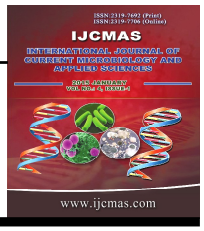
Int.J.Curr.Microbiol.App.Sci (2015) 4(1): 183-200

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 1 (2015) pp. 183-200**
http://www.ijcmas.com



Original Research Article

Decolorization of dye by alginate immobilized laccase from *Cercospora* SPF-6: Using compact 5 stage plug flow reactor

Vikram Thakur, Pradeep Kumar, Anita Verma and Duni Chand*

Department of Biotechnology, Himachal Pradesh University,
Summer Hill, Shimla 171005, India
**Corresponding author*

A B S T R A C T

In the present study, extracellular laccase enzyme was produced from *Cercospora* sp. SPF-6, which was isolated in our department and identified by IMTECH, Chandigarh. Laccase was entrapped in calcium alginate beads with maximum immobilization efficiency of 72.42% and enzyme activity of 0.617 U/bead. Based on optimization experiments, maximum decolorization of methyl orange dye was observed at 30˚C with citrate phosphate buffer (0.025M) at pH 2.5 in 30 min. Laccase mediator system constituting 12.34U (20 beads) of enzyme dose and 160µM pyrogallol as mediator and decolorize 200µM dye in the presence of Zn^{2+} which enhance total decolorization to 84.31%. Immobilized enzyme system maintained 50% of its efficiency even after 7 successive reaction cycles. When optimized parameters scaled up to the level of 5 stage plug flow reactor, 89.09% of dye decolorization was achieved in 1st reaction of 5th reactor. Difference in the retention time of crude dye and metabolites along with shift in absorption spectrum from 508nm to 400nm using HPLC and spectroscopy, respectively, validated the decolorization of dye. These preliminary results suggested that the plug flow reactor can potentially be adopted for dye decolorization and perhaps can be further developed as potential for large scale bioremediation of other toxic dyes.

K e y w o r d s

Laccase, *Cercospora* SPF-6, Plug flow reactor, HPLC, Methyl orange, Immobilization

Introduction

Laccases (E.C.1.10.3.2), p-diphenols-dioxygenoxidoreductase, are part of a group of enzymes termed the multicopper enzymes. These enzymes contain 15–30% carbohydrates and have a molecular mass of 60–90kDa, containing 4 copper ions, one molecule carry out one electron oxidation of phenol and its related compound and reduce oxygen to water **(**Couto and Herrera, 2006). The use of molecular O_2 as oxidant and fact that H_2O is only byproduct are very attractive catalytic features rendering laccases as an excellent 'Green Catalyst' **(**Riva, 2006). Laccases was 1st detected in exudates of Japanese lacquer tree *Rhus vernicifera.* Among fungi, Basidiomycetes and Deuteromycetes can produce laccase and White Rot Fungi have been found to be the most efficient laccase producers.

Current technologies such as coagulation, zonation and activated carbon can efficiently remove only restricted classes of dyes (Dubrow *et al.,* 1996, Hassan and Hawkyard., 2002, Matsui, 1996). So combination of physical, chemical and biological processes are most efficient for dye decolorization but can be expensive (Hai *et al.,* 2007, Robinson *et al.,* 2001). Activated carbon is used to remove acidic dyes but not reactive dyes (Reife and Freeman, 1996, Robinson *et al.,* 2001a). Electro coagulation has been used to remove dyes and pigments aggregates from textiles wastewater (Zidane *et al.,* 2008).

Worldwide, waste water from textile industries are discharged in large quantities into natural water bodies on a daily basis since are visible at low concentration of 0.005 ppm (mg/ml) (O' Neill *et al.,* 1999**)**. It is estimated that approximately 50,000 different types of dyes are lost to the environment worldwide and 30 % of reactive dye stuffs are lost and discharged with the effluents (Lewis, 1999). Several dyes, particularly azo dyes are mutagenic as parent molecule or when they are metabolized (Moawad *et al.,* 2003).

Materials and Methods

Fungal strains and culture growth condition

The fungal strain *Cercospora* sp. SPF-6 was already isolated in our lab from the soil of Himachal Pradesh as a laccase producing fungus. Fungus was maintained on PDA plates at 4˚C and subcultured by point inoculation with incubation at 30˚C for 96 hrs.

Inoculum preparation

Laccase producing *Cercospora* sp. SPF-6 was point inoculated from the master plate on potato dextrose agar (PDA) plates containing 20% infusion from potato, 2% dextrose and 1.5% agar (pH 6.2) at 30˚C for 96 hrs. These pre-cultures were used in production medium for the production of extracellular laccase. Five drops of veratryl alcohol (1 mM) was added as inducer before inoculation (Figure 21).

Fungal culture discs (6mm diameter) from growing edges of mycelium was transformed in 100ml of production medium containing 1.25% soya meal, 1.8% fructose, 1% yeast extract, 3µM $CuSO_4.5H_2O$, 10 µM $CaCl_2.2H_2O$, 10 µM $MgCl_2.2H_2O$ (pH 6.0) and was kept at 30˚C at 150 rpm for 108 hrs. After incubation, medium was filtered with Whattman No. 1 filter paper. The laccase was found to be extracellular in nature and used for the enzyme assay.

Assay condition and protein estimation

Laccase activity was assayed following the modified method of Nagai *et al.* (2003). The assay included 100µM ABTS, 890µl McIlvaine buffer at pH 3.0 and 10µl enzyme solution in a total volume of 1 ml at incubation period of 5 min at 30˚C. The reaction was stopped by adding 500µl sodium azide (3mM) and activity was measured spectrophotometrically at 420nm. Protein estimation was done using Bradford method. Standard curve of protein was prepared using the 20–200 µg/ml of bovine serum albumin.

Immobilization of enzyme on different matrices

Entrapment of laccase in calcium alginate and sodium alginate:

Laccase enzyme was immobilized by method of alginate bead immobilization using entrapment. Concentrations were optimized with respect of optimal laccase

activity. 2.5% sodium alginate and 0.2M $CaCl_2$ and 0.2M Cu_2SO_4 was prepared separately. Equal volume of sodium alginate and enzyme was mixed and beads were prepared using BUCHI Switzerland B-390 Encapsulator with following description as amplitude 5, pressure 450 mbar, temperature 30˚C, 1000V electrode and 1000 Hz frequency (Fig. 20). Mixture was dropped into a 0.2M ice cold solution of calcium chloride and copper sulphate respectively and was incubated overnight for curing in stirring condition using magnetic stirrer. The cured beads were washed with sterile distilled water.

Immobilization of laccase by adsorption on Silica:

Silica (4g) was dissolved in minimum amount of sodium phosphate buffer (0.1M, pH 7) and left overnight at room temperature for activation. After 24 hrs it was filtered with Whattman No.1 filter paper, 5 ml of crude enzyme was added in 1 g of silica (pellets). Binding time was provided by keeping in incubator shaker,then it was filtered. Protein estimation and enzyme activity was measured in pellet and supernatant broth (pellet contained immobilized enzyme).

