

Chapter 5

Applications of Cerrena unicolor MTCC 5159 in Bioremediation



ABSTRACT

Whole culture of *Cerrena unicolor* MTCC 5159 and its lignin-degrading enzymes (LDEs) especially laccases along with its exopolymeric substance (EPS) have been implicated in the decolourization of coloured substances. The fungal mycelia and the EPS decolourize using processes such as active and passive adsorption and absorption whereas the LDEs actively degrade the colourants. Triarylmethane and triphenylmethane dyes are good substrates and also good inducers of these laccases. Diazo dyes act as laccase inducers and are preferentially degraded over monoazo dyes. However, the decolourization of some of the diazo dyes may not be proportional to laccase activity. When applied in biobleaching of sugarcane bagasse, it was found that these laccases are sufficient even at low concentrations. However, the timely addition of the appropriate mediator as soon as repolymerization begins not only reverses repolymerization but also hastens the biobleaching process. *Cerrena unicolor* MTCC 5159 has a great potential in decolourizing dyes and coloured effluents as well as biobleaching of lignocellulosic pulp.

5.1 INTRODUCTION

Industrial effluents especially coloured ones from textile & dye and paper & pulp industries are very difficult to treat. They contain compounds with complex aromatic structures which make them quite difficult to degrade. These dyes are classified as **anionic** dyes which include direct, acid and reactive dyes; **cationic** dyes which include the basic dyes and **nonionic** dyes which include the disperse dyes (Fu & Viraraghavan, 2001). They can also be classified according to the chromophore they contain (Table 1; Fig. 1).

Table 1: Classes of synthetic dyes according to Colour Index (C.I)
(Wesenberg *et al.*, 2003).

Code	Chemical Class	Code	Chemical Class	Code	Chemical Class
10000	Nitroso	42000	Triarylmethane	53000	Sulfur
10300	Nitro	45000	Xanthene	55000	Lactone
11000	Monoazo	46000	Acridine	56000	Aminoketone
20000	Disazo	47000	Quinoline	57000	Hydroxyketone
30000	Trisazo	48000	Methine	58000	Anthraquinone
35000	Polyazo	49000	Thiazole	73000	Indigoid
37000	Azoic	49400	Indamine/Indophenol	74000	Phthalocyanine
40000	Stilbene	50000	Azine	75000	Natural
40800	Carotenoid	51000	Oxazine	76000	Oxidation Base
41000	Diphenylmethane	52000	Thiazine	77000	Inorganic

The chromophores in anionic and nonionic dyes are mostly azo groups or anthroquinone types. The reductive cleavage of azo linkages results in the formation of toxic amines. Sulphonated azo dyes with their high water solubility make their removal difficult (Zhao *et al.*, 2005). Anthroquinone-based dyes are most resistant to degradation due to their fused aromatic structures and effluents containing such structures retain their colour for a longer time. Basic dyes have a high brilliance and intensity and are highly visible even at very low concentrations. The metal-complexed dyes are mostly based on carcinogenic chromium. Among these dyes, the azo dyes account for over 60 % of the total dyes manufactured (Fu & Viraraghavan, 2001).

Table 2: Details of the synthetic dyes used in this study<http://stainsfile.info/StainsFile/dyes>

Dye [CAS [#] No.] (Colour Index No.)	Synonyms	Chromophore	λ_{\max} (nm)
Brilliant Green [633-03-4] (42040)	Malachite Green G, Emerald Green, Solid Green JO, Diamond Green G, Aniline Green, Benzaldehyde Green, Fast Green J, Basic Green 1, Astradiamant Green GX, Ethyl Green	<i>Triphenylmethane</i>	623
Crystal Violet [548-62-9] (42555)	<i>p</i> -rosaniline hydrochloride, gentian violet, aniline violet, bismuth violet, hexamethyl violet, methyl violet, methyl violet 10B, paper blue R, oxyozyl, methylrosaniline chloride, adergon, aizen, atmonil, avermin, axuris, badil, brilliant violet 5B, calcozine violet C, gentacid, gentioletten	<i>Triphenylmethane</i>	589
Aniline Blue [8004-91-9] (42755)	Water Blue, Acid Blue 22, Soluble Blue 3M, Marine Blue V	<i>Triarylmethane</i>	585
Methylene Blue [61-73-4] (52015)	Basic Blue 9, Chromosmon	<i>Phenothiazine</i>	663
Reactive Orange 16 [12225-83-1] (17757)	Brilliant Orange 3R, Red Label 706, Apricot, Strong Orange LR202, Remazol Brilliant Orange 3R	<i>Azo</i>	499
Trypan Blue [72-57-1] (23850)	Diamine Blue, Chloramine Blue, Benzamine Blue, Dianil Blue, Niagara Blue B	<i>Diazo</i>	599
Congo Red [573-58-0] (22120)	Direct Red 28, Cotton Red	<i>Secondary Diazo</i>	486
Remazol Brilliant Blue R [2580-78-1] (61200)	Reactive Blue 19, Red Label 722, Royal Blue, Intense Blue LR406	<i>Anthroquinone</i>	597
Poly-R 478 [68550-77-6] Red poly (vinylamine) sulfonate- anthrapyridone		<i>Anthrapyridone</i>	513 ÷ 362

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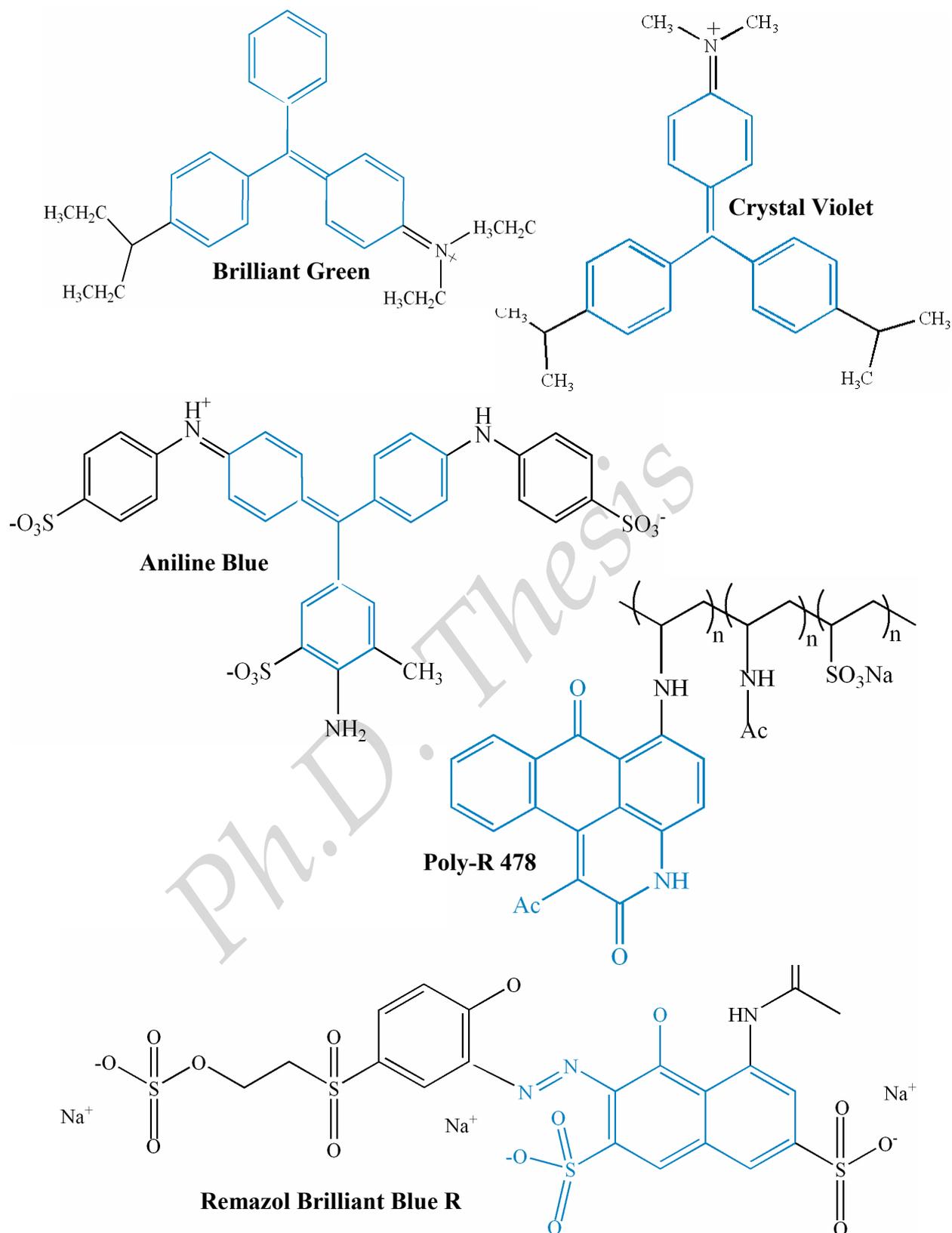


Fig.1: Molecular structure of the synthetic dyes used in this study.

The chromophore is in blue.

