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ORIGINAL PAPER

Assessing the tolerance of immobilized laccase from a salt-tolerant strain of *Trichoderma viride* Pers NFCCI-2745 to heavy metal ions, detergents and copper chelating agents

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Abstract The biotreatment of industrial effluents that contain heavy metals, detergents and metal chelating agents represents a great concern to industries as these compounds are either toxic to microbes or they act as non-competitive inhibitors/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. This study describes the immobilization conditions of laccase and analysis of the sensitivity of immobilized laccase from *Trichoderma viride* Pers NFCCI-2745 to heavy metals, detergent and copper chelating agents. The concentrations and combinations of cations influenced the activity of immobilized enzyme and its yield. The immobilized Cu–Ba alginate enzyme showed comparatively higher activity compared to the Cu-alginate enzyme. Most of the metal ions tested enhanced laccase activity at lower concentration. Immobilized laccase retained more than 70 % of its activity even in the presence of 20 mM copper chelating agents like EDTA and sodium thioglycolic acid. 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. Cu–Ba and Cu–Ni alginate enzyme exhibited comparatively higher tolerance than Cu-alginate beads to EDTA and sodium thioglycolate. Tween 20 and cetyl trimethyl ammonium bromide significantly increased the activity of all the beads. These properties of the immobilized laccase may be aptly considered and suitably 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.

Keywords Copper chelating · Heavy metals · Immobilization · Laccase · *Trichoderma* sp.

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Introduction

Laccases (benzenediol oxygen oxidoreductases, EC 1.10.3.2) are considered as one of the most promising enzymes for future industrial applications (Xu et al. 2000). Due to their low substrate specificity and strong oxidative abilities, laccases have a number of industrial applications such as textile dye bleaching, enzymatic conversion of chemical intermediates, biopulping, prevention of wine decolouration, the production of valuable compounds from lignin, soil bioremediation and biodegradation of environmental phenolic pollutants and removal of endocrine disruptors (Abadulla et al. 2000; Cuoto and Herrera 2006; Divya et al. 2013a, b, c; Fukuda et al. 2001; Hemmat and Mazaheriassadi 2013; Mayer and Staples 2002; Nayanashree and Thippeswamy 2014; Rama et al. 1998; Sole et al. 2012; Tyagi et al. 2014). The ideal laccases for industrial use would exhibit stability at high temperature and pH conditions (Niladevi et al. 2008).

In order to extend the applications of laccase more efficiently in industrial processes and biotreatment, their immobilization is necessary as it will potentiate its biochemical stability and reusability (Couto et al. 2004; Delanoy et al. 2005; Peralta-Zamora et al. 2003). 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. 2000; Teerapatsakul et al. 2008). The biotreatment of industrial effluents that contain heavy metals, detergents, copper chelating agents and phenolic compounds arises 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. Studies describing the effect of metal ions, surfactants and copper chelating agents have not been studied in detail. A recent study has described the effect of a non-ionic surfactant, merpol, on decolorization of dyes by immobilized laccase (Champagne et al. 2013), but not about anionic or cationic surfactants. 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. Thus, this study has been designed to immobilize laccase and to investigate the effects of heavy metal ions, copper chelating agents and detergents on the potential of immobilized laccase. Laccase-producing phenol and salt-tolerant strain of *Trichoderma viride* Pers NFCCI-2745 used for the study were isolated from the coconut husk retting ground, a highly phenolic and saline region in the back waters of Palayad region, near Kannur University, Thalassery campus, North Kerala, during December 2009, and the entire immobilization study was carried out at the Department of Biotechnology and Microbiology, Kannur University during June 2012 to December 2012.

Materials and methods

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 and Merck, India.

