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Comparison Study of Textile Dye Decolorization by Wild and Mutant Strain of White Rot Fungi from Various Geographical Regions in Tamilnadu

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ABSTRACT *Many natural dyes have been known for a long time. They were obtained from animal and vegetable sources. Organic compounds have an ability to impart their color to the material to be dyed, in an aqueous medium. Dyes can be classified according to their chemical constitution or on the basis of application to fibers. Fungi from the Basidiomycetes group are known as white rot fungi are heterogenous group of microorganisms but have in common the capacity to degrade lignin as well as other wood components. The white rot fungi are by far the most efficient liginiolytic microorganisms. They are able to degrade a wide variety of recalcitrant pollutants including various types of dyes. In this study white rot fungi from various geographical regions were collected and screened for laccase production. Potential strains were selected and classical mutation study was carried out and finally dye decolorization was compared between wild and mutant.*

Key words : Textile Dye, White Rot Fungi, Laccase, decolorization assay, UV mutation

Introduction

Fungi are known to degrade, or cause to deteriorate a wide variety of materials and compounds. They can degrade different type of wood, stored paper, textiles, plastics, leather and various wrapping materials. They can assist in deterioration of concrete or can cause decay of wall paintings or can even attack ancient and medieval glass surfaces. Bioremediation of textile effluents using microorganisms can transfer toxic dyestuffs into non toxic (Varsha Zope *et al*., 2007). Moreover, the discovery of the value of white-rot fungi in bioremediation has brought a great success in this field. A significant progress has been achieved in the area of the white-rot fungi growth and enzyme production with the aim of enhancement the enzyme production.

The designation "white rot fungi" refers to the members of the *Basidiomycetes,* a class of fungi that degrade lignin more rapidly than carbohydrates during the decay of wood under aerobic conditions. As the colored lignin pigments are degraded, the decaying wood acquires a white appearance. During early development, the filamentous organisms consist of undifferentiated, non-pigmented generative hyphae that grow through cell enlargement, and tip extension before developing into specialized hyphae. An ecological advantage of fungi over bacteria pertains to their ability to secrete enzymes into the environment from the growing tips of their filamentous hyphae. Various *Basidiomycete* species have been investigated for their capacity to produce ligninolytic enzymes within industrial processes or for the treatment of contaminated effluents, soil, or aquifers.

Due to rapid industrialization and urbanization, a lot of chemicals including dyes are manufactured and used in day-to-day life. Dyes are synthetic and aromatic molecular structural compounds. According to their dissociation in an aqueous solution, dyes can be classified as acid, direct reactive dyes (anionic), basic dyes (cationic) and disperse dyes (nonionic). Colored industrial effluents from the dyeing industries represent major environmental problems. Unbound reactive dyes undergo hydrolysis due to temperature and pH values during the dyeing processes (Sathya moorthy P**.,** *et al.*, 2006).

Disposal of untreated effluent to the surroundings often leads to the following consequences (a) makes the water bodies colored and creates aesthetic problem (b) limits the re oxygenation capacity of the receiving water and cuts-off sunlight which in turn disturbs the photosynthetic activities in the aquatic system and (c) causes chronic and acute toxicity. Thus it is mandatory to treat dye bath effluents prior to discharge into the surrounding aquatic systems (Srikumaran N**.,** *et al.,* 2001).

Once in the environment, certain dyes, containing azo linkages, have the potential to form carcinogenic breakdown products; whilst concerns have been raised over the toxicity of certain metal complex dyes. Textile effluent is a cause of significant amount of environmental degradation and human illnesses. About 40 percent of globally used colorants contain organically bound chlorine a known carcinogen. All the organic materials present in the wastewater from a textile industry are of great concern in water treatment because they react with many disinfectants especially chlorine. Chemicals evaporate into the air we breathe or are absorbed through our skin and show up as allergic reactions and may cause harm to children even before birth (Rita Kant, 2012). Due to increasingly stringent environmental legislation the textile industry in the UK and elsewhere is seeking to develop wastewater remediation technologies.