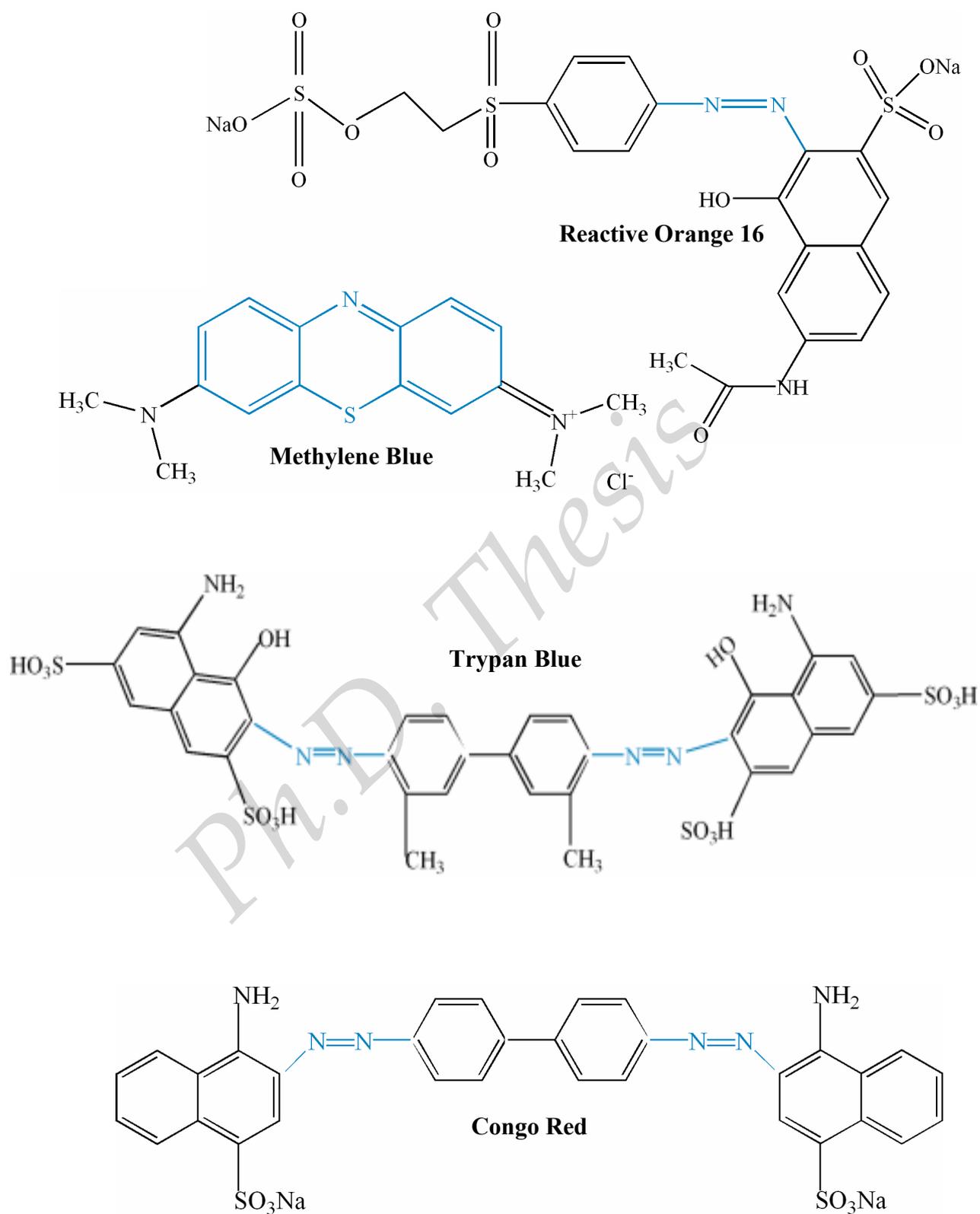


Fig.1 (contd): Molecular structure of the synthetic dyes used in this study.

The chromophore is in blue.

Table 3 : Advantages and disadvantages of the current methods of dye removal from industrial effluents (Robinson *et al.*, 2001b).

Physical/Chemical methods	Advantages	Disadvantages
<i>Fentons reagent</i>	Effective decolouration of soluble and insoluble dyes	Sludge generation
<i>Ozonation</i>	Applied in gaseous state, no alteration of volume	Short half-life (20 min)
<i>Photochemical</i>	No sludge production	Formation of by-products
<i>Sodium hypochlorite</i>	Initiates and accelerates azo-bond cleavage	Release of aromatic amines
<i>Cucurbituril</i>	Good sorption capacity for various dyes	Expensive
<i>Electrochemical destruction</i>	Break-down compounds are non-hazardous	High electricity consumption
<i>Activated carbon</i>	Good removal of a wide variety of dyes	Very expensive
<i>Peat</i>	Good adsorbent due to cellular structure	Specific surface areas for adsorption are lower than activated carbon
<i>Wood chips</i>	Good sorption capacity for acid dyes	Requires long retention times
<i>Silica gel</i>	Effective for basic dye removal	Side reactions prevent commercial application
<i>Membrane filtration</i>	Removes all dye types	Concentrated sludge production
<i>Ion exchange</i>	No adsorbent loss due to regeneration	Not effective for all dyes
<i>Irradiation</i>	Effective oxidation at laboratory scale	Requires high concentrations of dissolved oxygen
<i>Electrokinetic coagulation</i>	Economically feasible	High sludge production

Besides producing an aesthetically unacceptable intense colouring of water and soil bodies, they also result in decreased gas solubility in water bodies as well as block the passage of light to the lower depths of the aquatic system. This results in cessation of photosynthesis, leading to anaerobic conditions, which in turn results in the death of aquatic life causing foul smelling toxic waters. More importantly many dyes have carcinogenic origins (Fu & Viraraghavan, 2001).

Due to the low biodegradability of dyes, conventional effluent treatment systems are inefficient in treating waste water. It is usually treated by physical or chemical treatment processes (Table 3). These include physical or chemical flocculation combined with flotation, electro-flotation, flocculation with Fe^{+2} / $\text{Ca}(\text{OH})_2$, membrane-filtration, electro-kinetic-coagulation, electrochemical-destruction, ion-exchange, irradiation, precipitation, ozonation and Katox treatment method involving the use of activated carbon and air mixtures. However besides being inefficient, these technologies are expensive and have low adaptability to diverse types of dye effluents. Adsorption has been observed to be an effective process for colour removal from such effluents. Adsorption on activated charcoal is an effective method but is prohibitively expensive. Low-cost adsorbents have generally low adsorption capacities mediating the need for economical and easily available yet highly effective adsorbents (Fu & Viraraghavan, 2001).

In order to be economically viable, bioremediation methods however require to be safe and less expensive than conventional treatments. Bacteria and fungi along with their products such as enzymes especially the lignin-degrading enzymes (LDEs) and exopolymeric substances (EPS) have been the object of study for quite some time for use in bioremediation (D'Souza *et al.*, 2006). Bioadsorption is a means of bioremediation and includes the use of both living and dead cells of bacteria, fungi, algae, plant biomass or a combination thereof (Robinson *et al.*, 2001b). When only enzymes are involved, maintaining the optimum catalytic activity for a long period under controlled conditions for the treatment of massive volumes of effluents is a rather difficult task (Pritchard *et al.*, 2006). However the use of live cells especially fungal mycelia mediates remediation through several ways. In most cases the dyes adsorb onto the mycelial surface, which is the primary mechanism of decolourization, after which the LDEs come into play. Physical adsorption of the dyes onto the fungal hyphae, and degradation by extracellular

LDEs and / or absorption into the mycelia and subsequent degradation by intracellular LDEs and their associated enzymes are the means of colour removal. The dye-saturated mycelia can be regenerated and used for repeated dye adsorption. This sequential adsorption and degradation of dye molecules makes bioremediation through adsorption a feasible process. Batches of industrial effluents are not identical to each other. They are extremely varied, the adaptability of biological methods to varied conditions gives bioremediation an edge over the physico-chemical methods (Selvam *et al.*, 2003).

The LDEs include the laccases and the peroxidases. These LDEs act on lignin and lignin-analogous compounds since they share common features such as broad substrate specificity, high redox potential and are mostly extracellular in nature. The high redox potential and broad substrate specificity increases the range of pollutants that the enzymes are capable of degrading. These features combined with the fact that LDEs are mostly expressed under nutrient deficient conditions (which is usually the case in the nature) and their ability to oxidize substrates with low solubility have made them the preferred candidates for bioremediation along with the fungi, responsible for their production.

The process of bioremediation can be monitored by measuring any of the following factors: (1) by measuring the redox potential, together with pH, temperature, oxygen content and concentrations of electron acceptor (s) / donor (s) and the breakdown products such as carbon dioxide or (2) by measuring chemical oxygen demand (COD) and biological oxygen demand. Biological oxygen demand (BOD) represents only the organic matter which is capable of being degraded / oxidized by microbes whereas COD represents all the oxidizable matter, including organic matter in any particular effluent. For coloured effluents, bioremediation is measured by estimating the reduction in colour and percentage of detoxification achieved (Marmagne & Coste, 1996).

Industrial effluents also create effluent-specific problems besides the ones mentioned earlier. Mentioned below are problems specific to the effluents that have been used in this study.

Textile effluents are highly coloured effluents released by the textile and dye manufacturing industries that contain large amounts of mixtures of dyes. These dyes are mostly resistant to degradation leading to deleterious effects on aquatic life. Textile

effluents besides containing dyes also contain salts, often at very high ionic strength and have extreme pH values as well. Marine fungi may find application in the decolourization of industrial effluents that contain large amounts of salt and extreme pH values (Raghukumar *et al.*, 2008).

Molasses Spent Wash (MSW) is a by-product of sugar mills and alcohol distilleries, where the starting material is molasses. It contains mostly dark brown coloured recalcitrant compounds formed by the Maillard amino-carbonyl reaction, collectively termed as melanoidin. These compounds are toxic to many microorganisms including those generally involved in wastewater treatment processes. Anaerobic digestion of MSW-containing effluents is one of the treatments followed by distilleries and the resulting dark brown sludge is used as fertilizer. Dark brown colour of these effluents remains a major problem for the distilleries. Laccase and MnP along with glucose oxidase and sorbose oxidase have been used in the decolourization of MSW. The H₂O₂ produced by the oxidases may act as a bleaching agent by itself or assist the peroxidases in the decolourization process (Dehorter & Blondeau, 1993).

Black Liquor (BL) is an intensely coloured effluent released in large volumes by the paper & pulp industry. These effluents contain toxic chlorinated lignin-degradation products which include chlorolignins, chlorophenols and chloroaliphatics (Ali & Srekrishnan, 2001). Besides, these highly alkaline effluents with a pH ranging from 8 – 11, alter the pH of the soil and water bodies into which they are discharged. Among the LDEs, the peroxidases especially MnP, play a major role in the decolourization of this effluent. Since most of the LDEs have optimum activity between pH 3 – 6 and fungi in general show preferential growth under acidic conditions, BL is usually acidified prior treatment with these fungi. Decolourization of such effluents without the requirement of prior acidification would be useful both economically and environmentally, since acidification results in the water and soils becoming acidified after their discharge (Wu *et al.*, 2005).