Immobilization of laccase by entrapment in agar discs:

Agar discs (4%) were prepared. 2 g of agar was added in 50ml of distilled water, and this solution was allowed to autoclave and then in 15ml of agar solution, 5ml of enzyme (77.5 IU) was added and mixed properly and then this mixture was poured in Petridish and allowed for solidifying. Discs (6mm) were prepared with the help of borer and used to check enzyme activity and immobilization efficiency. Agar discs containing immobilized enzyme (9.78 U/g) were used. 500µl of buffer, 500µl of enzyme (15.544 U/ml) and 500µl of dye were taken and incubated at 35°C for 30 min to check enzyme activity.

Immobilization in k-carrageenan with tricalcium phosphate by encapsulation:

k-carrageenan solution 4% (w/v) with tricalcium phosphate was prepared in physiological saline. Solution was heated to 60°C to completely dissolve the k-carrageenan. After cooling to 40°C, crude enzyme was added, and the resulting matrix was pumped into a 2% KCl solution to induce gelation. The resulting bead size depended on the tube diameter, typical bead diameter were 5.0–6.0 mm. Beads with tricalcium phosphate can also be placed in an acidic 1% KCl solution to dissolve either all or part of the crystals out of the gel to enhance porosity of the beads.

Effect of buffer, pH and molarity of entrapped laccase for dye decolorization

Four buffer systems citrate phosphate buffer (pH 2.5–4.5), sodium acetate buffer (pH 4.0–5.5), sodium citrate buffer (3.0–5.0), sodium phosphate buffer (pH 7.0–8.0) with pH ranging from 2.5 to 8.0 were prepared (1 mM) and analyzed for best combination of buffer. Molarity of selected buffer was varied from 0.025 to 0.1M.

Effect of reaction temperature and incubation time for dye decolorization

The reaction mixture with immobilized enzyme was incubated at temperature ranging from 25˚C to 55˚C.Incubation time ranging from 10min to 360min to determine the optimum reaction time for maximum dye decolorization.

Effect of dye concentration and enzyme dose

The dye concentration and enzyme dose was varied from 20mM–200mM and 3.09–18.09 IU respectively in the reaction mixture containing immobilized enzyme.

Effect of mediators and its different concentration

Different mediators like ABTS, HOBT, PHBA, PABA, hydroquinone, pyrogallol, etc., at a concentration of 1 mM was used to check decolorization of methyl orange dye. These mediators concentration were varied from 20µM–200µM.

Reusability of immobilized enzyme

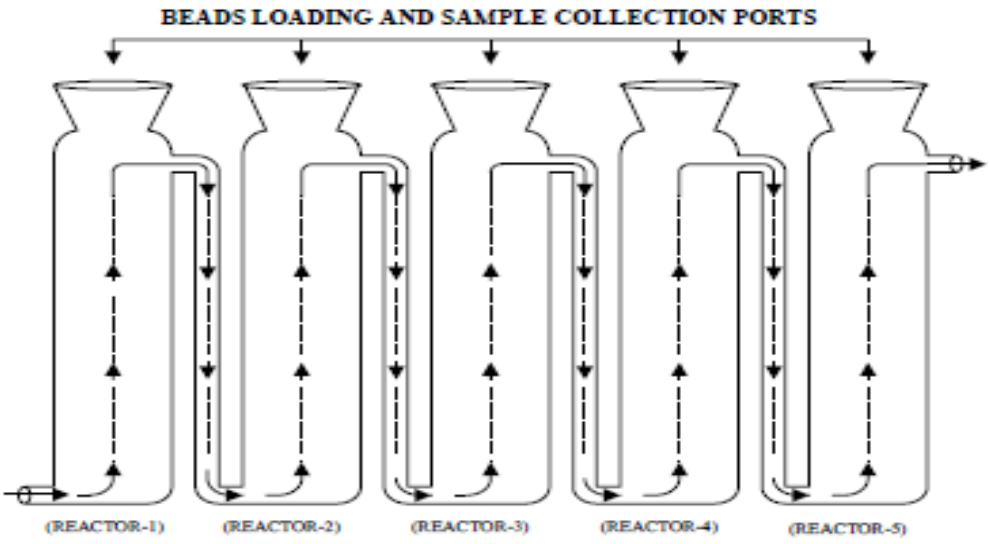
Calcium alginate beads with immobilized laccase enzyme were again and again used till the beads show 50% or less percent decolorization. This will show the ability and efficiency of the immobilized enzyme for the decolorization of methyl orange dye.

Five stage plug flow reactor

A highly compact five stage packed bed plug flow reactor was fabricated for the treatment of the simulated waste water (methyl orange 200µM and 500µM), Specification of each reactor of 5 stage plug flow reactor is as: Total volume 240ml, Void volume, 110ml, Beads volume 130ml, Bead (wt.) 0.0257g,

Diameter of bead 3.5mm, dye flow rate 2ml/min, dye used per cycle 110ml, operational temp 35°C. Decolorization of dye was carried out by compact five stage packed plug flow reactor. The immobilized beads were loaded in each glass column (25cm X 4.5cm), leaving a headspace of 6 cm. The dye solution was passed through the reactor using peristaltic pump at a flow rate of 2 ml/min for 28 hrs and the treated sample were collected at the outlet was subjected to dye decolorization at regular intervals and was compared with the untreated dye sample. The reactor temperature was maintained at 35°C. The schematic diagram of the packed bed reactor system used is shown in Figure 1

Fig.1 Sketch of the compact five-stage packed bed plug flow reactor (Chand *et al.*, 2004)



Analysis of metabolites formed after decolorization of dye

When effluent reached the top of a reactor, samples were taken for HPLC and spectroscopic analysis of degraded metabolites of pure azo dye methyl orange. The decolorization was monitored at λmax of respective dye using a UV/VIS spectrophotometer. In order to elucidate the degraded product of dye decolorization, degraded metabolites of pure azo dyes methyl orange were analysed using spectrophotometer and HPLC.

Spectrophotometric analysis

Dye decolorization was monitored at 508 nm using LabIndia UV 3000^{+} UV/VIS spectrophotometer and compared with the untreated sample dye.

High Performance Liquid Chromatography (HPLC)

HPLC was performed with Perkin Elmer System (Series 200 Ic pump, US Instruments Division, Norwalk, USA), Applied Biosystems 785A programmable Absorbance Detector equipped with a Lichro CART 125-4 HPLC C-18 reverse phase catridge (Merck KGaA 64271 Damstad, German) with a mobile phase of HPLC grade methanol at a flow rate of 1 ml min^{-1} using isocratic method with a 10 min run time.

The temperature was between 20°C and 25°C. The 5µl of sample volume was injected using microliter chromatography syringe (microliter #702 Made in USA, Hamilton Co, RENO, NEVAD, 25µl) and compounds were detected at 280nm and quantified by the area under curve of the chromatogram. HPLC calibration standard curve were prepared for methyl orange (200µM).