Enzymes Produced By White Rot Fungi:

White-rot fungi posses a great range of different enzymes such as hydrolytic enzymes (cellulase, pectinase, xylanase) and extracellular ligninolyticenzymes (lignin peroxides, manganese peroxidase and laccase). The expression pattern of these enzymes depends on the organism itself: some white-rot fungi produce lignin peroxidase and manganese peroxidase, but not laccase, while the other produces manganese peroxidase and laccase, but not lignin peroxidase. Therefore, among different types of white-rot fungi, some can equally decompose all of the three lignocellulose components in wood material, while some can degrade lignin and hemicellulose leaving cellulose intact. Most of these enzymes are industrially important and have the great potential in processes of bioremediation, biodegradation, biopulping, degradation detoxification of recalcitrant substances (Marina Tisma *et al*., 2010).

Some white rot fungi produce all three LME while others produce only one or two of them. LME are essential for lignin degradation, however for lignin mineralization they often combine with other processes involving additional enzymes. Such auxiliary enzymes (by themselves unable to degrade lignin) are glyoxal oxidase and superoxide dismutase for intracellular production of H_2O_2, a co -substrate of LiP and MnP, as well as glucose oxidase, aryl alcohol oxidase and cellobiose dehydrogenase involved in feedback circuits and linking lignolysis with cellulose and hemicellulose degradation in nature (Dirk Wesenberg *et al*.,2003).

Laccases (benzenediol:oxygen oxidoreductases, E.C. 1.10.3.2) are an interesting group of multicopper enzymes produced by higher plants and fungi that catalyze the oxidation of a wide range of organic compounds, such as phenols, in the presence of molecular oxygen. The most important applications discussed for laccases include pharmaceutical and food industries, textile effluent transformation, and wastewater detoxification biosensors. The unique properties of laccases, such as high stability in solution, mild reaction conditions, and selectivity for phenolic structure, make them attractive for use

in chemical synthesis. The mechanism of phenolic ring oxidation by laccase has been previously described. Releasing molecular nitrogen, instead of the formation of low molecular weight aromatic amines that are easily absorbed through the skin and known as powerful carcinogens and mutagens, could be considered as an advantage for using laccase for detoxification of synthetic dyes especially aromatic azo dyes. Wastewater released by various industries that use synthetic dyes because of their low cost, ease of synthesis, and color variety can pollute and harm the aquatic environment (Marina Tisma *et al*., 2010)

Laccase only attacks the phenolic subunits of lignin, leading to Cα oxidation, Cα-Cβ cleavage and aryl-alkyl cleavage. Laccases are able to reduce one molecule of dioxygen to two molecules of water while performing one-electron oxidation of a wide range of aromatic compounds, which includes polyphenols, methoxy-substituted monophenols and aromatic amines. This oxidation results in an oxygen-centred free radical, which can then be converted in a second enzyme-catalysed reaction to quinone. The quinone and the free radicals can then undergo polymerisation (Johannes *et al*., 2000).

MATERIALS AND METHODS

Collection of samples and processing

The sample was collected from natural habitat such as Dead wood, Textile effluent, Living tree, Soil and Living tree from various places of Tamilnadu.

The sample was serially diluted and inoculated in Potato Dextrose Agar medium plate, and was identified as per the standard procedure. Mycelium was isolated by aseptically transferring the upper unexposed part of the basidiocarp on Potato Dextrose Agar (PDA). The plates were incubated at 28 - 30 °C for 7-10 days under dark conditions. Distinct fungal colonies were isolated and repeatedly sub cultured until pure cultures were obtained. The cultures were maintained on PDA slants at 5 °C. Wood sample collected under aseptic conditions were transported to the laboratory as soon as possible for fungal examination especially for the presence of White rot fungi.

Preservation and maintenance

Pure fungal isolates were obtained on the PDA plates; these isolates were further sub-cultured on PDA slants and incubated at room temperature. After sufficient growth was obtained, the slants were stored in refrigerator and served as stock cultures. Subcultures were routinely made every 30 to 60 days.