It has been reported that effluents from paper and pulp mills and textile dye wastewaters are toxic and mutagenic besides being coloured. In these effluents, laccases have been shown to render phenolics, less toxic via degradation or polymerization

reactions or by cross-coupling of aromatic pollutants with naturally occurring phenols. Besides decolourization, detoxification of wastewaters is an important parameter which also requires monitoring (Raghukumar *et al.*, *in press*).

Paper is manufactured from wood, for this, a pulp of the woody (lignocellulosic) substrate requires to be prepared after which, the required cellulose fibers have to be separated from the hemicellulose and lignin. These cellulose fibers have to be then freed from all traces of lignin so that no traces of colour are present and finally it undergoes finishing producing the final product, paper.

The main processes for paper-pulp production that are currently in use are mechanical and chemical. Using fungi to convert wood chips into paper pulp is termed as '**biopulping**'. The fungus currently used in biopulping by 'BioPulping International' is *Ceriporiopsis subvermispora* and is reportedly easy to maintain. The wood is treated with steam creating a ventilation system that provides a good environment for the fungus to thrive in. Fungi decay the woody substrate to alter the lignin in the cell walls of the wood, which therefore 'softens' the wood chips. This process is non-polluting since it is chemical-free and since the wood is already softened, the remaining steps of the process require less energy. Biopulping provides a solution to the problems of chemical and mechanical paper production. It also reduces electrical energy needed by an average of 25 - 30 % it also saves about \$ 9 - \$ 20 per ton of pulp (<http://www.biopulping.com/2.html>).

The paper-pulp is then subjected to alkali-treatment to free the cellulose fibers from the hemicellulose and lignin. This is then bleached to reduce the colour due to residual lignin still present in the cellulose. Bleaching is done using chlorine, this process however is not environmental friendly. Fungi that live on decaying plant material specially the white-rots, on the other hand produce enzymes which break down hemicellulose and lignin and provide an environmental-friendly alternative to chlorine bleaching and is termed '**biobleaching**'. At present, this has not yet completely replaced chlorine but only augments the bleaching process, thereby decreasing the total residual chlorine load in the effluents. In theory, it should be possible to use the LDEs and laccase-mediator systems (LMS) in the pulping and bleaching phases of paper manufacture resulting in what is described as biopulping and biobleaching. Economical substrate for the fungi would include wood, fiber crops and agricultural residues, due to the variable nature of these substrates the rate of growth of the fungi, the LDE yield and

the type of LDEs produced would vary from batch to batch. Although this would result in decreased repeatability in the biobleaching process, it also means that there is increased adaptability.

Mediators are low molecular weight compounds that aid in lignin-degradation by assisting the LDEs in increasing the effective redox potential. The LMS involves use of these mediators which are oxidized by laccase to stable radicals which in turn oxidize other compounds which could not have been acted upon by laccase directly (Fig. 2). Lack of direct correlation between laccase activity and degradation of xenobiotics further supports the role of LMS. The redox potential of laccase alone is not high enough to break the carbon-hydrogen aliphatic bonds of certain compounds, the presence of a redox mediator allows for the oxidation of such bonds. The enzyme oxidizes the mediator, which can diffuse away from the enzyme and oxidize a substrate. The reduced mediator is then ready for the next cycle.

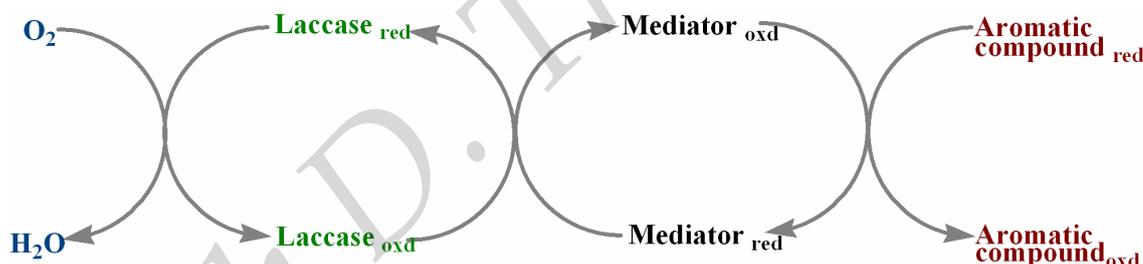


Fig. 2: Mediator-mediated oxidation of aromatic compounds by laccase. The aromatic compounds include lignin, synthetic dyes, effluents and other pollutants.

In nature, these mediators are synthetic compounds or natural compounds produced by fungi or plants. The synthetic compounds include ABTS, 1-hydroxybenzothiazole, violuric acid and N-hydroxyacetanilide (Camarero *et al.*, 2005). Natural mediators include the fungus-produced, 3-hydroxyanthranilic acid and degradation products of lignocellulose such as acetosyringone, *p*-coumaric acid, syringaldehyde and vanillin. Natural mediators have also been extracted from BL of eucalyptus-based kraft pulping. Laccase-mediator system has found applications in paper pulp delignification, degradation of Polycyclic Aromatic Hydrocarbons (PAHs) and industrial dyes (Camarero *et al.*, 2007).

5.2 OBJECTIVE

The objective of this chapter is to determine the bioremediation potential of *Cerrena unicolor* MTCC 5159 in the decolourization of synthetic dyes and industrial effluents and its laccase in the biobleaching of sugarcane bagasse.

5.3 MATERIALS AND METHODS

5.3.1. MICROORGANISM AND CULTURE CONDITIONS

Cerrena unicolor MTCC 5159 was raised as inoculum in the either LNM or B&K medium depending on whether LNM or B&K was used in the proceeding experiment. The 6 day-old culture, was then rinsed with distilled water and homogenized for 5 sec, all under sterile conditions. The mycelial suspension at 5 % v/v was used as inoculum. The day of inoculation in all experiments was considered to be day 0. Cultures were incubated at 30°C in the dark under static conditions.

5.3.2 LACCASE ASSAY

Laccase activity was estimated spectrophotometrically with the ABTS method ([Appendix 8.2.2.1.1](#)). Laccase was expressed as either U L⁻¹ or U mL⁻¹. An abiotic control without laccase had been used in every experiment and all the assay components had been brought to reaction temperature before assay. Protein concentration was estimated spectrophotometrically using Bradfords reagent (Sigma, St. Louis, Montana, U.S.A), ([Appendix 8.2.1.4](#)). All spectrophotometric measurements were carried out using UV-Vis 2450 spectrophotometer (Shimadzu, Japan). All values represent the mean of three measurements of two independent experiments each.

Decolourization of dyes and effluents by whole culture (*in vivo*)

This fungus was tested for its ability to decolourize various dyes and effluents every alternate day after the addition of the dye / effluent over a period of 6 days. Synthetic dyes such as trypan blue, aniline blue and remazol brilliant blue R (RBBR) were added at 0.04 %, methylene blue, crystal violet, brilliant green, Poly-R 478 and congo red at 0.02 % and reactive orange 16 (RO 16) at 0.015 % final concentration. All the four industrial effluents were each added at 10 and 20 % final concentrations, they included textile effluent A (TeA), textile effluent B (TeB), molasses spent wash (MSW) and black liquor (BL).

The dye / effluent was added to a 6 day-old culture grown in both B&K and LNM prepared in 25 ppt seawater. However, the results presented below are only from the B&K medium, since LNM was found to be toxic (Ramsay & Nguyen, 2002). The colour intensity of the dye in the medium at the time of addition was considered as 100 %. The extent of decolourization was recorded as % residual colour. Mean values from triplicate cultures were used for comparing the extent of decolourization of various dyes and effluents.

Decolourization of the dyes and effluents was estimated by monitoring them at their absorbance maxima. Trypan blue, aniline blue, RBBR, methylene blue, crystal violet, brilliant green, congo red and RO 16 were monitored at 599, 585, 597, 663, 589, 623, 486 and 499 nm respectively. Decolourization of Poly-R 478 was determined by the ratio of absorbance at 513 nm versus 362 nm. Industrial effluents such as TeA, TeB, MSW and BL were monitored at 505, 663, 663 and 317 nm respectively. The details of TeA, TeB, MSW and BL are given in [Appendix 8.4.1 - 8.4.4](#) respectively.

Decolourization of effluents by fungus free-culture filtrate (*in vitro*)

Cell-free culture filtrate containing 9 U of laccase was added to effluents such as TeA, TeB, MSW and BL, each at 10, 20 and 50 % final concentrations. These were then incubated at pH 6 and 60°C for 12 h. The percentage of decolourization achieved was calculated with reference to the control samples that were not treated with the enzyme and contained only the effluent at the respective concentration.

LACCASE PRODUCTION MEDIUM FOR BIOBLEACHING

The laccase production medium contained a mineral salts base consisting of 5 % (v/v) basal salts solution ([Appendix 8.1.1.1](#)), 10 % (v/v) trace elements solution ([Appendix 8.1.1.2](#)) and 1 mL of 0.005 % (w/v) thiamine. To this basal medium, 3 % glucose, 0.1 % glycine, 0.25 % Tween 80 and 0.1 % sodium chloride were added. The pH was adjusted to 4.5 using citrate-phosphate buffer and the final volume was made with distilled water. This is a modified version of the low nitrogen medium by [Tien and Kirk \(1988\)](#) and the medium optimized in the previous chapter. This is referred to as LNM in this chapter, to avoid ambiguity, with various versions of the LN medium.

For large-scale cultivation, 3-L Haffkine flasks with 1 L of LNM were inoculated with 100 mL of mycelial suspension and incubated as described above. On day 6, CuSO₄ at 2 mM was added to the cultures aseptically to stimulate laccase production. On day 12 when laccase activity reached its maximum, the cultures were vacuum-filtered to separate the mycelium. The culture filtrate was collected after passing through a series of filter papers namely; Whatman no. 1, GF/C, 0.45 µm and finally 0.22 µm (GV, Millipore, USA). This filtrate was then concentrated using ultrafiltration with positive pressure (Amicon) at 4°C. This concentrate was used as the crude laccase preparation for the biobleaching experiments. This was examined for the presence of cellulase ([Appendix 8.2.2.4](#)).

