Chapter 8 -

Immobilization of laccase from T. viride
8.1. Introduction

In order to increase the potential use of laccase in bioremediation processes, their immobilizations are necessary for biochemical stability and reusability (Couto et al. 2004; Delanoy et al. 2005; Peralta-Zamora et al. 2003). Selection of immobilization conditions is essential to design a system appropriate to each particular purpose and enzyme. Entrapment in alginate beads is one of the simplest methods of enzyme immobilization (Kierstan and Bucke 1977). Laccase is a copper-dependent enzyme and the enzyme immobilized in copper alginate is likely to retain more activity than laccase immobilized using other methods. Laccase of Pleurotus ostreatus and Ganoderma sp were successfully entrapped in copper alginate beads and decolorized some synthetic dyes efficiently (Palmieri et al. 2005; Teerapatsakul et al. 2008). But in some circumstances pore size of the alginate beads and the low physical stability of the beads in the presence of chelating agents can be problematical.

The biotreatment of industrial effluents that contains heavy metals, detergents, copper chelating agents and phenolic compounds arise a great concern to industries as these compounds are either toxic to microbes or they act as non-competitive inhibitors or as denaturing agents for several enzymes. So it is essential to know the effect of various agents usually present in the treatment plant on activity of the enzyme, for extending its applications in in-situ treatment processes. However very few studies are available on the optimization of
laccase entrapment in copper alginate beads. Also the effect of various agents present in the biotreatment plant on the enzymatic activity of immobilized laccase was not previously examined. This study describes the immobilization conditions of laccase from a newly isolated phenol and salt tolerant strain of *Trichoderma viride* Pers NFCCI-2745 and analysis of the effects of heavy metal ions, detergents and copper-chelating agents on immobilized enzyme.

### 8.1.1. Objectives of the study

The main objective of the study is to optimize the immobilization conditions of laccase and analyze of the effects of various agents present in the biotreatment plant on the enzymatic activity of immobilized enzyme.

### 8.2. Materials and Methods

#### 8.2.1. Chemicals and enzyme

Sodium alginate was purchased from Himedia, India. All other metal salts and chemicals used were of analytical grade and obtained from Himedia, SRL and Merck, India. *Trichoderma viride* Pers NFCCI-2745 was used as a source for laccase.

#### 8.2.2. Enzyme immobilization

Sodium alginate powder (3 % w/v) was added to the crude enzyme solution (10 U/ml) and then the mixture was stirred thoroughly to ensure complete mixing for 20 minutes. The mixture was added drop-by-drop by
Chapter 8-Immobilization of laccase

means of a peristaltic pump equipped with a syringe into 50 mL of 50-300 mM CuSO₄ used as cross linker solutions, dissolved in distilled water (flow rate 10 mL min⁻¹), corresponding metal-alginate beads were formed. After 30 minutes the spherical beads were washed with distilled water. The immobilization yield was determined as residual laccase activity found after dissolution of beads (by incubation in 50 mM sodium acetate buffer of pH 4.0, temp- 32 °C for 15 minutes) compared with the laccase activity added to the alginate solution. The same procedure was used to study effects of crosslinking agents such as CaCl₂, ZnSO₄, SrCl₂, BaCl₂, NiCl₂, MgCl₂ and HgCl₂ and the combination of each ions with CuSO₄ (each of 0-75 mM). The selected enzyme alginate beads were used for following study; pH and temperature optima, analyzing the tolerance of immobilized laccase to heavy metal ions, detergent and copper-chelating agents.

8.2.3. Optima pH and temperature of immobilized alginate laccase

Cu-Ba 75 mM (7.5: 2.5) alginate beads, Cu–Ni 75 mM (7.5: 2.5) alginate beads, Cu-alginate beads (75 mM) and Cu-Sr 75 mM (7.5: 2.5), were selected for following study; pH and temperature optima, analyzing the tolerance of immobilized laccase to heavy metal ions, detergent and copper-chelating agents.