Spore suspension preparation

A mycelium disc of 1.2 cm diameter obtained from a 4 to 5 days old culture plates of fungus were transferred to 25 ml PDA in a 250 ml conical flask and incubated at room temperature for 4 to 5 days. At the end of the incubation period 30 ml sterile water was added to each culture and the flasks were shaken with shaker. Then the content of each conical flasks were filtered through glass wool. The filtrate contained spores and were used for spore count on PDA. The same spore suspension was used in the experiments described below.

Identification of White rot fungi:

The WRF is identified by its morphological growth of mycelial pad using lacto phenol cotton blue staining procedure.

Lacto phenol cotton blue staining:

Placed a drop of lacto phenol cotton blue on a clean glass slide and removed the small portion of colony on PDA agar plate with a sterile inoculation wire and placed it in a drop of LPCB. Placed the cover glass and above the stain apply gentle pressure. Allowed the preparation for staining for 15minutes. Examined the preparation microscopically and observe the mycelial pad.

Collection of Commercial dyes and chemicals

The textile dyes yellow, blue and red were obtained from Tirupur textile dye industry, Tamilnadu, India.

Decolorization study using wild and mutant white rot fungi

Mutagenesis study using UV

UV mutation

Cell suspension was used for UV mutation. 4 ml of cell suspension was poured in 5 Petri plates. These plates were placed in UV light at distance of 30 cm away from UV lamp. The different time exposures are 5, 10, 15, 20 &25 minutes. Each plate was stored in dark over night. The strain growth was better in 5 minutes exposed plate than the other plates .Hence the strain from the 5 minute exposed plate was taken for decolorization study. This was followed by Bhargavi Moturi, *et al*., 2010.

Decolorization study using wild and mutant strain:

In 100 ml of Potato Dextrose Broth 10 ml of WRF both wild and mutant strain was added individually in two different flasks and 10 ml of textile dye was added and incubated at 37˚C. The 5ml treated textile dye was centrifuged at 10,000 rpm for 10 min and decolorization was assessed by measuring the absorbance of the supernatant at 520nm using spectrophotometer. The percentage of decolorization was calculated using the following formula: % of Decolorization = Initial OD-Final OD x 100/Initial OD. This was done according to Bhargavi Moturi, *et al.*, 2010.

Determination of Decolorization:

The 5ml treated textile dye was centrifuged at 10,000 rpm for 10 min and decolorization was assessed by measuring the absorbance of the supernatant at 520nm using spectrophotometer. The percentage of decolorization was calculated using the following formula;

% of Decolorization = Initial OD-Final OD x 100/Initial OD.

Estimation of Laccase by Lowry method:

The protein was estimated according to the method of Lowry. Pipette out various Concentration of working standard solution and serially of test tubes. Made-up the volume of 0.2ml with distilled water (20,40,60,80&100ul).

To each test tube add 1 ml of the mixed Reagent **(Solution1: Alkaline sodium carbonate+Solution2: Copper sulphate + solution3: Sodium potassium tararate).** Mix thoroughly and allow standing at room temperature for 10-15. Add 0.3ml of diluted folin-ciocalteau rapidly and Mix properly. Incubate at tubes for 60 mins in a water bath at 50^{0}C. Measure OD at 660nm.

Result and discussion

Lignin is an aromatic heteropolymer of phenyl-propanoid units which confers structural rigidity to woody plant tissues and protects them from microbial attack. To depolymerize and mineralize lignin, white rot fungi have developed a nonspecific oxidative system including several extracellular oxidoreductases, low-molecular-weight metabolites, and activated oxygen species. The ability of white rot fungi to degrade a wide number of organopollutants is in part due to the action of this nonspecific system. Extracellular enzymes involved in the degradation of lignin and xenobiotics by white rot fungi include several kinds of laccases, peroxidases , and oxidases producing H_2O_2.