BIOBLEACHING ASSAY

Sugarcane bagasse was the substrate used for the biobleaching experiments. The lignin content of the untreated and treated bagasse samples was estimated using the 'Kappa number' method, given in [Appendix 8.2.1.3](#). Before assay, the enzyme-treated sample was rinsed with distilled water to remove the enzyme, filtered to remove the excess moisture and then immediately placed at 90°C, to inactivate any residual laccase completely dry the sample after which, the lignin content was estimated. The lignin content was calculated in terms of % Klason lignin. Biobleaching is calculated in terms of % biobleaching which is the difference between the lignin content (% Klason lignin) of the untreated or the 0 h sample and enzyme-treated bagasse samples at that sampling interval.

Effect of mediators on biobleaching at 12 and 24 h sampling intervals

Crude laccase containing 1000 U prepared in citrate phosphate buffer (pH 3) was added to 0.1 g of bagasse and incubated at 60°C in a dry bath. Mediators such as 1-hydroxybenzotriazole (HBT), veratryl alcohol (VA), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), syringic acid (SA), hydroquinone (HQ), 3',5'-dimethoxy-4'-hydroxy acetophenone (DMHAP) and 4-hydroxy-2,2,6,6 tetra-methyl piperidine-1-oxyl (HTMP) at 2 mM were added separately to the bagasse. Biobleaching was determined at 12 and 24 h. The control contained only laccase and no mediator.

Effect of ABTS on biobleaching after repolymerization had begun

Crude laccase containing 1000 U prepared in citrate phosphate buffer (pH 3) was added to 0.1 g of bagasse and incubated at 60°C in a dry bath. The biobleaching was determined at 12-h time intervals up to 96 h. The mediator, ABTS was added at 2 mM separately when repolymerization just began, to determine how this addition would affect the repolymerization process. The control contained only laccase.

Effect of ABTS on biobleaching at various time intervals.

Crude laccase containing 10, 20 and 30 U prepared in citrate phosphate buffer (pH 3) was added to 0.1 g of bagasse in the presence of 2 mM ABTS and incubated at 60°C using a dry bath. Biobleaching was determined at 12-h time intervals up to 72 h. The control contained 10, 20 and 30 U of crude laccase only.

Effect of mediators on biobleaching at various time intervals

Crude laccase containing 20 U prepared in citrate phosphate buffer (pH 3) was added to 0.1 g of bagasse and incubated at 60°C in a dry bath. Mediators such as HBT, VA, ABTS, SA, HQ, DMHAP and HTMP at 2 mM concentration were added separately to the

bagasse at 6 h. The biobleaching was determined at 12-h time intervals up to 72 h. The control contained only laccase.

Impact of time of early addition of mediators on biobleaching.

Crude laccase containing 20 U prepared in citrate phosphate buffer (pH 3) was added to 0.1 g of bagasse and incubated at 60°C in a dry bath. Mediators such as HBT, ABTS and HTMP at 2 mM concentration were added separately at 6 h. The biobleaching was determined at 12 and 24 h. The control contained only laccase.

Comparison of two different methods for estimation of biobleaching.

Crude laccase containing 45 effective laccase U prepared in citrate phosphate buffer at either pH 3 or pH 6 on the biobleaching of bagasse (0.1 g) at 60°C was determined at various time intervals. Since the crude laccase was roughly twice as active at pH 3 than at pH 6, the laccase U calculated were 45 effective laccase U at a particular pH, i.e. the actual amount used of crude laccase used when incubated at pH 3 was roughly half that used at pH 6. Here two methods of estimating biobleaching were compared, the kappa number method ([Appendix 8.2.1.3](#)) and the brightness index method. For the brightness index method, a mat of the dried bagasse was prepared and placed against a spectrophotometer (Premier Colour Scan, model SS 5100A, India) and the reflectance reading recorded. The control was untreated bagasse for both the estimation methods.

5.4 RESULTS

The B&K medium prepared in 25 ppt seawater had an initial pH of around 7.5, which did not drop below 6 even after the decolourization experiment was completed. Presented below (Fig. 3) are decolourized synthetic dyes prepared in both LNM and B&K medium.

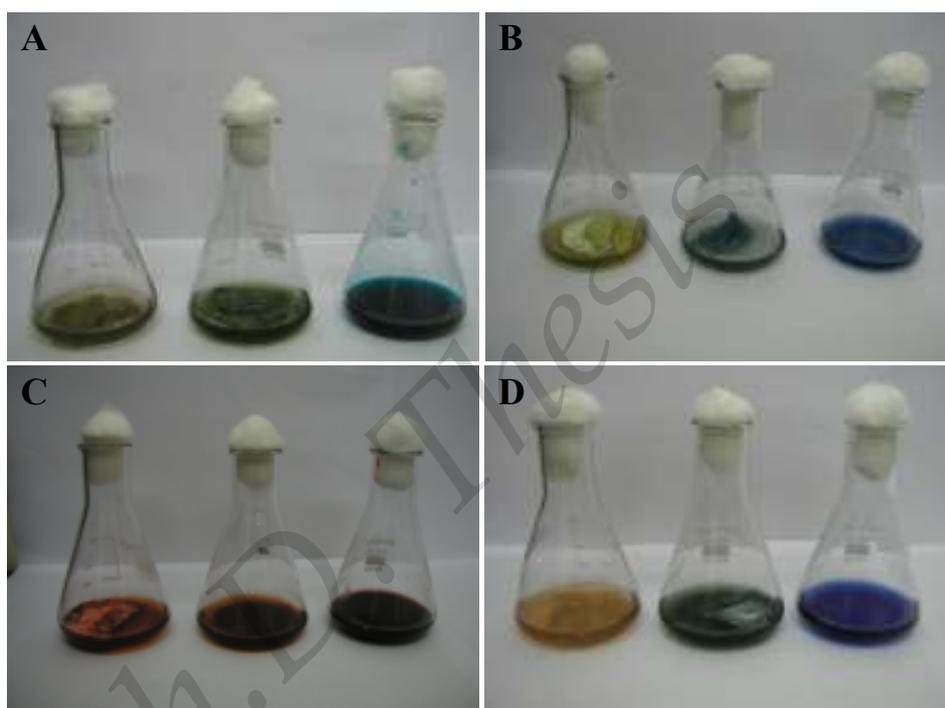


Fig. 3 *Cerrena unicolor* MTCC 5159 after 6 days in the presence of synthetic dyes. (A) Brilliant green (0.02 %) (B) Aniline blue (0.04 %) (C) Congo red (0.02 %) (D) Remazol brilliant blue R (RBBR) (0.04 %). The flask on the left-hand side in each figure contains the culture with dye prepared in B&K medium; in the center, it contains the culture with dye prepared in LNM and on the right-hand side is the control which contains only dye without culture.

Although good amount of decolourization was achieved using LNM, the medium itself is known to be toxic (Ramsay & Nguyen, 2002), defeating the purpose of bioremediation. Henceforth, the results presented or discussed are obtained using only B&K medium prepared in 25 ppt seawater.

The synthetic dye, brilliant green was decolourized to the maximum within 4 days whereas RO 16 was least decolourized (Fig. 4 A₁ - A₂).

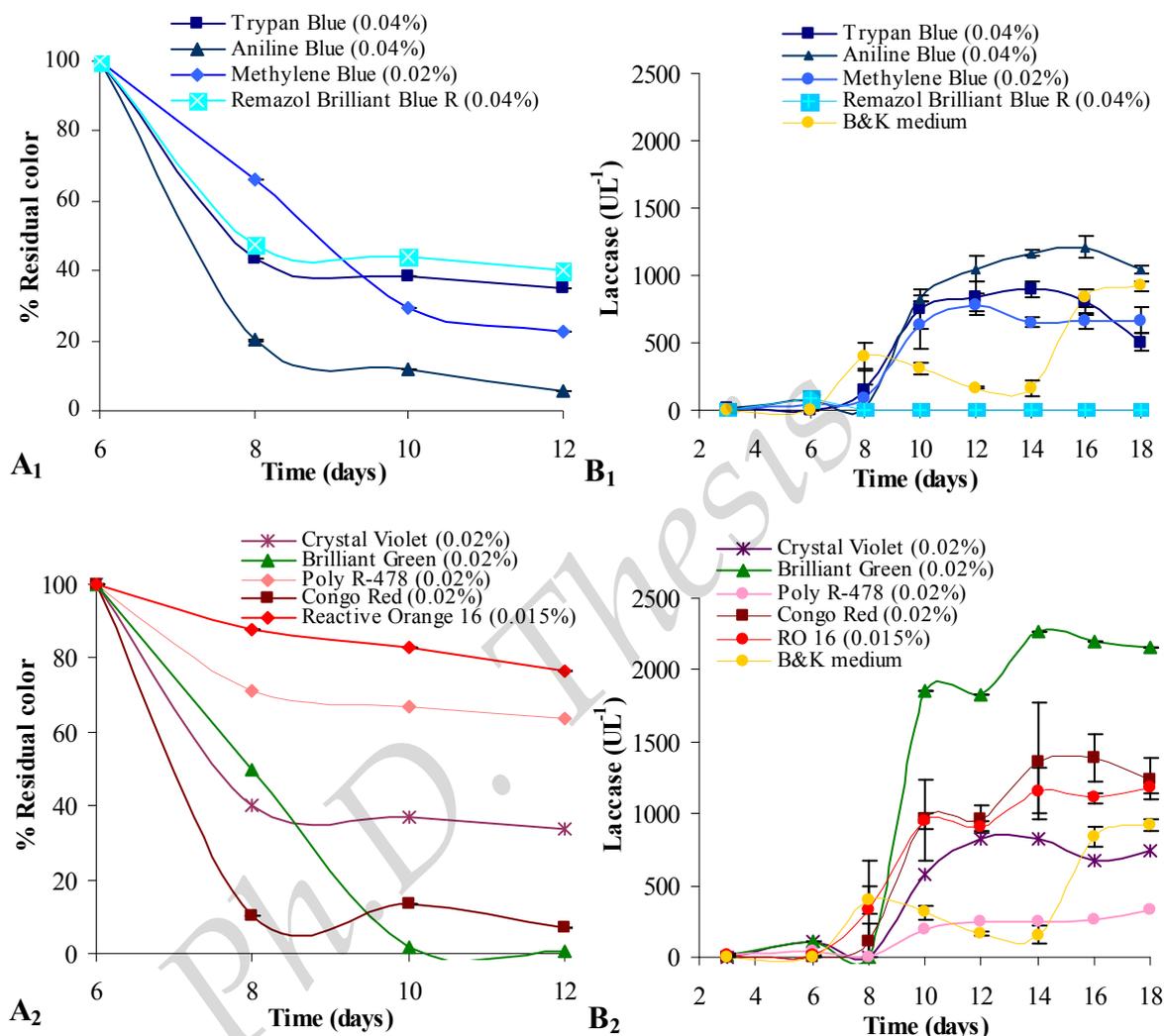


Fig. 4: *Cerrena unicolor* MTCC 5159's (A₁₋₂) Decolourization of synthetic dyes and (B₁₋₂) Induction of laccase in B&K medium with and without dye supplements.