Results and Discussion

Immobilization of laccase by different methods

Cercospora sp. releases laccase, which permits several possible interactions with the support surface. In the present study, the laccase from *Cercospora* sp. was immobilized on 4 different matrices by different methods such as entrapment in calcium and copper alginate, adsorption on silica matrix, immobilization on carrageenan and agar gel disc. Immobilization of laccase on calcium alginate was seemed to be the most efficient.

Alginate is a natural polymer and can be easily converted into hydrogels via cross-linking with divalent cations. Calcium alginate is the most commonly used support for enzyme immobilization (Palmieri *et al.*, 1994) as shown in Table 1. The formation, mechanical and structural properties of alginate beads depend upon different parameters such as the alginate concentration, nature and the concentration of the cations (Ouwerx *et al.*, 1998).

Initially, several supports were tested to immobilize the enzyme and a close correlation between enzymatic immobilization and dye adsorption capacity was invariably observed and until now, laccase has been immobilized on various supports including immobilization on oxirane acrylic beads, porosity glass**,** copper alginate gel (Palmieri *et al.*, 1994) and activated carbon. Laccases immobilization can see on inorganic supports like alumina (Kandel bauer *et al.*, 2004; Zille *et al.*, 2003), and silica (Peralta-Zamora *et al.*, 2003).

Optimization of immobilization conditions

Maximum laccase binding efficiency was observed at 2.5% calcium alginate. Lower concentration of alginate resulted in beads with poor gel strength and increased enzyme leaching. The beads were more transparent and the bead shape was also affected at lower concentration of alginate. Increasing the alginate concentration beyond 2.5% failed to enhance the laccase binding efficiency which indicated that 2.5% sodium alginate concentration was the optimum for proper bead formation. The nature of cation is one of the other crucial factors that determine the functional properties of the beads. Extensive studies have been carried out on the type of cations and their interaction with alginate during bead formation (Ouwerx *et al.,* 1998; Rodrigues and Ricardo 2006). In case of calcium alginate beads, the maximum laccase binding efficiency was observed at 0.2M $CaCl_2$ concentration. Also, due to alginate's biocompatibility and simple gelation with divalent cation such as Ca^{2+}, it is widely used for cell immobilization and encapsulation.

When the overall efficiency of all the immobilized systems were compared, calcium alginate beads proved to be the better support for the immobilization of laccase, exhibiting higher binding efficiency and enzyme activity as shown in Figure 2. This was probably because calcium having high affinity for alginates was strongly complexed in the polymer network as reported by Ouwerx *et al.* (1998). The operational efficiency (efficiency at which the system performs) of any immobilized system depends upon the amount of enzyme retained by the system (Laccase binding efficiency). Hence calcium alginates were selected as best immobilization matrix for further studies.

Selection of buffer system and pH optimization

Among four buffers tested (pH ranging from 2.5 to 8.0), maximum decolorization of the dye i.e. 83.02% was recorded in citrate phosphate buffer with pH 2.5 as shown in (Fig. 3). *G. lucidum* laccase prefers acidic range for higher decolorization extent. The maximal decolorization was observed at pH 4.0 for both RB-5 and RBBR (Deveci *et al.,* 2004). The percentage decolorization falls as pH was altered. The reason is that the active site loses its affinity towards substrate at extreme pH. These findings were in correlation with several earlier reports for *P. ostreatus* laccase, showing pH optima of 3.0–3.6 for ABTS (Palmieri *et al.,* 2003).

Optimization of buffer molarity

To find out the optimum molarity of buffer for achieving maximum dye decolorization by alginate gel entrapped enzyme, the molarity of citrate phosphate buffer was varied from 0.025M–0.1M. The maximum decolorization of 72.33% was obtained with 0.025M concentration of citrate phosphate buffer as shown in Figure 4. The further increase or decrease in buffer molarity has adversely effected the dye decolorization. Lu *et al.* (2007) also reported same buffer phosphate citrate at slightly higher concentration (0.1M) for alginate- chitosan immobilized laccase system.

Optimization of reaction temperature for decolorization of methyl orange

A gradual increase in the decolorization of methyl orange by immobilized enzyme was observed when temperature was increased from 25°C–55°C and maximum decolorization of 75.05%was recorded at 35°C. Increase in temperature up to 45°C showed little or no change in decolorization and further increase in temperature resulted

in slight decrease in the dye decolorization. These results were in concurrence with the experiment performed for mediator based decolorization of azo dyes by laccase from *Streptomyces psammoticus* by Niladevi *et al.* (2008).

Optimization of incubation time for dye decolorization

Immobilized laccase has shown maximum decolorization of dye (72.14%) after 30 min of incubation. Further increase in incubation time led to slight decrease in dye decolorization as shown in Figure 5. It might be either due to product inhibition or denaturation of enzyme, when incubated for a longer period of time. Similarly, de Araújo *et al.* (2005) performed assay of laccase of *Botryosphaeria* sp., *Pleurotus ostreatus* and *Aspergillus* sp. with ABTS at 55°C.

Optimization of dye concentration for decolorization of methyl orange

Decolorization rate was influenced by concentration of dye. Immobilized laccase with 12.34U (20 alginate beads) at 35°C showed maximum decolorization of (65.75%) at 200 µM/1.1ml of reaction mixture as shown in Figure 6. The result revealed that the dye decolorization was decreased or remains constant with increasing dye concentration. Asgher *et al.* (2012) immobilized *Pleurotus ostreatus* laccase by entrapping in a sol-gel matrix which had optimum substrate concentration 0.5mM for immobilized laccase similarly to one studies.

Optimization of enzyme dose for decolorization of methyl orange by immobilized laccase

Enzyme dose (IU) of immobilized enzyme was optimized for the maximum dye decolorization. Decolorization increases with increase in enzyme dose from 3.08 IU to 12.34 IU as showed in Figure 7.

However, the optimum enzyme dose required to obtain maximum dye decolorization (61.7%) was 12.34 IU (20 Beads). Further increasing the enzyme amount did not enhance decolorization level, this might be due to zone of equivalence between enzyme and substrate reached. A similar observation has been reported in other studies using the crude or purified laccase (Soares *et al.,* 2001; Murugesan *et al.,* 2006; Saito *et al.,* 2003).

Mediators selection

Generally, mediators were reported to enhance the activity of laccase enzyme. So the effect of various mediators like PABA, HOBT, PHBA, Pyrogallol, ABTS etc were checked for maximum dye decolorization by immobilized laccase. Maximum dye decolorization (77.96%) was recorded in the presence of Pyrogallol as showed in Figure 8. Similar results for decolorization of reactive dyes by immobilized laccasefrom (*Trametes versicolor*), were reported by Peralta-Zamora *et al.* (2003).

Optimization of pyrogallol concentration for decolorization of methyl orange

Varying concentrations of selected mediator (pyrogallol) were used in the reaction mixture for decolorization of dye by immobilized enzyme. Immobilized laccase showed maximum decolorization (78.46%) with 160µM pyrogallol of 1mM stock solution as shown in Figure 9.