Collection of fungal samples

The present study identified Tirupur, Namakkal, perundurai, Salem and Ercode areas of Tamil Nadu state, India wherein; an abundance of textile industry and white rot fungi. A total of 130 samples were collected from which 87 strain of white rot fungi were isolated. The result revealed that there was no correlation drawn between the number of samples obtained and isolation of white rot fungi strains. A total of 35 textile effluent samples were collected from Tirupur, 27 dead wood samples were obtained from Namakkal, 20 living tree samples from Perundurai, 23 soil samples from Salem and 25 living tree samples were collected from Ercode districts of Tamil Nadu for the present study (Table 1 and Figure 1).

Microbes isolated from various Geographical area of Tamil Nadu

Out of 130 samples collected from five different districts of northern part of Tamil Nadu only 87 isolates were found to be fungi species. Highest number of fungi were isolated from samples collected from Tirupur (26 isolates) which are from textile effluent, followed by 19 isolates from dead wood samples collected from Namakkal and 17 isolates from living tree samples of Ercode. Least number of fungi isolates was found to be in living tree samples collected from Perundurai isolates (11 numbers) (table 2).

Identification of White rot fungi:
Lacto phenol cotton blue staining:
Generally lacto phenol cotton blue is used for staining the fungal culture. Jhadav *et al.* had performed lacto-phenol cotton blue staining method for the fungal culture. The white rot fungus isolated in the present study was stained with lacto phenol cotton blue and observed under microscope. The result obtained is presented in Figure 2. The blue colored stain as observed under microscope confirmed that the isolate organism is the white rot fungi species.

Mutagenesis study using UV
The potent strain was mutated by exposing to UV at different periods of 5,10,15,20 and 25 minutes. The mutants that were exposed to expensive period of 10 to 25 minutes radiation showed reduced growth rate. Only in 5 minutes exposed plate shows mild increase in growth rate. Similar procedure was carried out by Bhargavi Moturi *et al* (2010) were the strains that were exposed to UV radiation for a time period of 3, 6 and 9 min did not vary much from the wild strain in mycelial morphology as well as in the growth pattern, instead they showed mild increase in growth rate, but the mutants that were exposed to extensive period of time such as 12 and 15 min to UV radiation showed reduced growth rate as well as reduction in extension and branching of hyphae.

Decolorization Assay
Determination of Decolorization
The 5ml treated textile dye was centrifuged at 10,000 rpm for 10 min and decolorization was assessed by measuring the absorbance of the supernatant at 520nm using spectrophotometer. The percentage of decolorization was calculated using the following formula and the rate of decolorization had been presented in figure 3.

% of Decolorization = Initial OD-Final OD x 100/Initial OD.

Estimation of Laccase enzyme produced during decolorization
As a result of Lowry's method, total concentration of protein was found to be high in mutated strain as (870 µg) when compared to synthetic media (550 µg). The standard curve was drawn based upon the optical density values measured at 660nm in which BSA was used as standard. The protein react with copper in an alkaline medium and the Phosphomolybdate present in Folin's Ciocalteau reagent react with aromatic amino acids such as tyrosine and tryptophan, reduces phosphomolybdate to a blue color which as sharp absorption maximum of 660 nm. The intensity of the color depends on the amount of these amino acids present. The Lowry's method has been previously used by Risna *et al*., 2002 for estimating the total enzyme concentration (figure 4).

Discussion
Laccase were first described in 1883 from the Japanese lacquer tree *Rhus vernicifera* . Since then, several laccase have been studied with respect to their biological function, substrate specificity, copper binding structure, and industrial applications.

It supports the degradation of various xenobiotic compounds and has emerging applications in production of biosensors and biofuels cells. Hence it is no surprise that such an enzyme will be of considerable market value. However, as this enzyme is secreted in very low amounts by the organism, a lot of work is being carried out in increasing the production output and optimization.