The immobilized enzymes (15 beads/ tube) were assayed for laccase activity using 20 mM guaiacol as substrate in 100 mM buffers pH ranging from 2.0 to 11 at 32°C for 10 min in comparison to free soluble enzyme.
pH optima was studied using the following buffers (100 mM): Glycine-HCl, pH- 2.5, 3; Acetate buffer, pH-3.5, 4, 4.5, 5, 5.5 and 6; Phosphate buffer, pH 6, 6.5 and 7; Tris-HCl buffer, pH 7.5, 8.0 and 9.0 and Glycine-NaOH buffer, pH-9, 10 and 11. For optima temperatures of each immobilized enzymes, the assay reactions were performed in 50 mM sodium phosphate buffer, pH 6.5 at various temperatures ranging from 30 to 90 °C for 10 min with 20 mM guaiacol substrate.

8.2.4. Effect of different pH and temperature on immobilized alginate laccase

pH sensitivity was checked by incubating the beads and the free enzyme with the respective buffers, pH ranging from 4 to 7 for 96 hours at 4 °C and beads were assayed every 24 hour for laccase activity using guaiacol (20 mM) as substrate at 32 °C in comparison to free soluble enzyme. pH sensitivity was studied using the following buffers (50 mM): Acetate buffer, pH- 4, 5 and 6; Phosphate buffer, pH 6, 6.5; Tris-HCl buffer, pH 7.0. For temperature optimization of each immobilized enzymes, the assays reactions were performed in 50 Mm sodium phosphate buffer, pH 6.5 at various temperatures ranging from 30 to 70 °C for 10 min with guaiacol (20 mM) substrate.

8.2.5. Effect of metal ions on the activity of immobilized laccase

Since metal ions can act as non-competitive inhibitors of enzymes, their presence in treatment plants usually posse a threat to biotreatment
processes. So the effect of metal ions on the activity of immobilized laccase was checked. Immobilized laccase was incubated for 10 minutes with 0 - 25 mM metal ion solution and then assayed for laccase activity at standard assay condition. The metal chloride used in the present study are; Hg$^{2+}$, Cd$^{2+}$, Ba$^{2+}$, Sr$^{2+}$, Ni$^{2+}$ and Ca$^{2+}$, Mg$^{2+}$.

**8.2.6. Effect of copper chelating agents on the activity of immobilized laccase**

Since laccase is a copper dependent protein the presence of copper chelating agents in treatment plants usually pose a threat to its stability and enzymatic action. Also in some circumstances the low physical stability of the beads in the presence of chelating agents can be problematical (Teerapatsakul et al. 2008). So the effect of copper chelating agents on the activity of immobilized laccase was checked. Immobilized laccase was incubated for 10 minutes with 1- 50 mM copper chelating agents and then assayed for laccase activity at standard assay condition. The copper chelating agents used in the present study are; EDTA, Sodium thioglycolic acid and Sodium azide.

**8.2.7. Effect of Detergent on the activity of immobilized laccase**

Since most detergents are known to denature enzymes, their presence in treatment plants usually pose a threat to biotreatment processes. So the effect of detergents on the activity of immobilized laccase was checked. Immobilized laccase was incubated for 10 minutes with 0.1 to
10 mM detergent solution and then assayed for laccase activity at standard assay condition. The detergents used in the present study are; SDS, an anionic detergent; CTAB a cationic detergent and Tween 20, non ionic detergent.

8.3. Results and Discussions

In this study, crude laccase enzyme secreted by a salt tolerant strain of T. viride NFCCI-2745 isolated from coconut husk retting ground, a highly saline and phenolic rich zone, in the backwaters of Kerala was investigated for alginate entrapment with different cation types and their combinations. The cation concentrations on enzyme entrapment were optimized.

The effect of pH and temperature, heavy metal ions, detergents and copper-chelating agents on catalytic reaction of the immobilized enzymes was also studied.