Among the other synthetic blue dyes, aniline blue was decolourized almost completely within 4 days from addition of the dye. Trypan blue, methylene blue and RBBR were decolourized by 60 – 70 % within the same time. However, Poly-R 478 and RO 16 showed low levels of decolourization. The dyes that were decolourized also acted as inducers for laccase (Fig. 4 B₁- B₂) and in turn were further decolourized by the laccase produced. Laccase activity in the presence of Poly-R 478 was lower than equal to the

control and its decolourization was very low as well. Whereas, even though RBBR showed good decolourization, corresponding laccase activity was not observed.

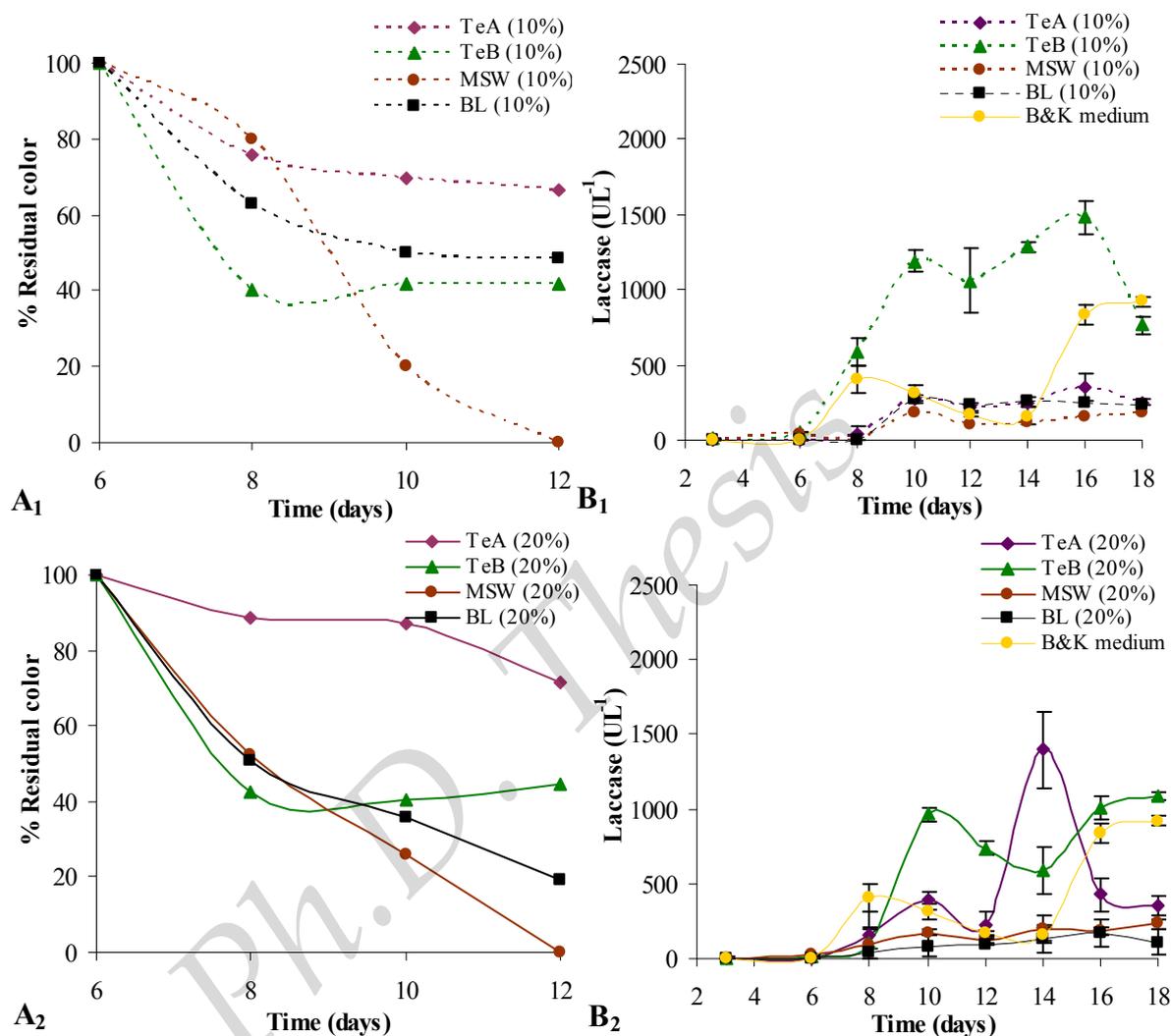


Fig. 5: *Cerrena unicolor* MTCC 5159 (A₁₋₂) Decolourization of industrial effluents and (B₁₋₂) Induction of laccase in B&K medium with and without effluent supplements.

Among the effluents (Fig. 5 A₁ - A₂), ~ 60 % decolourization of BL (10 %) was achieved by day 6 but surprisingly at 20 %, it was decolourized to a greater extent. MSW at 10 and 20 % was completely decolourized within 6 days. Among all the effluents, TeA at both concentrations was least decolourized (Fig. 5 B₁ - B₂). Textile effluent B at both 10 and 20 % was capable of consistently inducing laccase more than the control. However, on day 8 after of addition of dye, TeA at 20 % induced laccase activity comparable to that of TeB at 10 %, which was the maximum among the effluents tested.

The decolourization of synthetic dyes and effluents such as brilliant green, methylene blue, Poly-R 478, TeB (10 %), BL (10 %), aniline blue, trypan blue and crystal violet were proportional to laccase activity with r^2 values of 0.99, 0.99, 0.96, 0.96, 0.95, 0.95, 0.94 and 0.93 respectively. The decolourization of the remaining dyes and effluents such as black liquor (20 %), TeA (10 %) and RO 16, MSW (10 %), TeB (20 %), MSW and TeA at 20 % were not proportional to laccase activity, with r^2 values of 0.79, 0.73, 0.63, 0.59, 0.07, 0.04 and 0.02 respectively. The synthetic dye, congo red also did not show any correlation with laccase activity even though an appreciable laccase titer and significant decolourization was obtained. With RBBR, no laccase activity was observed, yet considerable decolourization occurred.

Two days after the dyes / effluents were added to 6 day-old culture, only TeB at 10 % induced more laccase than control. By the end of the experiment, 6 days after the addition of the dyes / effluents, only MSW at 10 % and 20 % and BL at 20 % still did not induce laccase despite getting completely decolourized. Poly-R 478, TeA at 10 % and 20 % were non-responsive in terms of laccase production, since the laccase titer induced in their presence was less than equal to the control. The remaining dyes and effluents had a positive effect on laccase production, with brilliant green showing maximum influence on laccase production, followed by TeB at 10 %.

Table 3: Effect of crude laccase[#] on effluent-decolourization after 12 h of incubation.

Effluent	Effluent (%)	% Decolourization (SD)
TeA	10	26 (5)
	20	23 (4)
	50	5 (0.5)
TeB	10	43 (6)
	20	73 (8)
	50	18 (2)
MSW	10	58 (5)
	20	50 (5)
	50	0 (0)
BL	10	8 (0.4)
	20	23 (3)
	50	38 (7)

[#] contains 9 U laccase.

The % decolourization of MSW and TeA decreased with increasing concentrations of effluent, whereas the opposite occurred with BL (Table 3).

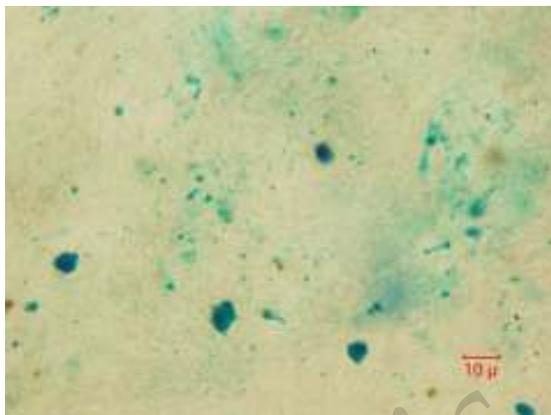


Fig. 6: A 21 day-old culture of *Cerrena unicolor* MTCC 5159 stained with Alcian blue. The hyphae are unstained whereas the surrounding exopolymeric substance stained blue.

A microscopic view of *Cerrena unicolor* MTCC 5159, under 100 X magnification, demonstrating the presence EPS, stained blue with Alcian blue around the unstained mycelium (Fig. 6). The exopolymeric substance (EPS) was very successful in the decolourization of dyes and effluents, even when used alone (D'Souza *et al.*, 2006).

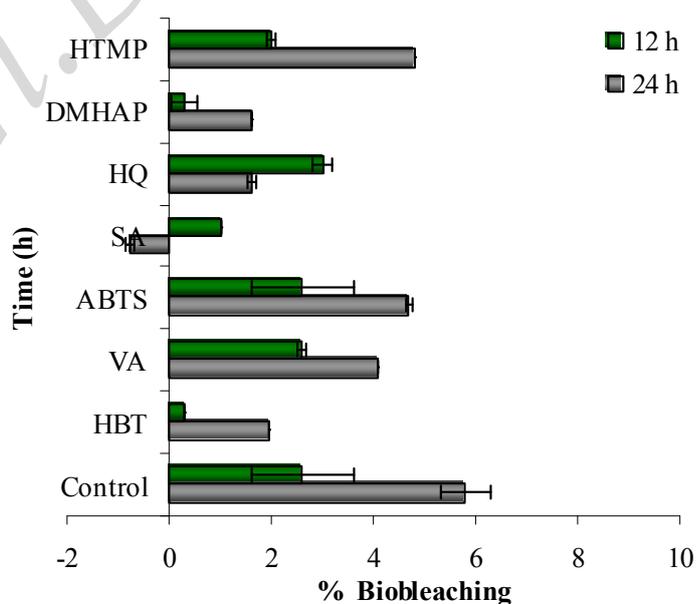


Fig. 7: Biobleaching (%) of sugarcane bagasse using 1000 U of crude laccase in the presence of the mediators was determined after 12 and 24 h, at pH 3 and 60°C. The control contained only crude laccase.