The laccase-producing salt and phenol-tolerant fungi, *T. viride* Pers NFCCI-2745 isolated from the coconut husk retting pile, a highly saline and phenolic rich environment in the estuarine waters of North Kerala, India, were used as a source for laccase. Laccase being an extracellular enzyme may also have the potential to tolerate this harsh environmental condition. So selecting such strain may be advantageous to treat the phenolic effluents like textile and coir industry which are highly phenolic and saline in nature. Identification of the strain was done based on Inter transcribed spacer (ITS) sequencing of rDNA of fungal genome from the National Fungal Culture Collection of India (NFCCI), Pune, India, and the strain was deposited under the accession number: NFCCI-2745, and the ITS sequence of the fungus deposited at GenBank, NCBI under the accession ID: KF638399.1. For immobilization of extracellular laccase, *Trichoderma* sp NFCCI-2745 was cultivated on a defined medium (Divya et al. 2013c) with composition (in gL^{-1}): glucose—10; peptone—1.0; yeast extract—0.5 using 0.3 mM copper to stimulate laccase formation. Mycelia were separated by centrifugation (20 min; 10,000*g*) after 96 h cultivation when laccase activity reached its maximum and vacuum filtered with 0.22 μm membrane (PVDF Durapore GV 0.22 μm, Millipore). Laccase activity was determined by the oxidation of guaiacol (20 mM) buffered with 50 mM sodium phosphate buffer of pH 6.5, temperature −32 °C at 460 nm (Divya et al. 2013c).

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 min. The mixture was added drop-by-drop by means of a peristaltic pump equipped with a syringe into 50 mL of $CuSO_4$ used as cross-linker solutions, dissolved in distilled water (flow rate 10 mL min^{-1}). Experiments were conducted in triplicate with different concentrations of $CuSO_4$ solutions ranging between 50 and 300 mM (50, 75, 100, 125 up to 300 mM); the corresponding metal alginate beads were formed. After 30 min, the spherical beads were washed with distilled water. The same procedure was used to study effects of different concentrations of cross-linking agents such as $CaCl_2$, $ZnSO_4$, $SrCl_2$, $BaCl_2$, $NiCl_2$, $MgCl_2$ and $HgCl_2$ and the combination of each ions with $CuSO_4$, each of 0–75 mM (0 mM cation:75 mM Cu, 25 mM cation:50 mM Cu, 37.5 mM cation:37.5 mM Cu, 50 mM cation:25 mM Cu and 75 mM cation:0 mM Cu,) to get a final concentration of 75 mM. The immobilization yield was determined as residual laccase activity found after dissolution of beads (by incubation in 50 mM sodium phosphate buffer of pH 6.5 temperature −32 °C for 15 min) compared with the laccase activity of free soluble enzyme. The beads which gave maximum enzyme activity were selected for further experiments. The selected enzyme alginate beads were further used for determining the pH and temperature optima and analyzing the tolerance of immobilized laccase to heavy metal ions, detergent and copper chelating agents.

Optima pH and temperature of immobilized alginate laccase

Cu-alginate beads, Cu–Ba alginate beads, Cu–Ni alginate beads and Cu–Sr alginate beads were selected for the following study; pH and temperature optima, and stability and

Table 1 Buffers used for optimization study

Buffers	pH
Glycine–HCl	2.5 and 3
Sodium acetate	3.5, 4, 4.5, 5, 5.5 and 6
Sodium phosphate	6 and 6.5
Tris–HCl	7.0, 7.5, 8.0 and 9.0
Glycine–NaOH	9, 10 and 11

assessing their potential in treating phenolic industrial effluent. All the selected beads were prepared in cross-linker solution of 75 mM of respective cationic solution. For combination beads, the Cu:Ba/Sr/Ni which were maintained in the ratio 7.5:2.5, respectively, to get a final concentration of 75 mM cross-linker solution gave maximum laccase activity and hence selected for further experiments.

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 with free soluble enzyme. pH optima was studied using the buffers (100 mM) given in Table 1.

For optimum temperature 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.

Effect of metal ions on the activity of immobilized laccase

Immobilized laccase was incubated for 10 min with 0–25 mM metal ion solution and then assayed for laccase activity at standard assay condition. The metal chlorides used in the present study are: Hg^{2+}, Cd^{2+}, Ba^{2+}, Sr^{2+}, Ni^{2+}, Mg^{2+} and Ca^{2+}.

Effect of copper chelating agents on the activity of immobilized laccase

Immobilized laccase was incubated for 10 min with different concentration of copper chelating agents ranging between 1 and 10 mM and then assayed for laccase activity at standard assay conditions. The copper chelating agents used in the present study are: EDTA, Sodium thioglycolic acid and Sodium azide. Each experiment was conducted in triplicate.