For this current research work, wood decay fungal sample was collected from Berijam forest, Kodikanal. The collected sample was cultured on PDA media supplemented with Benomyl and Chloramphenicol. Particularly Benomyl was used to select wood decay fungi and Chloramphenicol was used to inhibit the unwanted bacterial growth. In order to find laccase producing fungi from the fungi isolated from decayed wood samples, a simple screening method was followed using solid media containing indicator compound guaiacol.

The isolated white rot fungi were screened for laccase production on PDA media supplemented with Guaiacol. If the isolated white rot fungus produces laccase, it catalyzes the oxidation of Guaiacol to form reddish brown color in the medium.

The positive fungal strain was cultivated by submerged fermentation method using both wild and mutant type. By using Guaiacol and acetate buffer enzyme activity was measured in u/ml which is defined as the amount of enzyme catalyzing the production of one micromole of colored product per min per ml. As a result of this assay laccase production was found to be Volume activity (0.00038u/ml). As a result of Lowry's method, total concentration of protein was found to be (Yellow-10 mg/ml, Blue- 9.1 mg/ml, Red- 9.8mg/ml) when compared to wild strain (Yellow-6.5 mg/ml, Blue- 7.0 mg/ml, Red- 7.2mg/ml).

The standard curve was drawn based upon the optical density values measured at 660nm in which BSA was used as standard. The protein react with copper in an alkaline medium and the Phosphomolybdate present in Folin's Ciocalteau reagent react with aromatic amino acids such as tyrosine and tryptophan, reduces phosphomolybdate to a blue color which as sharp absorption maximum of 660 nm. The intensity of the color depends on the amount of these amino acids present.

Figure 1.Collection of sample.

Table 1: Collection of Samples from various geographical areas for fungal.sps.

Sl.No.	Place	Month and year	Number of samples	Source
1.	Tirupur	May 2011	27	Textile effluent
2.	Namakkal	Aug 2011	35	Dead wood
3.	Perundurai	Oct 2011	20	Living tree
4.	Salem	Dec 2011	23	Soil
5.	Ercode	Mar 2012	25	Living tree

Table 2: Fungal isolated from various Geographical area

Sl.No	Geographical Area	Collected samples	Number of fungal. sps
1.	Tirupur	27	19
2.	Namakkal	35	26
3.	Perundurai	20	11
4.	Salem	23	14
5.	Ercode	25	17
Total		130	87

Fig.2: Lacto phenol cotton blue staining

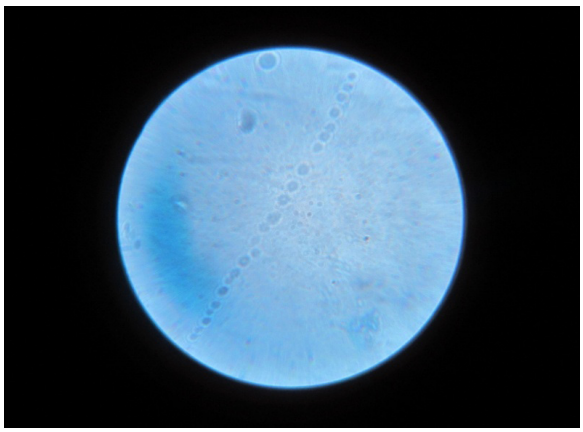
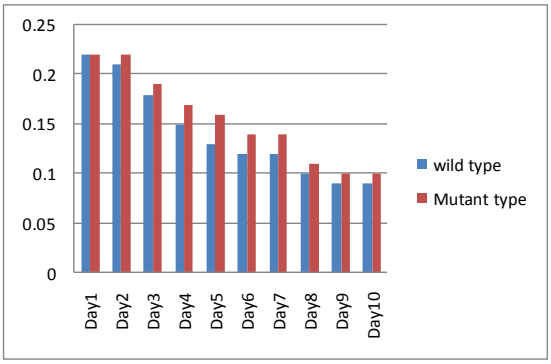
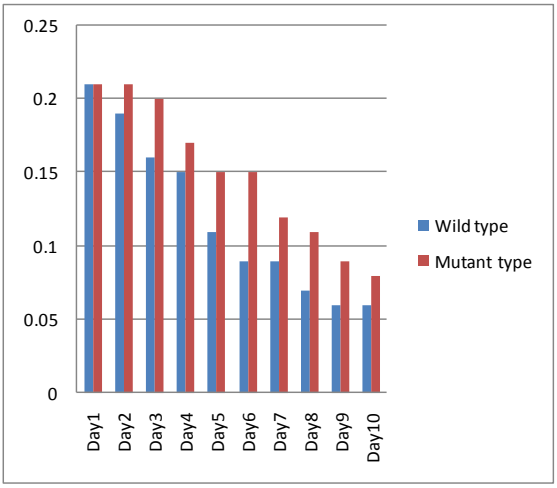