The medium employed did not induce the production of cellulases. The HQ-supplemented bagasse showed maximal biobleaching after 12 h (Fig. 7), however at 24 h a high degree of repolymerization was observed. From the mediators; HQ and SA-supplemented bagasse showed repolymerization whereas although the other mediators showed continued depolymerization, they were not efficient enough as compared to the control containing only laccase, showed a stable trend of delignification up to 24 h which was more than any of the mediator supplemented samples, with the maximum amount of biobleaching. However, the mediators VA & ABTS showed roughly similar, albeit lower biobleaching than the control.

It was observed that the biobleaching continued steadily up to 24 h followed by mild repolymerization till 48 h, and then continued depolymerization which stabilized at 60 h and remained so till 96 h (Fig. 8).

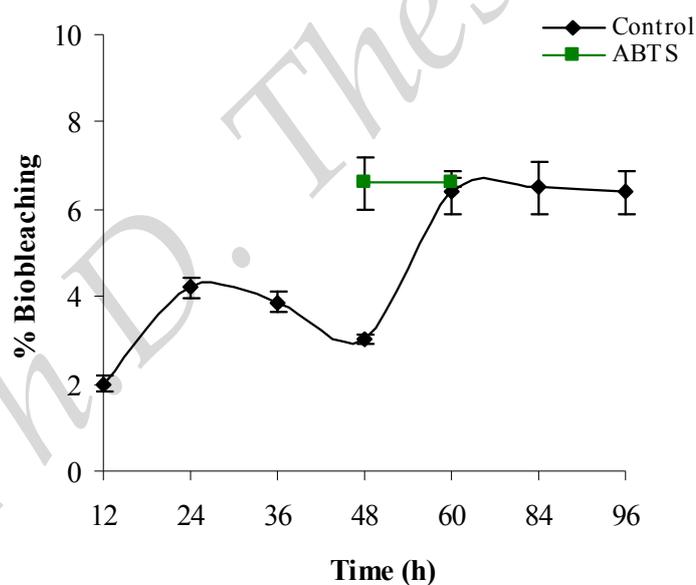


Fig. 8: Biobleaching (%) of sugarcane bagasse using 1000 U of crude laccase determined at pH 3 and 60°C at various time intervals. The mediator, ABTS was added at 36 h, after the repolymerization reaction began after 24 h.

Since repolymerization was first observed at 36 h, ABTS was immediately added as a mediator supplement to curb the repolymerization process. It was chosen among the mediators due to its maximal and consistent aid in depolymerization. Addition of ABTS helped to curb repolymerization and decreased the time required to achieve maximal

biobleaching by 12 h. Laccase at 10, 20 and 30 U with and without ABTS supplementation were screened for their ability to delignify bagasse, at 12 h intervals (Fig. 9). The ABTS-supplemented 10 laccase U showed maximal biobleaching in half the time as compared to that of the control (only 10 laccase U, no ABTS).

Where 20 laccase U were used, the control (only 20 laccase U) was able to biobleach the bagasse to a greater extent than the ABTS-supplemented 20 laccase U. After which, slight repolymerization was experienced which got stabilized by 60 h. The control and the ABTS-supplemented laccase observed the same trend for biobleaching, however the control was more efficient than the ABTS-supplemented laccase.

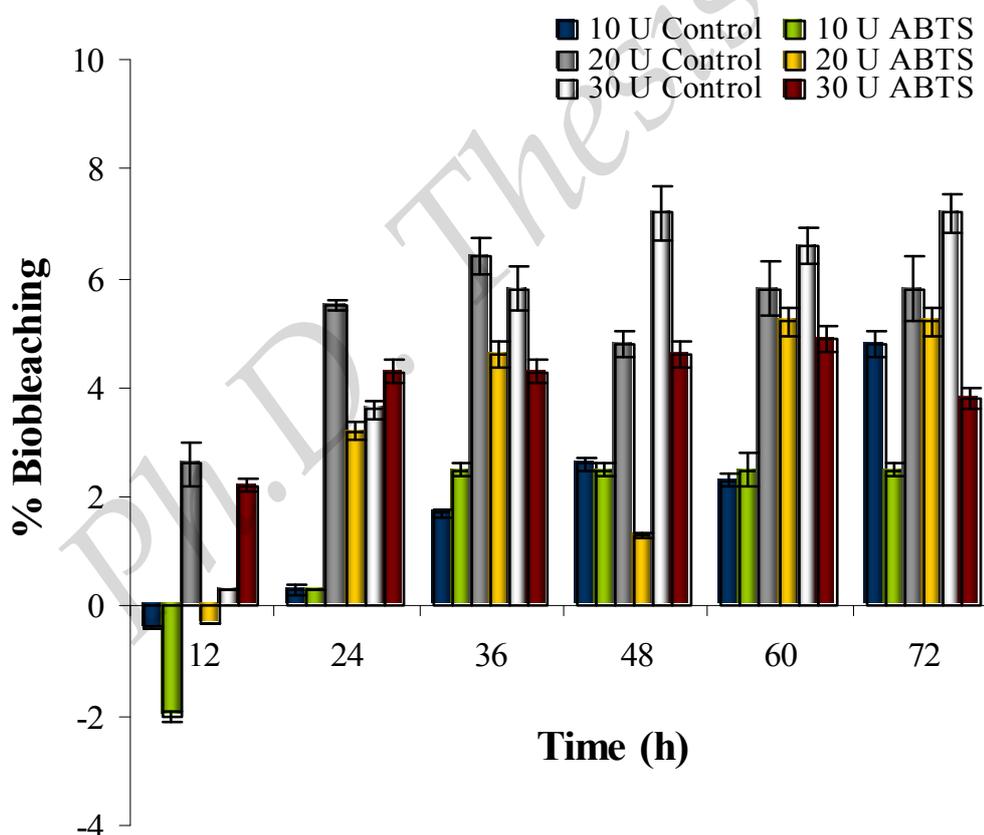


Fig. 9: Biobleaching (%) of sugarcane bagasse determined at various time intervals using 10, 20 and 30 U of crude laccase with and without the mediator ABTS, at pH 3 and 60°C.

When 30 laccase U were used, the control (only 30 laccase U, no ABTS) and the ABTS-supplemented 30 laccase U behaved in similar fashion to the 20 laccase U and its ABTS-supplemented 20 laccase U. Here however, the difference between the control and the ABTS-supplemented 30 laccase U was more pronounced.

HTMP-, HBT- and ABTS-supplemented 20 laccase U initially supported minimal biobleaching which later got reversed (Fig. 10). In general, the mediators that were capable of bringing effective biobleaching were HBT and DMHAP. Among these, HBT showed repolymerization after 12 h followed by an increase in biobleaching.

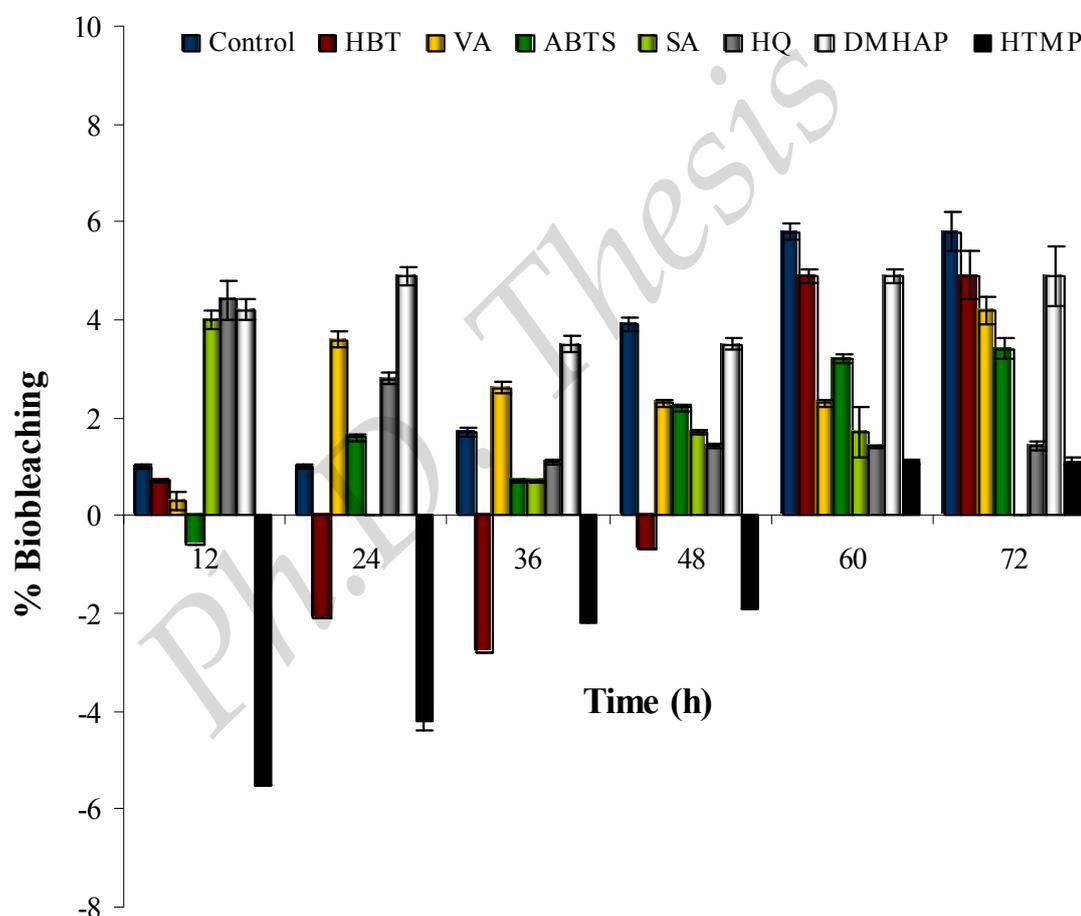


Fig. 10: Biobleaching (%) of sugarcane bagasse determined at various time intervals using 20 U of crude laccase in the presence of mediators added at 0 h, at pH 3 and 60°C. The control contained only crude laccase.