Effect of metal ions and other inhibitors on the decolorizationof methyl orange by immobilized laccase

Generally, metal ions act as cofactors for many enzymes. In order to establish the role

of metal ion as a cofactor in dye decolorization, various metal ions at concentration of 1mM was added to the reaction mixture. From Figure 10, we can conclude that maximum decolorization of 84.31% was observed with Zn^{2+}. None of the metal ions showed strong inhibitory effect. Although with SDS only 52.3% dye decolorization was achieved. Leontievsky *et al.* (2001) also reported that the immobilized enzyme was more tolerant to the effect of the strong inhibitor sodium azide.

Reusability of immobilized laccase

Reusability of the immobilized enzyme for dye decolorization was assayed at 35ºC for 30 min repeatedly till the activity decreased to about 50 percent or less or till it show constant percent dye decolorization. The experiment showed in Figure 11, up to 7th reaction cycles the activity of immobilized alginate entrapped laccase was reduced only to 50%. So this is the advantage of immobilized laccase enzyme for the dye decolorization.

Batch and Fed batch decolorization of methyl orange at laboratory scale (50 ml) by alginate entrapped laccase

Optimized parameters were used at 50 ml scale to study batch and fed batch decolorization of methyl orange. In batch more dye was added in a single feeding at the time of incubation and in fed batch mode, total three feedings of dye was added to the reaction mixture.

Samples were taken at the regular intervals of the time and percent decolorization was measured as shown in Figure 12 & 13. With batch mode of decolorization, maximum dye decolorization (77.23%) was recorded and with fed batch, slight decrease of 0.73% in decolorization, maximum decolorization of 76.50% was achieved.

Decolorization of methyl orange by alginate entrapped laccase using five stage plug flow reactor

The experiments were performed in the reactor that contained laccase entrapped in calcium alginate beads as shown in Figure 14. It was observed that 89.09% dye decolorization was achieved in the 5th reactor 1st run after 15 hrs of reactor running time.

The operational stability of the immobilized laccase was estimated by reusing the same beads for successive runs. A total of 5 runs, each with duration of 2 hrs were performed. The dye model solution of same composition was used for the successive catalytic cycles. Enzyme leaching has been reported as the major problem with alginate beads. However, in the present study it was observed that the system maintained 50% of operational efficiency even after 5 successive runs. The results indicated that the enzyme leaching was prominent only during the initial run that reduced the dye color from 46% to 13% within 1st reactor and varies in different reactors stage and runs. After the initial run, the enzyme leaching was relatively low, resulting in 50% operational efficiency after 5 runs (28 hrs) as shown in Figure 15. This was probably due to the fact that the enzyme coated on the surface was leached out easily during the initial run while the enzyme entrapped deep within the alginate beads is retained relatively for long duration.

Dye decolourization was also studied by taking into consideration all five stages of single run with respect to Standard Control (S.C.) and Relative Control (R.C.) as shown in Figure 16. There was about 67.32% dye decolorization in reactor stage No-5 during the first run. A gradual increase in

decolorization of dye in first run of all five subsequent reactor stages is due to the fact that the latter reactor stage always gets the decolorized dye from the previous reactor, that's why 5th reactor showed 67.32% decolorization of dye in comparison to 1.75% of 1st reactor as showed in Figure 17.

Analysis of metabolites formed after decolorization of dye

High Performance Liquid Chromatography (HPLC): The residual amount of by product formed were determined for each cycle by HPLC.HPLC analysis shown in Figure 18, of methyl orange showed 2 peaks at 0.57 min and 0.53 min, whereas metabolites after decolorization showed peaks at 0.74, 1.73, 2.93 and 4.73 min. The difference in retention time between methyl orange and metabolites formed after decolorization implies the biodegradation of methyl orange into different metabolites. The decolorization patterns of dye by the five stage plug-flow reactor are shown by HPLC chromatograms.

Spectrophotometric analysis: The decolorization was monitored at λ_{max} of dye i.e 508 nm using Labindia UV 3000+ UV/VIS spectrophotometer. UV/VIS spectrum of non degraded dye and degraded dye at each stage of reactor was analysed at wavelength in range of 700.00 nm–400.00 nm with Y-axis range of 0.00–2.00 at the interval of 0.5. Although dye and the decolorized dye products showed out of range spectrum, yet we can see the shift of spectrum towards left as compared to untreated dye spectrum as shown in Figure 19. The untreated dye showed maximum absorbance at 480–508 nm range but the decolorized dye sample from each stage of reactor showed max absorbance at 400 nm. This shift in spectrum may be due to the alteration in the structure of azo dyes. At each stage of reactor, there was steadily increase in percentage of dye decolorization as compared to previous stage, with overall decolorization of 81.36% at 5th stage with comparison to untreated dye.

Table.1 Immobilization of laccase by different methods and its activity

Immobilization methods	**Enzyme activity (IU/g matrix)**	**Immobilization efficiency (%)**
Agar Discs	9.78	51.36
Ca-alginate	19.16	72.42
Cu- alginate	18.76	71.78
Carrageenan (Irish moss)	8.69	48.23
Silica 60 (0.060-0.2mm)	12.09	56.12

Fig.1(a) Alginate Beads (b) Silica immobilized enzyme (c) Agar Discs

Fig.2 Selection of buffer and optimization of pH for dye decolorization of methyl orange by alginate gel entrapped laccase from *Cercospora* sp.

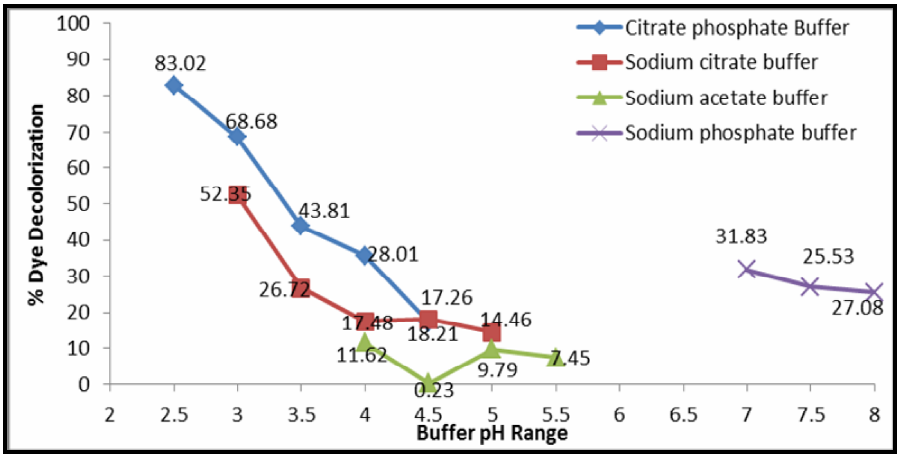


Fig.3 Effect of citrate phosphate buffer molarity on methyl orange decolorization