Effect of detergents on the activity of immobilized laccase

Immobilized laccase was incubated for 10 min with different concentration of detergent solutions, ranging between 0.1 and 10 mM and then assayed for laccase activity at standard assay condition. The detergents used in the present study are: sodium dodecyl sulfate (SDS), an anionic detergent; cetyl trimethyl ammonium bromide (CTAB), a cationic detergent; and Tween 20, non-ionic detergent. Each experiment was conducted in triplicate.

Statistics

All experiments were repeated at least three times, and each value was measured in triplicate. The results are expressed as means ± SDs. To analyze the statistical significance among the different experimental conditions, two-factor ANOVA with a Bonferroni post-test multiple comparisons and a two-tailed paired Student's *t* test was preformed at 95 % confidence interval using GraphPad Prism software.

Results and discussion

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 effects of pH and temperature, heavy metal ions, detergents and copper chelating agents on catalytic reaction of the immobilized enzymes were also studied.

Enzyme immobilization on alginate

Different kinds of alginate beads were used to entrap laccase from *T. viride* NFCCI-2745. Spherical round beads of 2 mm diameter with laccase activity were formed when entrapped in Cu–Ba, Cu–Sr and Cu-alginate beads. While Zn and Cu–Ni alginate entrapment produced slightly irregular beads, Sr and Ba alginate produced higly irregular shaped beads, with comparatively lower laccase activity and Ni-alginate entrapment produced string-like alginate laccase, 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 $CuSO_4$. 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; Duran et al. 2002). It was earlier reported that Cu-alginate laccase from *Trametes villosa* gave better immobilization yield than Ca-alginate for laccase (Brandi et al. 2006). Our results support the earlier findings, and in addition, the combinations of Cu with other cations also gave promising results.

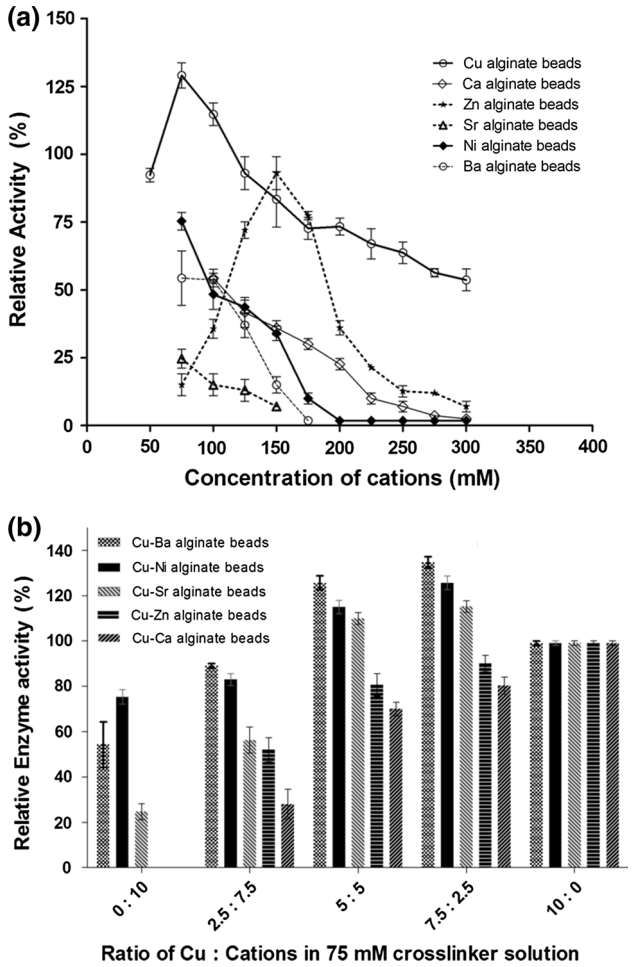

Fig. 1 Alginate entrapment of laccase from *T. viride* NFCCI-2745. **a** Different kinds of alginate beads. **b** Ratio of Cu^{2+} in the combination