Mediators such as HTMP, HBT and ABTS when added at 0 h, showed a high initial repolymerization, which decreased over a period of time (Fig. 10). The same mediators

when used with the same amount of crude laccase (20 U), did not show any repolymerization when added after 6 h of biobleaching had taken place with the enzyme alone (Fig. 11). This proved to be successful since the repolymerization was avoided.

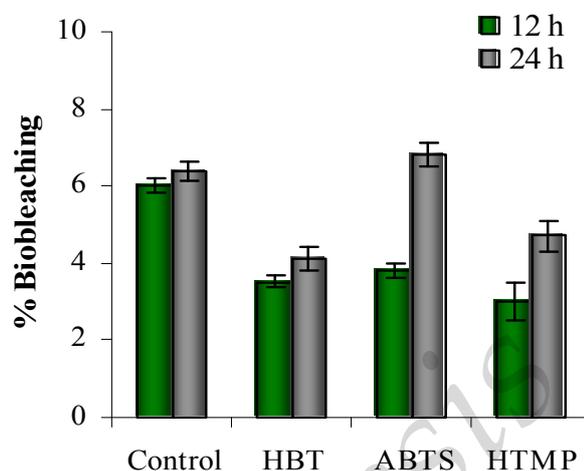


Fig. 11: Biobleaching (%) of sugarcane bagasse determined at 12 and 24 h at pH 3 and 60°C, using 20 U of crude laccase in the presence of mediators; HBT, ABTS and HTMP, added at 6 h. The control contained only crude laccase.

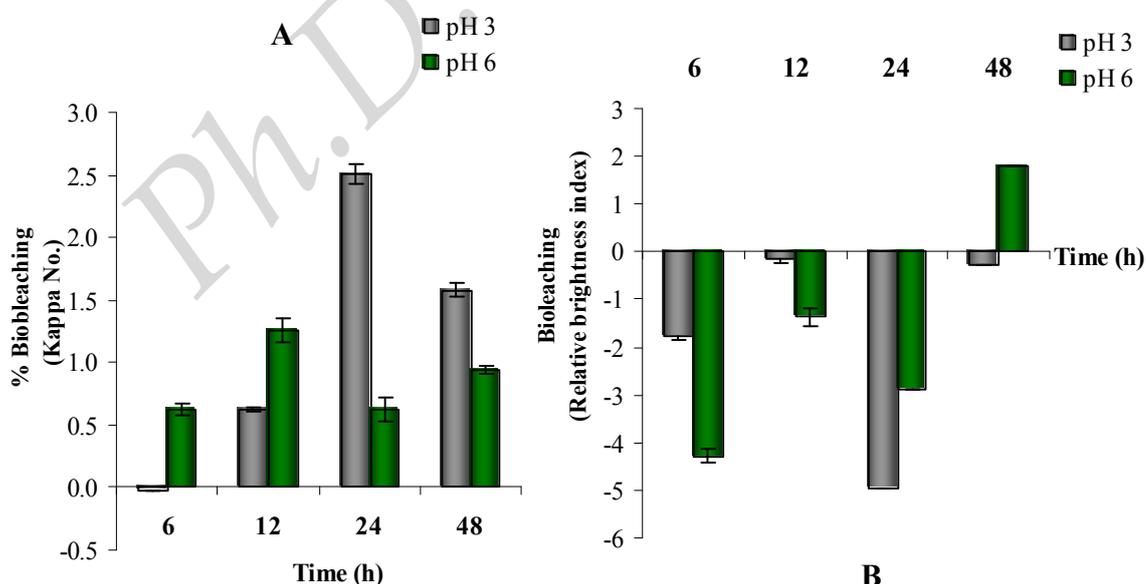


Fig. 12: The effect of 45 effective U of crude laccase on the biobleaching of sugarcane bagasse determined at various time intervals at 60°C at pH 3 and 6 and by (A) Kappa number and (B) Brightness index method. The control was untreated dry bagasse.

The two methods for biobleaching estimation, the kappa number method and the brightness-reflectance method gave opposing results (Fig. 12) with the exception of 48 h reading, although they reflected the same trend.

5.5 DISCUSSION

In the dye decolourization studies done by Ramsay and Nguyen (2002), the authors found that LNM was more toxic than the dye itself. An organic medium such as B&K yielded a very high titer of laccase even in the presence of 25 ppt seawater, which was not achieved in an inorganic and economically expensive medium such as LNM (as seen in Chapter 4). The inclusion of seawater was imperative since industrial effluents often contain large amounts of salts. To be able to function under such conditions and still produce appreciable decolourization was the objective of using a marine-derived fungus in these studies.

Even after active growth and decolourization of the dyes and effluents, the pH of the B&K medium which was initially 7.5 did not fall below 6, due to the buffering action of the seawater. This also indicated that the decolourization observed was not due to acidification of the medium, which is often the cause of many dyes to be converted into their leuco-form (they lose colour), which can readily be reconverted upon readjusting the pH of the medium. Several of the dyes tested for decolourization in fact induced laccase production.

Most of the dyes and effluents added to the culture medium were decolourized and simultaneously acted as inducers. Among these, brilliant green induced highest laccase production and its decolourization was proportional to laccase activity. This was followed by 10 % TeB which contained 8 reactive dyes induced maximum laccase production (among the effluents), it showed a high correlation between laccase activity and decolourization. The larger amount of TeB decolourization in comparison to TeA can be accounted for due to its reactive blue base and pH of 2.5. Reactive blue dyes include anthroquinone and diazo dyes, both of which have shown good decolourization by these laccases, its pH is also around the optimum pH for activity of these laccases

which is 3 whereas TeA was mainly composed of Azo dye-20 and had a pH of 8.9. From the results, it was observed that mono-azo dyes showed minimal decolourization and the alkaline pH was almost equal to the pH at which MTCC 5159 laccase was most stable (pH 9) with no activity. Here TeA did not induce laccase activity greater than the control even after 6 days, neither did a significant co-relation exist between its decolourization and laccase production. Any decolourization that occurred may be due the presence of enzymes working either alone or in conjunction with laccase. Even though TeA, induced considerable laccase activity on day 8 after effluent addition, it was unlikely that it would have made a difference in its decolourization, since this effluent was mainly composed of an azo dye which has shown to be more or less resistant to these laccases.

When TeB was used at 1 % (Chapter 4), laccase production was increased by ~100-fold, far greater than even brilliant green. Textile effluent B is mainly composed of reactive blue dyes which include anthroquinone and diazo dyes, both these dyes although get decolourized but anthroquinone dyes do not seem to induce laccase production whereas diazo dyes are good laccase inducers (Bhatt *et al.*, 2005). Hence, its acidic pH and the presence of diazo reactive blue dyes probably make TeB a good laccase inducer. What component of this mixture of dyes was actually involved in inducing such high laccase titers merits further investigation since the composition of effluents do not remain constant. The complete decolourization of MSW to some extent may be accounted for by its acidic pH of 4.3. High production of ligninolytic enzymes during treatment of paper mill effluents was reported in *Trametes versicolor* (Manzanares *et al.*, 1995).

The mechanism of laccase-catalyzed dye decolourization can differ depending upon dye structure. The white-rot fungus *Trametes versicolor* was shown to use anthroquinone dyes as direct substrates for its laccase. In this study however, although the anthroquinone dye, remazol brilliant blue R, got decolourized by 50 % within 6 days yet no laccase activity was detected, not even the basal amount induced by the medium itself. Obviously, its decolourization was due to enzymes other than laccase or by some other mechanism. Similar results have been obtained by (Vyas & Molitoris, 1995) who shown that a oxygenase called 'RBBR oxygenase' was implicated in the decolourization of RBBR which is distinct from MnP, LiP and laccase.

Azo and indigo dyes have been shown to be indirect substrates (non-substrate) i.e. their decolourization may be dependant upon small molecular weight

metabolites being secreted into the culture medium (Wong & Yu, 1999). These metabolites mediate interactions between laccase and dyes, which under normal conditions would not have been degraded by laccase. The decolourization rate of the non-substrate dyes was shown to be limited by the concentrations of mediating compounds rather than laccase activity in the culture filtrate (Wong & Yu, 1999). In the present study, among the synthetic dyes, it was found that brilliant green and aniline blue induced the highest laccase production and were decolourized to a greater extent than other dyes. Crystal violet on the other hand induced moderate amounts of laccase which was sufficient to bring about its decolourization which also showed good correlation to laccase activity. Poly-R 478 has been used for screening for the presence of peroxidases since it generally is decolourized by MnP and LiP whereas laccase only slightly modifies this dye (Wesenberg *et al.*, 2003) This fungus preferentially produces laccases which explains why Poly-R 478 was unable to induce laccase and whatever laccase was produced was probably due to the medium alone.

Although congo red, a secondary diazodye did not show any correlation with laccase activity, it still showed appreciable laccase induction. There are two explanations for this occurrence;

- 1) An enzyme other than laccase is induced by congo red and is responsible for its decolourization.
- 2) Although laccase may still be largely responsible for the decolourization of congo red, congo red might not be a direct substrate of laccase and its degradation may involve the production of a metabolite which is the substrate of laccase and effects the decolourization of congo red.

Similar results with congo red were obtained earlier (Wong & Yu, 1999). In the present study however, trypan blue, a diazo dye showed appreciable laccase induction and correlation with laccase activity. On the other hand, even though RO 16, a monoazo dye induced moderate laccase activity, its decolourization was negligible at ~ 20 %. (Svobodová *et al.*, 2007) have shown that laccase in combination with MnP was very successful in decolourization of RO 16. From these results, it is obvious that great variation exists within a single group of dyes such as the azo class in eliciting laccase

production and effecting its own decolourization. Apparently, the diazo group is far more successful in inducing laccase and mediating its own decolourization compared to the monoazo group. It has also been shown that batch decolourization processes of azo dyes using laccase cannot be attained. The application of laccases alone in bioremediation has been limited due to the occurrence of polymerization reactions. These authors have shown that laccase modifies azo dye structures by destroying their chromophoric structure, observed visually as a reduction in colour. However, over long periods of oxidation, the products obtained during the degradation reaction can undergo further reactions leading to couplings between themselves or with the unreacted dye, producing a large amount of coupled products leading to a darkening of the solution. This could explain the occurrence of repolymerization, within 4 days in congo red, a diazo dye and TeB (10 & 20 %), a textile effluent containing a mixtures of several dyes. Immobilization of the enzyme has been proposed to avoid such coupling reactions (Zille *et al.*, 2005).