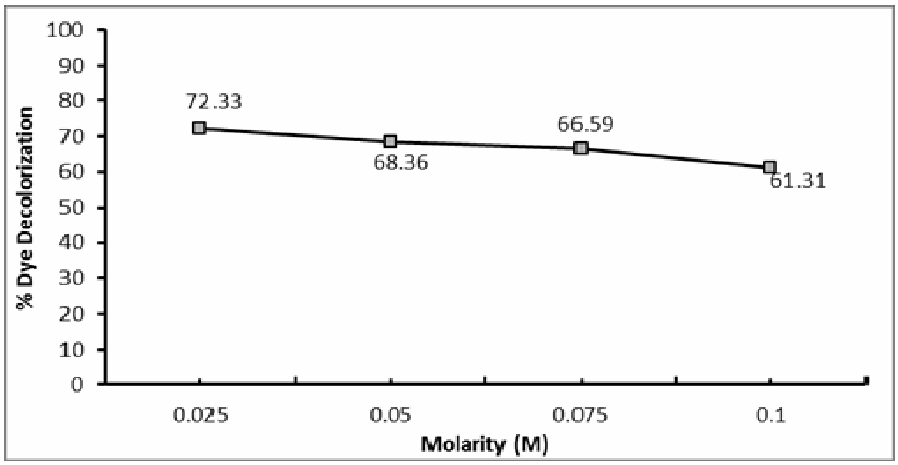


Fig.4 Optimization of incubation time for dye decolorization

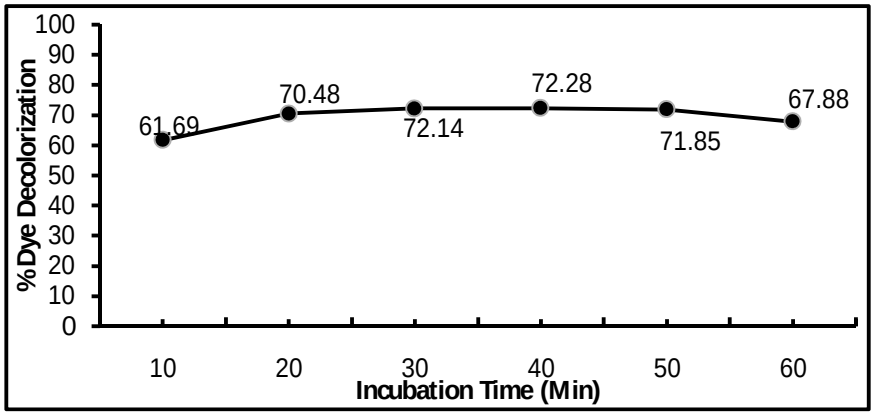


Fig.5 Optimization of dye concentration for dye decolorization

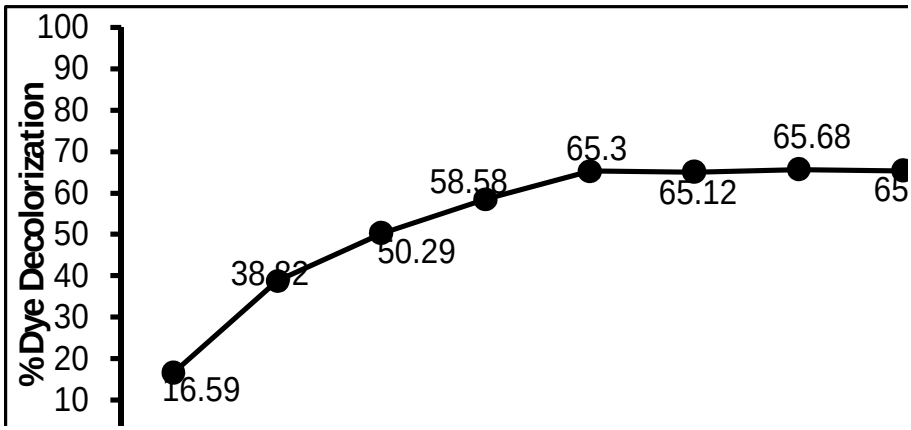


Fig.6 Optimization of enzyme dose for decolorization of methyl orange by alginate entrapped laccase

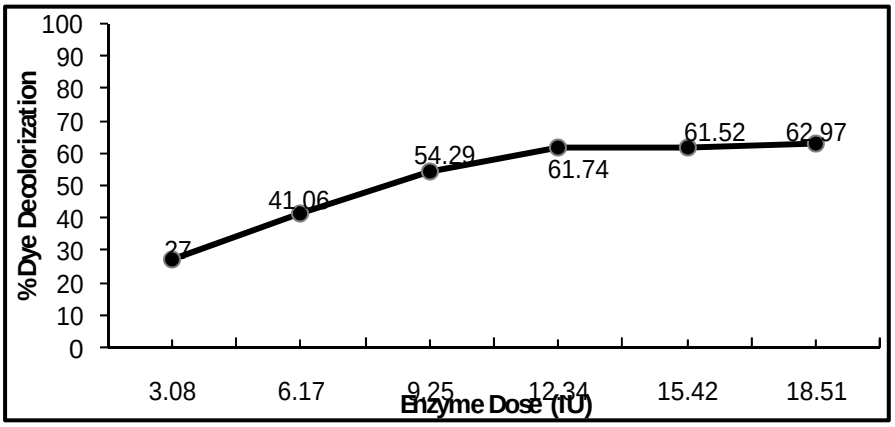


Fig.7 Effect of different mediators on decolorization of methyl orange by alginate entrapped laccase

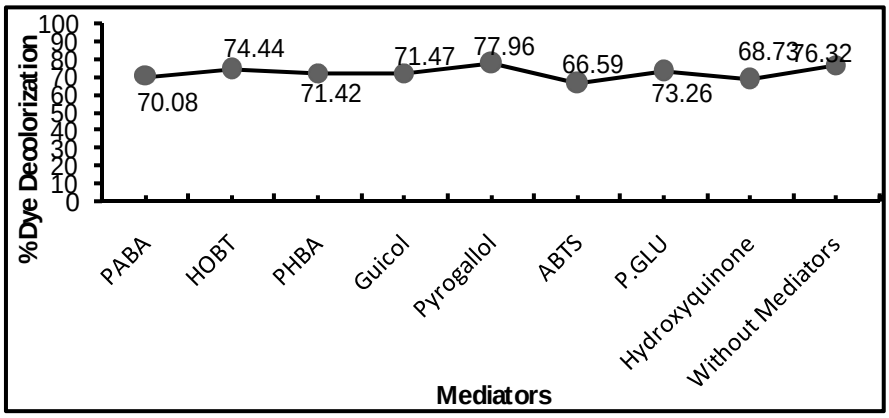


Fig.8 Effect of Pyrogallol concentration in decolorization of methyl orange by alginate entrapped laccase