The enzyme activity of the immobilized beads is plotted in relative percentage taking the activity of free enzyme (10 U/mL) as 100 %. Of the six metals tested as shown in Fig. 1a, the Cu-alginate beads at 75 mM concentration gave highest activity (*P* value <0.001) 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. Increasing the concentration of $CuSO_4$ and other cationic solutions had a negative effect on immobilization yield. It was earlier reported that increasing of the alginate or $CuSO_4$ concentration limits the substrate transfer into the alginate bead (Knezevic et al. 2002; Teerapatsakul et al. 2008). Also in the immobilisation of *Candida rugosa* lipase, increasing the alginate concentration decreased immobilisation yield, but increasing the concentration of the cross-linking agent,

Table 2 Two-factor ANOVA with Bonferroni's post-test indicating the significance of ratio of Cu in combination with Ba, Ni and Sr

Cu-alginate	Ratio of Cu	Difference	*T*	*P* value
Cu–Ba	5.000	40.33	13.57	<0.001
	7.500	42.07	14.16	<0.001
Cu–Ni	5.000	29.67	9.985	<0.001
	7.500	33.07	11.13	<0.001
Cu–Sr	5.000	24.67	8.302	<0.001
	7.500	22.73	7.651	<0.001

$CaCl_2$ had little effect (Won et al. 2005). Since 75 mM $CuSO_4$ gave maximum activity, all the beads were prepared in cross-linker solution of 75 mM of respective cationic solution for the experiments conducted thereafter.

As shown in Fig. 1b, beads where Cu is in combination with Ba, Ni and Sr gave higher activity than Cu-alginate beads, and combination of Cu with Zn/Ca produced beads with much lesser activity than Cu-alginate. The results of the statistical analysis using two-factor ANOVA with Bonferroni post-tests indicates that Cu in combination with Ba, Ni and Sr were significant in the ratio from 0.5 to 7.5 (*P* value <0.001) data shown in Table 2. Cu:Ba/Sr/Ni which were maintained in the ratio 7.5:2.5, respectively, to get a final concentration of 75 mM cross-linker solution gave higher immobilization yield than Cu-alginate beads (10:0), and beads prepared with Cu:Zn/Ca gave least activity (Fig. 1b). Increasing the concentrations of other cations in combination with Cu above 25 mM decreased the activity of immobilized laccase for all the metals/cations tested. In other words, decreasing the concentrations of Cu below 50 mM in the combination beads decreased the activity of immobilized laccase for all the metals/cations tested. Cu-alginate beads retained 95 % of the initial activity even after storing for 7 days at 4 °C (data not shown). Stimulation of laccase activity upon the addition of Cu^{2+} ions was reported in *P. ostreatus* and *Ganoderma* sp (Baldrian and Gabriel 2002; Teerapatsakul et al. 2008). To our knowledge, this is the first study describing the immobilization of laccase with different cations and their combinations.

pH and temperature optima of immobilized laccase

Cu-alginate beads, Cu–Ba alginate beads, Cu–Ni alginate beads and Cu–Sr alginate beads which showed maximum laccase activity were selected for further studies. In the case of combination beads, the Cu:Ba/Sr/Ni which were maintained in the ratio 7.5:2.5, respectively, to get a final

