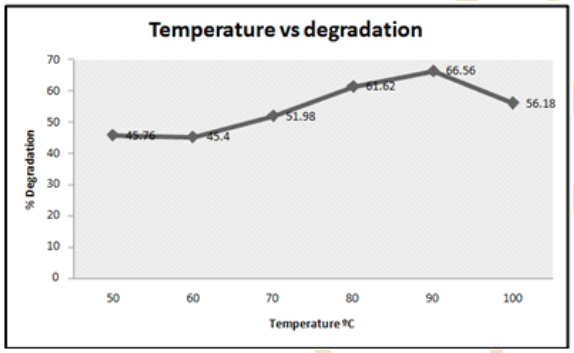

Fig. 15 : Temperature optimization for Acid Brilliant Black dye degradation using laccase enzyme

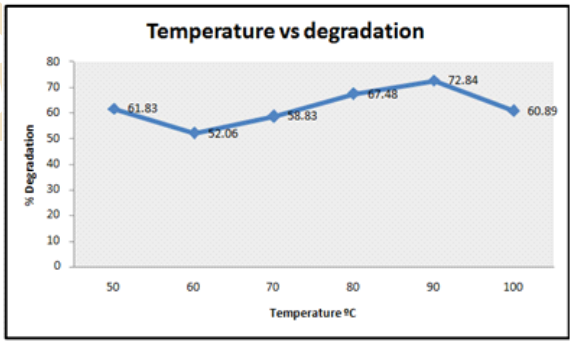

Fig. 16 : Temperature optimization for Direct Blue E dye degradation using laccase enzyme

All the above mentioned studies were affected by temperature. They all proved enzyme deactivation above optimal temperature (Munoz *et al.,* 1997). Laccase activity examined in the current study showed optimum temperature of 90°C and was stable at higher temperature than laccase purified through mesophilic fungi, such as *Pyncoporus cinnabarinus* (Eggert *et al*., 1996). Hence, laccase activity investigated in the present study revealed its thermostability.

The optimum pH for dyes Acid Violet 4BY, Acid Brilliant Black, and Direct Blue E was found to be 5.0, whereas Velu *et al.* (2011) reported 5.5 as optimum pH. The

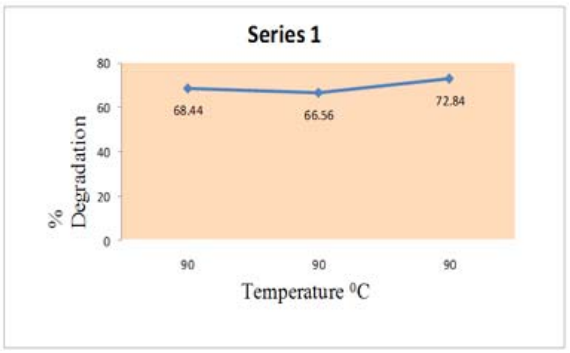

Fig. 17 : Comparison of percentage degradation of Acid Violet, Acid Brilliant Black and Direct Blue E dyes using laccase

results obtained from GC-MS examination revealed that among the indicated compounds, few of them were notable for their characteristic biological capacity, 0.29% (-) Loliolide, 0.91% 2,3-Dihydro-Bezofuran, 65.93% 4-O-Methylmannose. These compounds are building blocks of these three dyes and are responsible for characteristic decolurisation, depending on the dye's individual morphology and activity.

Therefore, the present comparative study showed the potential of laccase to decolorize three textile dyes- Acid Violet 4BY, Acid Brilliant Black and Direct Blue E. The results showed that the percentage degradation by Direct Blue E dye was found to be maximum as compared to other two dyes.

Direct Blue E showed maximum capability of degradation than Acid Violet 4BY and Acid Brilliant Black, due to greater impact of laccase enzyme on Direct Blue E dye. These results could be further implemented in product formation such as bar like stain removers, spray type products, addition in detergent, etc.

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