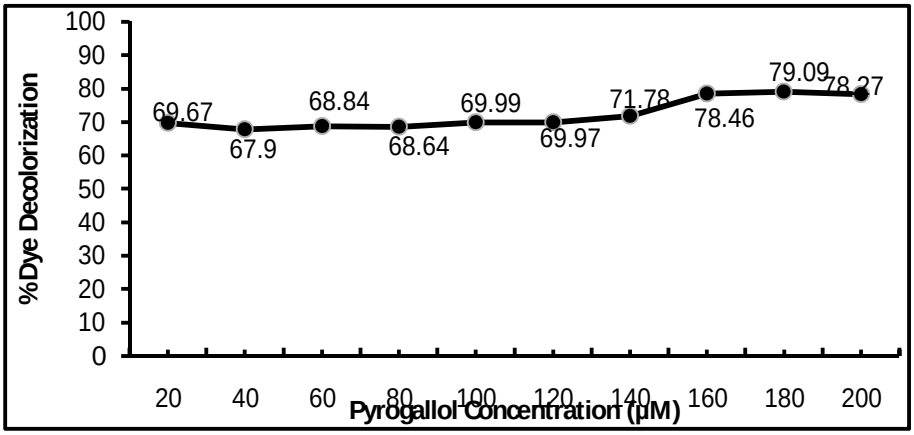


Fig.9 Effect of metal ions in dye decolorization

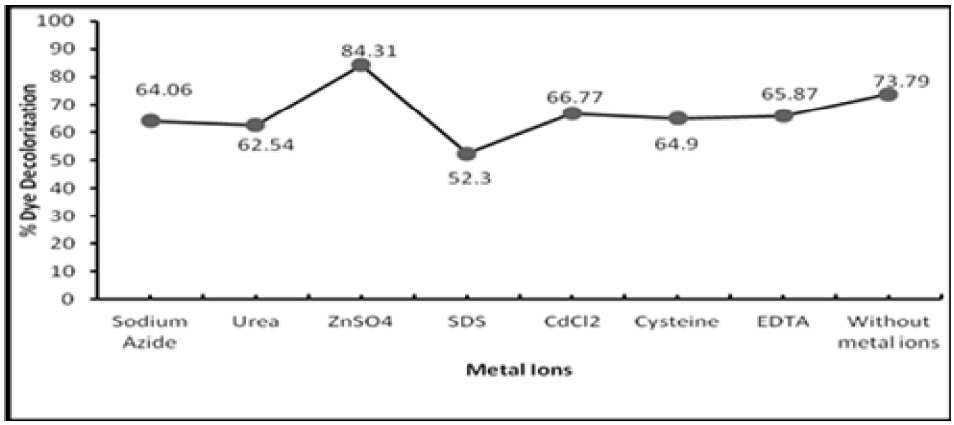


Fig.10 Reusability of immobilized enzyme

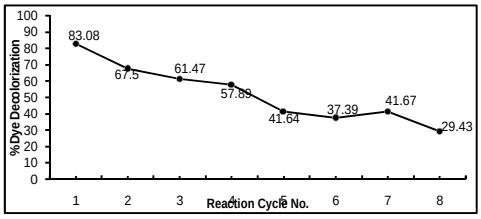


Fig.11 Batch & fed batch decolorization of methyl orange using immobilized laccase