8.3.1. Enzyme immobilization on alginate

Different kinds of alginate beads were used to entrap laccase from T. viride NFCCI-2745 (Figure. 8.1.a). Results are plotted in relative percentage taking the activity of free enzyme (10 U/ml) as 100 %. The Cu alginate beads at 75 mM concentration gave highest activity followed by 150 mM Zn-alginate beads, 75 mM Ni-alginate beads and 100 mM Ca-alginate beads which showed significantly lower activity compared to Cu-alginate beads.
Figure 8.1a: photograph showing the activity of certain immobilized beads without dissolution. 2-14: Beads incubated with guaiacol (20 mM) for 10 minutes

1, 15-20: Control (without guaiacol)

1. Cu (50 mM) Alginate beads
2. Cu (75 mM) Alginate beads
3. Sr (75 mM) Alginate beads
4. Ba (75 mM) Alginate beads
5. Ni (75 mM) Alginate beads
6. Cu:Ba (1:1) Alginate beads
7. Cu:Sr (1:1) Alginate beads
8. Cu:Ni (1:1) Alginate beads
9. Cu:Ba (7.5:2.5) Alginate beads
10. Zn (150 mM) Alginate beads
11. Zn (200 mM) Alginate beads
12. Cu: Ni (7.5:2.5) Alginate beads
13. Zn (150 mM) Alginate beads
14. Cu: Sr (7.5 : 2.5) Alginate beads
15. Cu (75 mM) Alginate beads
16. Ba (75 mM) Alginate beads
17. Ni (75 mM) Alginate beads
18. Cu:Ba (7.5 : 2.5) Alginate beads
19. Cu: Sr (7.5 : 2.5) Alginate beads
20. Cu: Ni (7.5:2.5) Alginate beads
Since 75 mM CuSO_4 gave maximum activity the combination with other cations was tried so as to get the ionic strength of the combinations to be maximum of 75 mM only. Of the beads used in combination with copper ions, Cu–Ba alginate beads (7.5: 2.5) gave the highest immobilization yield followed by Cu–Ni alginate beads (7.5: 2.5) and Cu–Sr (7.5: 2.5) respectively than Cu alginate beads (10: 0). Results are plotted in relative percentage taking the activity of free enzyme (10 U/ml) as 100 % (Figure 8.1.b).

It was earlier reported that Cu-alginate laccase from *Trametes villosa* was better support than Ca-alginate for laccase immobilization (Brandi *et al*. 2006). Our results support the earlier findings, and in addition the combinations of Cu^{2+} with other cations also gave promising results. Spherical round beads of 2 mm diameter with laccase activity were formed for 50 - 300 mM CuSO_4, 100 - 200 mM CaCl_2 cross linker solutions, Cu–Ba (75 mM) cross linker solution and Cu–Sr (75 mM) cross linker solution. Where as Cu–Ni (75 mM) cross linker and 150-200 mM ZnSO_4 produced slightly irregular beads. SrCl_2 and BaCl_2 when used alone as cross-linkers, produced irregular shaped beads, with comparatively lower laccase activity and NiCl_2 (75 mM ) when used alone, as cross linker solution, produced string-like alginate laccase, but with detectable enzymatic activity. No beads were formed for MgSO_4 and HgCl_2, when used as cross linkers either alone or in combination with CuSO_4.
The Cu alginate beads at 75 mM concentration gave highest activity followed by 150 mM Zn-alginate beads, 75 mM Ni-alginate beads and 100 mM Ca-alginate beads which showed significantly lower activity compared to Cu- alginate beads. Since 75 mM CuSO$_4$ gave maximum activity the combination with other cations was tried so as to get the ionic strength of the combinations to be maximum of 75 mM only. Of the beads used in combination with copper ions, Cu–Ba alginate beads (7.5: 2.5) gave the highest immobilization yield followed by Cu–Ni alginate beads (7.5: 2.5) and Cu-Sr (7.5: 2.5) respectively than Cu alginate beads (10: 0) (Figure 8.1.c). Increasing the concentration of CuSO$_4$ and other cationic solutions had a negative effect on immobilization yield. Increasing of the alginate or CuSO4 concentration limits the substrate transfer into the alginate bead (Knezevic et al. 2002, Teerapatsakul et al. 2008). In the immobilization of *Candida rugosa* lipase, increasing the alginate concentration decreased immobilization yield but increasing the concentration of the cross-linking agent, CaCl$_2$, had little effect (Won et al. 2005). In the presence of 75 mM CuSO$_4$ 95 % of the initial activity remained even after incubation for 7 days at 4°C. Stimulation of laccase activity upon the addition of Cu$^{2+}$-ion were reported in *Pleurotus ostreatus* and *Ganoderma* sp (Baldrian and Gabriel 2002; Teerapatsakul et al. 2008).