Figure 3. Decolorization Assay

3.1. Comparative study of Wild and Mutant type Textile dye degradation –Yellowcolor



3.2. Comparative study of Wild and Mutant type Textile dye degradation –Blue color



3.3. Comparative of Wild and Mutant type Texile dye degradation –Red color

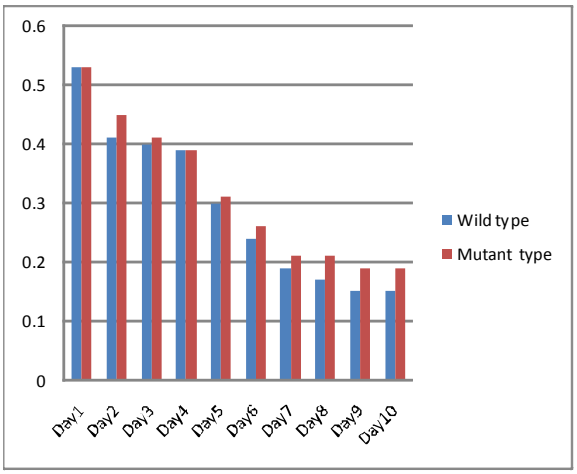
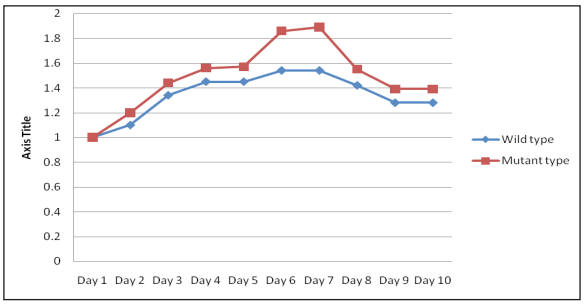
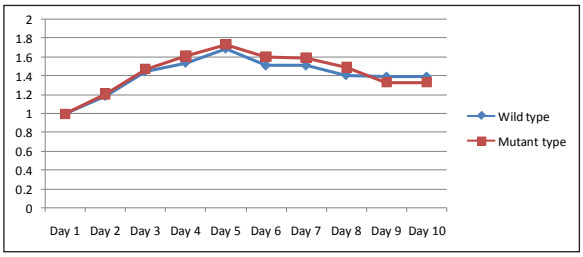


Figure 4. Comparative study of Wild and Mutant type estimation of laccase

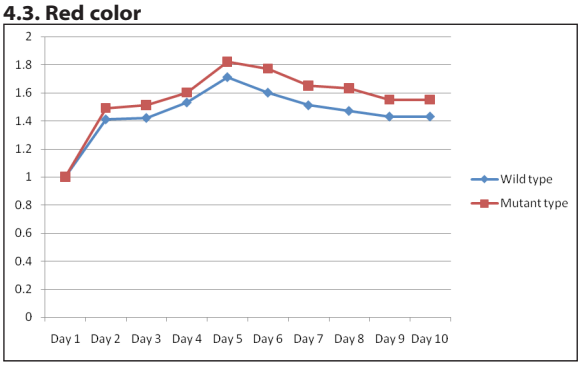
4.1. Yellow color



4.2. Blue color



4.3. Red color



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