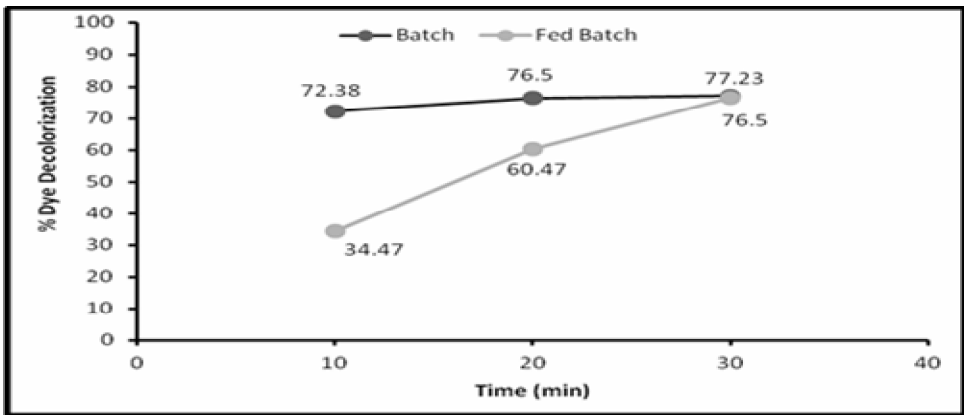


Fig.12 (a) Batch (b) Fed-batch

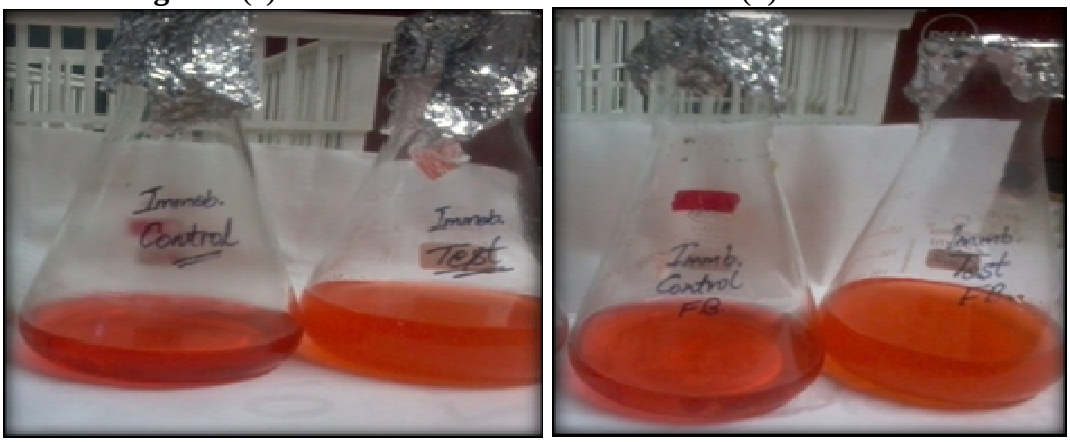


Fig.13 Five-stage plug flow reactor after dye decolorization

Fig.14 Dye decolorization pattern at 200 µM dye

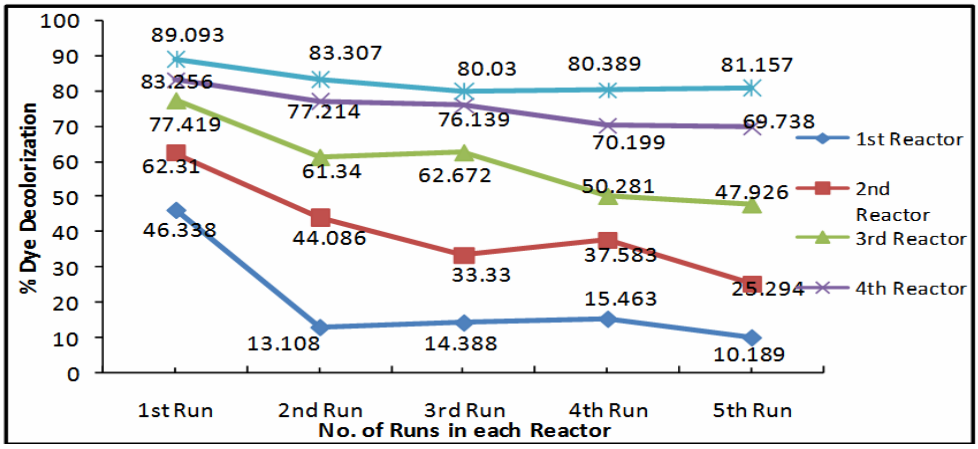


Fig.15 1st run with 200 µM dye

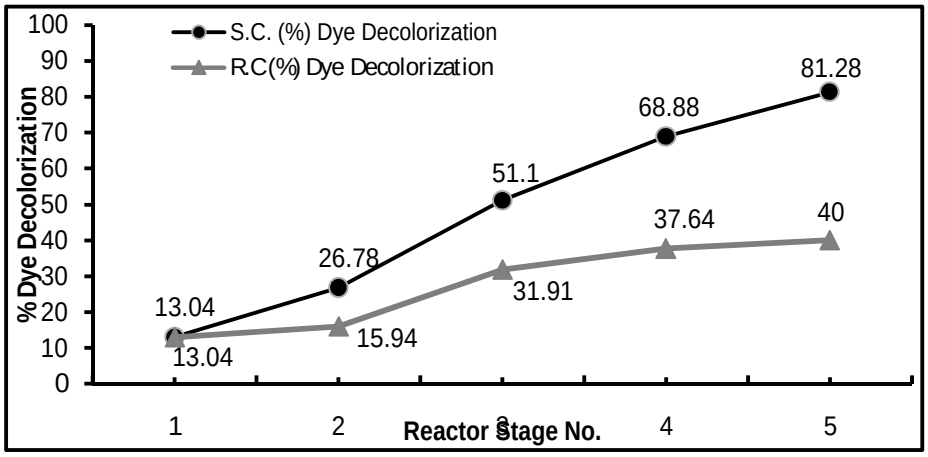


Fig.16 Dye decolorization pattern at 500 µM dye concentration

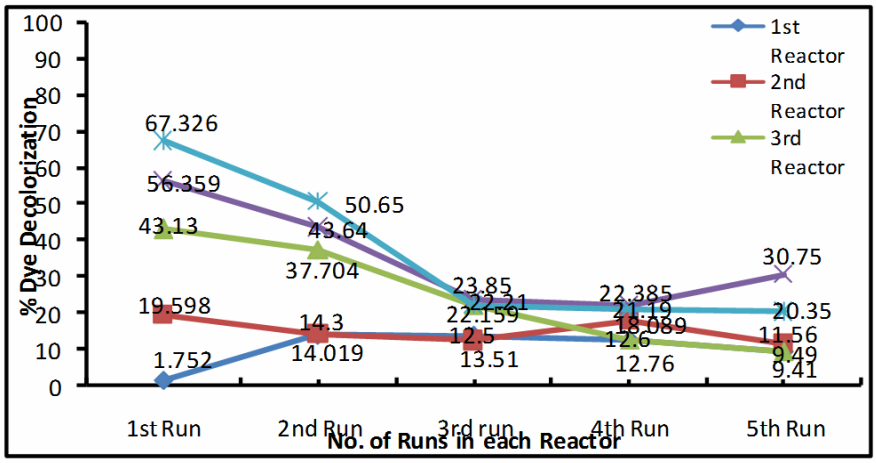


Fig.17 (a) Untreated dye (10X) (b) Decolorized dye (10X)

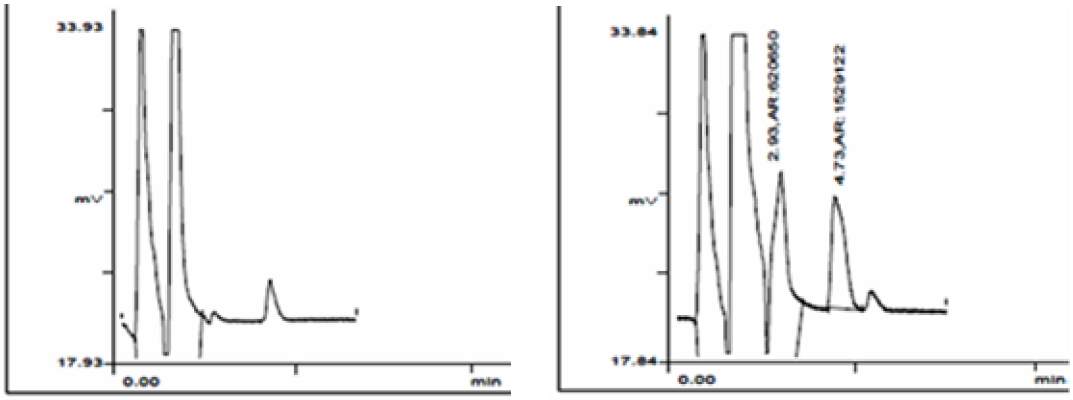


Fig.18 Spectrum of decolorized sample at different reactor stage

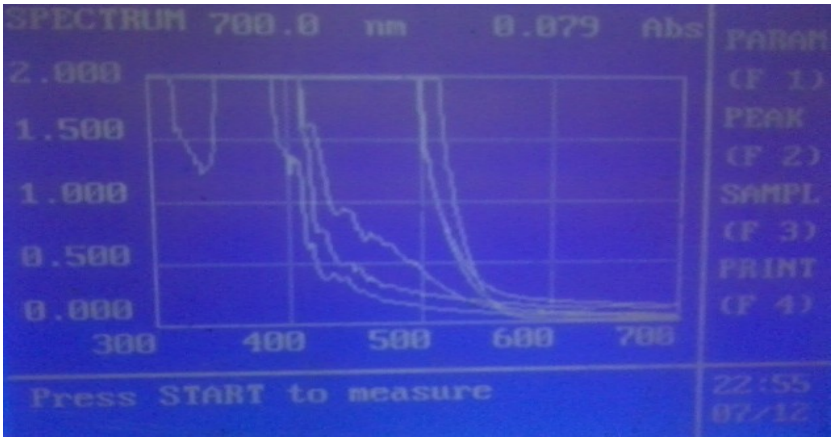


Fig.19 Decolorized dye samples

Fig.20 BUCHI Switzerland B-390 Encapsulator

Fig.21 *Cercospora* sp. SPF-6

Acknowledgement

We acknowledge the staff of the Department of Biotechnology, Himachal Pradesh University, who arranged excellent working conditions for us. We would like to thank University Grant Commission, New Delhi and Department of Biotechnology, Ministry of Science and Technology, GOI for providing funds to Department of Biotechnology, Himachal Pradesh University, Shimla India. Special thanks to Ms. Anita Verma for providing me her novel fungal strain and assisting me in my research work.

References

Asgher, M., Bhatti, H.N., Ashraf, M., Legge, R.L. 2008. Recent developments in biodegradation of industrial pollutants by white rot fungi and their enzyme system. *Biodegradation*, 19: 771–783.

Chand, D., Kumar, H., Sankhian, U.D., Kumar, D., Vitzthum, F., Bhalla, T.C. 2004. Treatment of simulated wastewater containing toxic amides by immobilized *Rhodococcus rhodochrous* NHB-2 using a highly

compact 5-stage plug flow reactor. *World J. Microbiol. Biotechnol.*, 20: 679–689.