Laccase is a copper dependent enzyme and thus Cu ions play an important role in maintaining the catalytic mechanism of laccase (Palmieri et al. 1997; Dura´n et al. 2002). To our knowledge this is
the first study describing the immobilization of laccase with different cations and their combinations.

![Figure 8.1b: Relative activity of the immobilized beads](image)

It was earlier reported that Cu-alginate laccase from *Trametes villosa* was better support than Ca-alginate for laccase immobilization (Brandi et al. 2006). Our results support the earlier findings, and in addition the combinations of Cu with other cations also gave promising results. Increasing the concentration of CuSO$_4$ and other cationic solutions had a negative effect on immobilization yield. Increasing of the alginate or CuSO4 concentration limits the substrate transfer into the alginate bead (Knezevic et al. 2002, Teerapatsakul et al. 2008). In the immobilisation of *Candida rugosa* lipase, increasing the alginate concentration decreased
immobilization yield but increasing the concentration of the cross-linking agent, CaCl$_2$, had little effect (Won et al. 2005).

**Figure. 8.1c:** Relative activity of the immobilized beads in the combination

In the presence of 75 mM CuSO$_4$ 95 % of the initial activity remained even after incubation for 7 days at 4 $^\circ$C (data not shown). Stimulation of laccase activity upon the addition of Cu$^{2+}$-ion were reported in *Pleurotus ostreatus* and *Ganoderma* sp (Baldrian and Gabriel 2002; Teerapatsakul et al. 2008). Laccase is a copper dependent enzyme and thus Cu ions play an important role in maintaining the catalytic mechanism of laccase (Palmieri et al. 1997;
Dura\’n et al. 2002). To our knowledge this is the first study describing the immobilization of laccase with different cations and their combinations.

**8.3.2. pH and Temperature Optima of immobilized laccase**

All the tested Immobilized enzymes displayed almost similar pattern of pH effects compared to that of the free enzyme. The immobilized enzyme was active in a broad pH range of 2.5 – 7.0 with maximum activities at pH 4.0 and pH 6.5 (Figure 8.2.a). In contrast to earlier reports no significant shift in the optimum pH was observed for immobilized laccase. The alginate is able to absorb H\(^+\) within beads that resulted in decreasing the H\(^+\) outside beads and shifting the optimum pH (Lu et al. 2007; Tischer and Kasche 1999; Yinghui et al. 2002). This problem could be resolved by using the solution with a high ionic strength (Tischer and Kasche, 1999). The Cu-alginate beads (75 mM), Cu-Ba- 75 mM (7.5: 2.5), Cu–Ni- 75 mM (7.5: 2.5), and Cu-Sr- 75 mM (7.5: 2.5) alginate beads showed.

Study on the effect of temperature on laccase activity of the immobilized -alginate enzymes compared to the free enzyme however revealed significant differences (Figure8.2.b).

All the immobilized enzymes analyzed, exhibited higher temperature optima than that of free enzyme. The immobilized alginate enzymes had the highest activity at 80 °C whereas the temperature optimum for free enzyme was 70 °C. A similar effect of
rise in the optimum temperature by 10 °C were reported for lipase in grey mullet and laccase of *Lentinus polychrous* (Aryee and Simpson, 2012; Phetsom *et al.* 2009).

![Figure 8.2a: Optimum pH of the immobilized enzymes](image-url)
8.3.3. pH and Temperature tolerance of immobilized laccase

The immobilized Cu-alginate enzymes were well stabilized than all other immobilized enzymes, when kept in tested buffer pH ranging from 4.0 to 7.0 for 96 hours with interesting increased in laccase activity (Figure 8.3.a,b,c,d).