Triarylmethane dyes are the parent structures of triphenyl methane dyes. In this study, the triaryl methane and the triphenyl methane dyes showed good laccase production and correlation between laccase titer and decolourization as well, indicating that these dyes are not only good inducers of laccases of MTCC 5159 but are good substrates as well. Similar results were seen with methylene blue which is a phenothiazine dye.

The cell-free culture filtrate was successful in decolourizing MSW and TeA, the amount of decolourization decreased with increasing concentrations of effluent. However for BL, increasing concentrations resulted in increased decolourization, this may be due to the increased presence of phenolics at higher concentrations of BL which acts as laccase inducers (Ali & Sreekrishnan, 2001). At lower concentrations of BL, the concentration of phenolics may not be sufficient to induce laccase production.

Presence of LiP and MnP, the other major lignin-degrading enzymes play an important role in dye decolourization. In *Cerrena unicolor* MTCC 5159, laccase was the major LDE produced. Similarly laccase as the major LDE was reported with *Pycnoporus cinnabarinus* (Eggert *et al.*, 1996b) *Phlebia tremellosa* (Robinson *et al.*, 2001a) and *Pleurotus sojarcaju* (Chagas & Durrant, 2001). The white-rot fungus *Clitocybula dusenii* producing laccase as its major LDE, also showed decolourization of industrial textile dye effluents (Wesenberg *et al.*, 2002).

D'Souza *et al.*, (2006) have showed that decolourization of several dyes and effluents could be achieved within 6 - 12 h by incubating with the laccase-containing mycelia-free culture supernatant. However, on incubation of the culture supernatant with TeA and TeB, reduction in colour was seen within the initial 2 h after which there was no further reduction in colour. Addition of redox mediators may improve this situation as was reported in the case of pure laccase from a commercial formulation (Soares *et al.*, 2001). The EPS produced by white-rot fungi is reported to play an active role in lignin degradation (Bes *et al.*, 1987). In concurrence in majority of the cases, the EPS produced by this fungus was more efficient in decolourization than the culture supernatant. Even RO 16 and Poly-R 478 that were not decolourized when added to the growing culture were totally decolourized by the EPS. This might be due to the combined effect of adsorption and enzymatic decolourization, since EPS is known to trap extracellular enzymes within its matrix (Wingender *et al.*, 1999).

The medium had been optimized to almost exclusively support laccase production (Chapter 4), cellulase was not induced under the present medium conditions, which makes this especially suitable for use in the biopluping process, where the lignin is selectively removed without weakening the cellulose fiber (Bernier *et al.*, 1992).

Biobleaching is an environmentally friendly alternative to harsh chemical treatments such as chlorination of paper pulp. Xylanases are one of the most prominent enzymes involved in this process and reduces the total requirement for chlorine (Raghukumar *et al.*, 2004). Laccase is also an enzyme that achieves this goal however, the economics of laccase production and stability need to be considered before applying this technology on a large scale. Mediators have been shown to increase the effectiveness of the enzymes. However, the problem encountered when dealing with laccase is that besides achieving biobleaching of pulp via depolymerization, it can also catalyze the reverse action, i.e. repolymerization, which is counter productive to the biobleaching process. While utilizing it for biobleaching, this fact requires to be taken into consideration and the entire process merits careful observation to determine the minimum yet effective amount of laccase required in bringing about large amount of biobleaching within the shortest possible period. This also includes the time at which repolymerization occurs and steps required to be taken to avoid it.

From the biobleaching experiments it was observed that if laccase was supplied in excess, the need of mediators to enhance biobleaching could be done away with. The economics of whether low amounts of laccase supplemented with effective low-cost mediators are better than larger amounts of laccase alone, needs to be investigated. If the former option is more economical then, the type and concentration of mediator and the time of its addition requires to be standardized along with laccase concentration prior to large-scale biobleaching.

From Fig. 7, it is clear that when crude laccase was used at 1000 U, the requirement of mediators became superfluous. None of the mediators could match the biobleaching brought by the enzyme alone, although ABTS and HTMP came comparatively closer. At such high amounts of laccase, mediators were either antagonistic or at the least, non-responsive.

The previous experiments were carried out using laccase at a 1000 U, which is not commercially viable whereas laccase at 10 to 30 U is. Thus, laccase at 10, 20 and 30 U with and without ABTS supplementation were screened for their ability to delignify bagasse. Here the ABTS-supplemented 10 laccase U and its respective control, on an average achieved similar levels of biobleaching. Even though at this concentration of laccase, the effect of mediators is clearly visible, the total biobleaching achieved was not sufficient. When 20 laccase U were used, the control was able to bleach the bagasse to a greater extent than the corresponding ABTS-supplemented laccase within the same period although both followed the same trend. This indicated a slight excess of enzyme. The difference between the control and the ABTS-supplemented 30 laccase U was more pronounced than with the 20 laccases U, indicating a definite excess of enzyme. Laccase at 20 U was the minimum concentration required to achieve sufficient biobleaching without becoming too superfluous.

Mediator-supplemented 20 laccase U such as HTMP, HBT and ABTS showed minimal biobleaching initially but this got reversed with time (Fig. 11). The mediators which resulted in effective biobleaching in majority of the cases, were HBT and DMHAP. Among these, HBT showed repolymerization after 12 h followed by a steady increase in biobleaching. To determine whether this repolymerization could be halted by manipulation of the time of addition of the mediators; HTMP-, HBT- and ABTS which showed high initial repolymerization were added after 6 h of biobleaching

had taken place instead of at 0 h. This change in time of addition of the mediators eliminated the problem of repolymerization. However, in most of the above experiments, regardless of the amount of laccase used, the presence of mediators seemed to be superfluous. Crude laccase performed well and for most of the time was better than if supplemented with mediators. Only when repolymerization began, the addition of mediator seemed to help in maintaining biobleaching by reversing the repolymerization action. Thus, we can infer that not only does the concentration of laccase determine at what time and to what extent biobleaching occurs but also the time of addition of the mediator is critical to avoid repolymerization and this too, is dependant of the type of mediator used.

In Fig. 12, although the effective laccase U were equal in pH 3 and 6, since at pH 3 the activity was double that at 6, the amount of enzyme used was roughly half (calculated by precise spectrophotometric measurements at both the temperatures). After the initial delay in removal of lignin, the greater efficiency of the enzyme at pH 3 is obvious in the latter half of the experiment (Fig. 12 A). However, this breakdown of lignin due to the presence of laccase does not translate into increased brightness of the pulp as observed in Fig. 12 B. This may be since the experiment was carried out for only 48 h and success in increasing the brightness was observed only at 48 h. Brightening of the pulp may have been achieved, if incubated for longer periods with the enzyme.

However, it is to be noticed that the pH that supported maximal biobleaching or removal of lignin at any given time (Fig. 12 A), supported minimum brightness (Fig. 12 B). Brightness and residual lignin in terms of kappa number are both assays for biobleaching and should in theory, if biobleaching had occurred, give at least a similar trend. However, this was not the case in the above experiment. These contradicting results are probably due to the fact that although lignin has been degraded by laccase, reflected in lower kappa number the degradation products which are also coloured aromatics have not been completely removed before 48 h which prevent the brightness of the pulp from increasing. Sometimes these products may repolymerize to give more intensely coloured products than the original lignin, thus decreasing brightness (Zille *et al.*, 2005). This repolymerization can be avoided by using laccase in conjunction with MnP (Svobodová *et al.*, 2007).

Biobleaching cannot be determined by the traditional 'kappa number' method alone, since even though this method is precise in monitoring the percent lignin in the sample and detects polymerization and depolymerization as well, it may not be a true reflection of the actual biobleaching that takes place. This is because some of the lignin molecules which may have been altered by the enzyme and hence not estimated by this method, through coupling reactions result in a darkening of the sample, this may or may not be recorded by the kappa number method. It would be better to confirm the action of biobleaching with the 'brightness index' test, which is a spectrophotometric reflectance reading.

Laccase is the major extracellular enzyme of *Cerrena unicolor* MTCC 5159 responsible for decolourizing majority of the dyes and effluents containing dyes of varying structures. Some dyes such as triarylmethanes and the triphenylmethanes are excellent substrates of laccase and they induce laccase production, as well. Many dyes are not the direct substrate of laccase such as some diazo dyes and their degradation depends on the presence of some small molecule metabolites or its own degradation products which bring about further decolourization. The production of the small molecule metabolites by MTCC 5159 may not be proportional to the release of laccase, the decolourization of such dyes cannot be correlated with laccase activity.

Industrial effluents contain a consortium of dyes, whose composition varies on a regular basis. These effluents may contain dyes which are good substrates or non-substrates or indirect substrates of laccase, however in such a consortium, the substrate dyes in industrial effluents may mediate the interaction between laccase and other non-substrate dyes effecting the decolourization of both, substrate as well as non-substrate dyes and also act as inducers for more laccase. The decolourization rates of non-substrate dyes, which otherwise would never have been decolourized could now be significantly improved by this mechanism. Further investigation should be conducted on the relationship between specific laccases which have commercial viability and dyes structures, since the preferences for substrates vary with the type of laccase. Also the potential of dyes to act as mediators and the kinetics of the mediator-involved dye degradation of non-substrate dyes and laccase also merit intense investigation (Wong & Yu, 1999).

In conclusion, the marine-derived *Cerrena unicolor* MTCC 5159 decolourized several synthetic dyes and effluents in the presence of sea salts. Cell-free

culture supernatant containing laccase or EPS precipitated from the culture medium were also very successful in the decolourization process. It was also able to bring about biobleaching without the need of large amounts of mediators. This fungus appears to be a good candidate for industrial application in bioremediation of coloured waste waters in the presence of chlorides and sulphates as well as in biobleaching.

Ph.D. Thesis