Couto, S.R., Toco-Herrera, J.L. 2006. Industrial and biotechnological applications of laccases: A review. *Biotechnol. Adv.*, 24(5): 500–513.

de Araújo, J.H.B., Uemura, V.O., de Moraes, F.F., de Melo Barbosa, A., Zanin, G.M. 2005. A comparative study on fungal laccases immobilized on chitosan Braz. *Arch. Biol. Technol.*, http://dx.doi.org/10.1590/S1516-89132005000400001

Deveci, T., Unyayar, A., Mazmanci, M.A. 2004. Production of remazol brilliant blue R decolorizing oxygenase from the culture filtrate of *Funalia trogii* ATCC 200800. *J. Mol. Catal. B: Enzyme*, 30: 25–32.

Dubrow, S., Boardman, G., Michelsen, D. 1996. Chemical pretreatment and aerobic anaerobic degradation of textile dye wastewater. John Wiley & Sons Inc, New York. Pp. 75–102.

Hai, F., Yamamoto, K., Fukushi, K. 2007. Hybrid treatment system for dye wastewater. *Crit. Rev. Environ. Sci. Technol.*, 37(4): 315–377.

Hassan, M.M., Hawkyard, C.J. 2002. Decolorization of aqueous dyes by sequential oxidation, treatment with ozone and fenton's reagent. *J. Chem. Technol. Biotechnol.*, 77: 834–841.

Kandel bauer, A., Maute, O., Kessler, R.W., Erlacher, A., G¨ubitz, G.M. 2004. Study of dye decolorization in an immobilized laccase enzyme-reactor using online spectroscopy. *Biotechnol. Bioeng.*, 87(4): 552–563.

Leontievsky, A.A., Myasoedova, N.M., Golovleva, L.A., Sedarati, M., Evans, C.S. 2001. Adaptation of the white-rot basidiomycete *Panus tigrinus* for transformation of high concentrations of chlorophenols. *Appl. Microbiol. Biotechnol.*, 59: 599–604.

Lewis, D. 1999. Coloration in the next century. *Rev. Prog. Color. Related Top.*, 29(1): 23–28.

Lu, L., Zhao, M., Wang, Y. 2007. Immobilization of laccase by alginate-chitosan microcapsules and its use in dye decolorization. *World J. Microbiol. Biotechnol.*, 23: 159–166.

Matsui, M. 1996. Environmental chemistry of dyes and pigments, Ch. Ozonation. John Wiley & Sons Inc: New York. Pp. 43–59.

Moawad, H., El-Rahim, W., Khalafallah, M. 2003. Evaluation of biotoxicity of textile dyes using two bioasays. *J. Basic Microbiol.*, 43(3): 218–229.

Murugesan, K., Arulmani, M., Nam, I.H., Kim, Y.M., Chang, Y.S., Kalaichelvan, P.T. 2006. Purification and characterization of laccase produced by a white rot fungus *Pleurotus sajor-caju* under submerged culture condition and its potential in decolorization of azo dyes. *Appl. Microbiol. Biotechnol.*, 72(5): 939–46.

Nagai, M., Kawata, M., Watanabe, H., Ogawa, M., Saito, K., Takesawa, T., Kanda, K., Sato, T. 2003. Important role of fungal intracellular Laccases for melanin synthesis: purification and characterization of an intracellular laccases from *Lentinula edodes* fruit bodies. *Microbiology*, 149: 2455–2462

Niladevi, K.N., Sheejadevi, P.S., Prema, P. 2008. Stratigies for enhancing laccase yield from *Steptomyces psammoticus* and its role in mediator-based decolourization of Azo dyes. *Appl. Biochem. Biotechnol.*, 151(1): 9–19. doi:

10.1007/s12010-008-8175-6.

O' Neill, C., Hawkes, F.R., Hawkes D., Lourenco, N.D., Pinheiro, H.M., Delee, W. 1999. Color in textile effluents- sources, measurement, discharge consents and simulations: A review. *J. Chem. Technol. Biotechnol.*, 74(11).

Ouwerx, C., Velings, N., Mestdagh, M.M., Axelos, M.A.V. 1998. Physico-chemical properties and rheology of alginate gel beads formed with various divalent cations. *Poly Gels Networks,* 6: 393–408.

Palmieri, G., Cennamo, G., Faraco, V., Amoresano, A., Sannia, G., Girdina, P. 2003. Atypical laccase isoenzymes from copper supplemented *Pleurotus ostreatus* cultures. *Enzyme Microb. Technol.*, 33: 220–230.

Palmieri, G., Giardina, P., Bianco, C., Scaloni, A., Capassoi, A., Sannia, G. 1994. A novel white laccase from *Pleurotus ostreatus*. *J. Biol. Chem.*, 272: 31301–31307.

Peralta-Zamora, P., Pereira, C.M., Tiburtius, E.R.L., Moraes, S.G., Rosa, M.A., Minussi, R.C., Duran, N. 2003. Decolorization of reactive dyes by immobilized laccase. *Appl. Catal. B Environ.*, 42: 131–144.

Reife, A., Freeman, H. 1996. Environmental chemistry of dyes and pigments, Ch. Carbon adsorption of dyes and selected intermediates. John Wiley & Sons Inc, New York. Pp. 3–29.

Riva, S. 2006. Laccases: Blue enzyme for green chemistry. *Trends Biotechnol.*, 24(5): 219–26.

Robinson, T., McMullan, G., Marchant, R., Nigam, P. 2001a. Remediation of dyes in textile effluent: A critical review on current treatment technologies with a proposed alternative. *Bioresour. Technol.*, 77(3): 247–255.

Robinson, T., Nigam, P.S. 2001b. Remediation of textile dye waste water using a white rot fungus *Bjerkandera adusta* through solid-state fermentation (ssf). *Appl. Biochem. Biotechnol.*, 151(23): 618–628.

Rodrigues, R.J., Ricardo, L. 2006. Copper ions binding in Cu-alginate gelation. *J. Carbohydr. Chem.*, 25: 219–232.

Saito, T., Hong, P., Kato, K., Okazaki, M., Inagaki, H., Maeda, S. 2003. Purification and characterization of an extracellular laccase of a fungus (family Chaetomiaceae) isolated from soil. *Enzyme Microb. Technol.*, 33: 520–6.

Soares, G.M.B, Costa-Ferreira, M., Amorim, M.T.P. 2001. Use of laccase together with redox mediators to decolorize Remazol Brilliant Blue R. *J. Biotechnol.*, 89: 123–9.

Zidane, F., Drogui, P., Lekhlif, B., Bensaid, J., Blais, J.F., Belcadi, S., Kacemi, K.E. 2008. Decolorization of dye containing effluent using mineral coagulant produced by electrocoagulation. *J. Hazard. Matter*, 161(23): 974–981.

Zille, A., Tzanov, T., G¨ubitz, G.M., Cavaco-Paulo, A. 2004. Immobilized laccase for decolorization of reactive black 5 dyeing effluent. *Biotechnol. Lett.*, 25(17): 1473–1477.