Ionic strength conditions have been reported previously to affect immobilization (Smalla et al. 1988; Grabski et al. 1995). Eupergit–laccase complex demonstrated a 60 % increase in activity for the immobilized laccase when the binding buffer concentration increased from 0.1 to 1.5 M (D’Annibale et al. 2000).
Figure 8.3 a): pH tolerance of Immobilized beads Cu-alginate beads

Figure 8.3 b): pH tolerance of Immobilized beads Cu-Ba alginate
Figure 8.3: pH tolerance of Immobilized beads c) Cu-Sr alginate; b) Cu-Ni alginate
Figure 8.3: pH tolerance of Immobilized beads c) Cu- Sr alginate; b) Cu-Ni alginate

As shown in Figure 8.4, immobilized laccase exhibited activity at broad temperature ranges (30-60°C), gradually decreasing its activity at higher temperature. There are reports on immobilized laccase showing a higher thermal stability than free enzyme at the same temperature (Reyes et al. 1999; Cho et al. 2008).

The immobilized-laccase activity was extremely lost up to 70 % at temperatures greater than 60°C. The highest thermal stability was observed at 50°C for all Cu alginate enzymes.
Figure 8.4: Temperature tolerance of Immobilized beads
8.3.4. Effect of Metal ions, copper chelating agents and Detergents on the activity of immobilized laccase

The stability exhibited by the immobilized laccase towards high concentrations of various metal ions, copper chelators and detergents signify its substantial potential in biotechnological applications. To calculate relative activities, the actual activity in the absence of these agents under the same experimental conditions was set to 100%.

**Effect of Metal ions**

All the immobilized enzymes displayed almost similar pattern of activity with metal ions (Figure. 8.5). The effect of metal ions on the immobilized enzymes is expressed in relative activity. To calculate
relative activities, the actual activity in the absence of these agents under the same experimental conditions was set to 100 %. The immobilized laccases exhibited an increased activity in the presence of metal ions at lower concentrations. With increase in the concentration of metal ions, laccase activity reduced gradually but retained 50 - 70 % of its activity even at 10 mM concentrations of all the metal ions tested. With further increase in the metal ion concentration above 20 mM, laccase activity decreased but had detectable activity even at 25 mM.
Figure 8.5: Effect of Metal ions on Immobilized beads
Figure 8.5 c & d: Effect of Metal ions on Immobilized beads

**Effect of Detergents**

CTAB and Tween 20 significantly increased the activity of all the beads (Figure. 8.6). All beads expressed almost similar pattern of activity with Ba-Cu alginate exhibiting somewhat higher activity than rest. Of all detergents analyzed, SDS alone decreased the enzymatic activity at concentrations higher than 2 mM.
Figure 8.6.a: Effect of detergents on Immobilized beads
Figure 8.6 d: Effect of detergents on Immobilized beads; y axis: Concentration of detergents in mM

**Effect of copper chelating agents**

Cu-Ba and Cu-Ni alginate beads retained 90% of its initial activity even in the presence of 20 mM EDTA and Sodium thioglycolic acid, whereas Cu alginate beads could retain only 70% of its initial activity under the same conditions (Figure 8.7). This may be due to the effect of copper chelating agents on the physical stability of the...
Cu-alginate beads (Teerapatsakul et al. 2008). Sodium azide on the other hand inhibited 80% of the activity of all the beads even at a very low concentration of 0.5 mM.

Figure 8.7 a: Effect of Cu-Chelators on Immobilized beads. Y axis: Concentration of Cu Chelators (mM)
To our knowledge this is the first study describing the immobilization of laccase with different cations and their combinations. Further the effects of various metal ions, copper chelating agents and detergents on the enzymatic activity of immobilized laccase were not previously examined. The stability exhibited by the immobilized laccase towards a broad range of pH, temperature, high concentrations of various metal ions, copper chelators and detergents signify its substantial potential in
biotechnological applications. These properties of the immobilized laccase may be carefully considered and appropriately utilized in treating phenolic effluents that may contain heavy metals, detergents and certain copper chelating agents which may otherwise pose a threat to enzymatic activity.