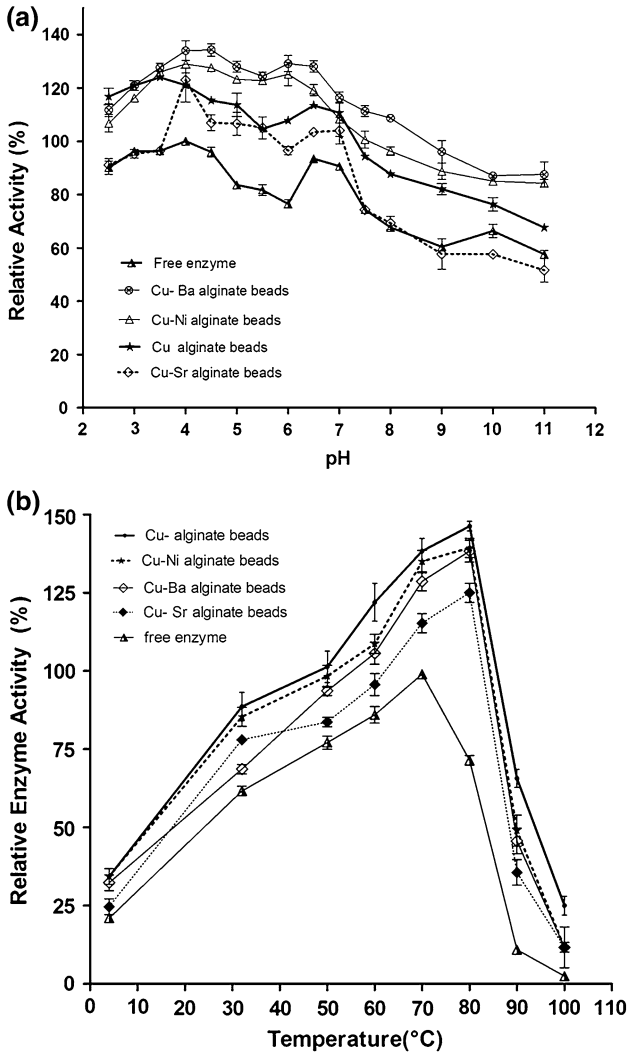

Fig. 2 pH and temperature optima of immobilized laccase. **a** pH Optima. **b** Temperature optima

concentration of 75 mM cross-linker solution exhibited maximum activity.

All the tested immobilized enzymes displayed almost similar pattern of pH effects compared to that of the free enzyme. The immobilized enzyme was most active in a broad pH range of 2.5–7.0 with maximum activities at pH 4.0 and pH 6.5 (Fig. 2a). The two peaks observed for the enzyme activity may be due to the presence of laccase isozymes in the crude culture filtrate. 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 a solution with a high ionic strength (Tischer and Kasche 1999). Thus, in contrast to earlier reports, no significant shift in the optimum pH was observed for immobilized laccase.

Table 3 Values of two-tailed paired *t* test compared at 95 % confidence interval between free enzymes and different metal alginates

Metal alginate	*df*	T	*P* value
Cu	7	5.174	0.0013
Cu–Ba	7	3.493	0.0101
Cu–Ni	7	4.421	0.0031
Cu–Sr	7	3.091	0.0175

Study on the effect of temperature on laccase activity of the immobilized alginate enzymes namely Cu-alginate beads, Cu–Ba alginate beads, Cu–Ni alginate beads and Cu–Sr alginate beads compared to the free enzyme, however, revealed significant differences ($P < 0.05$) data shown in Table 3. All the immobilized enzymes analyzed as shown in (Fig. 2b) exhibited higher temperature optima than that of free enzyme *P* value <0.001. The Cu–, Cu–Sr, Cu–Ni and Cu–Ba alginate enzymes had optimum temperature 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 was reported for lipase in gray mullet and laccase of *Lentinus polychrous,* respectively (Aryee and Simpson 2012; Phetsom et al. 2009).

Effect of metal ions, copper chelating agents and detergents on the activity of immobilized laccase

The effects of various metal ions, copper chelating agents and detergents on the enzymatic activity of immobilized laccase were not previously examined. In the present study, Cu-alginate beads, Cu–Ba alginate beads, Cu–Ni alginate beads and Cu–Sr alginate beads were selected for assessing their tolerance to heavy metal ions, copper chelating agents and detergents. All the selected beads were prepared in a cross-linker solution of 75 mM of a respective cationic solution. In the case of combination beads, the Cu:Ba/Sr/Ni were maintained in the ratio 7.5:2.5, respectively, to get a final concentration of 75 mM cross-linker solution.

Effect of metal ions

Metal ions can act as non-competitive inhibitors of enzymes; their presence in treatment plants usually poses a threat to biotreatment processes. So, the effect of metal ions on the activity of immobilized laccase was checked. To calculate relative activities, the actual activity in the absence of these agents under the same experimental conditions was set to 100 %. All the immobilized enzymes displayed almost a similar pattern of activity with metal ions (Fig. 3). The immobilized laccases exhibited an increased activity in the presence of metal

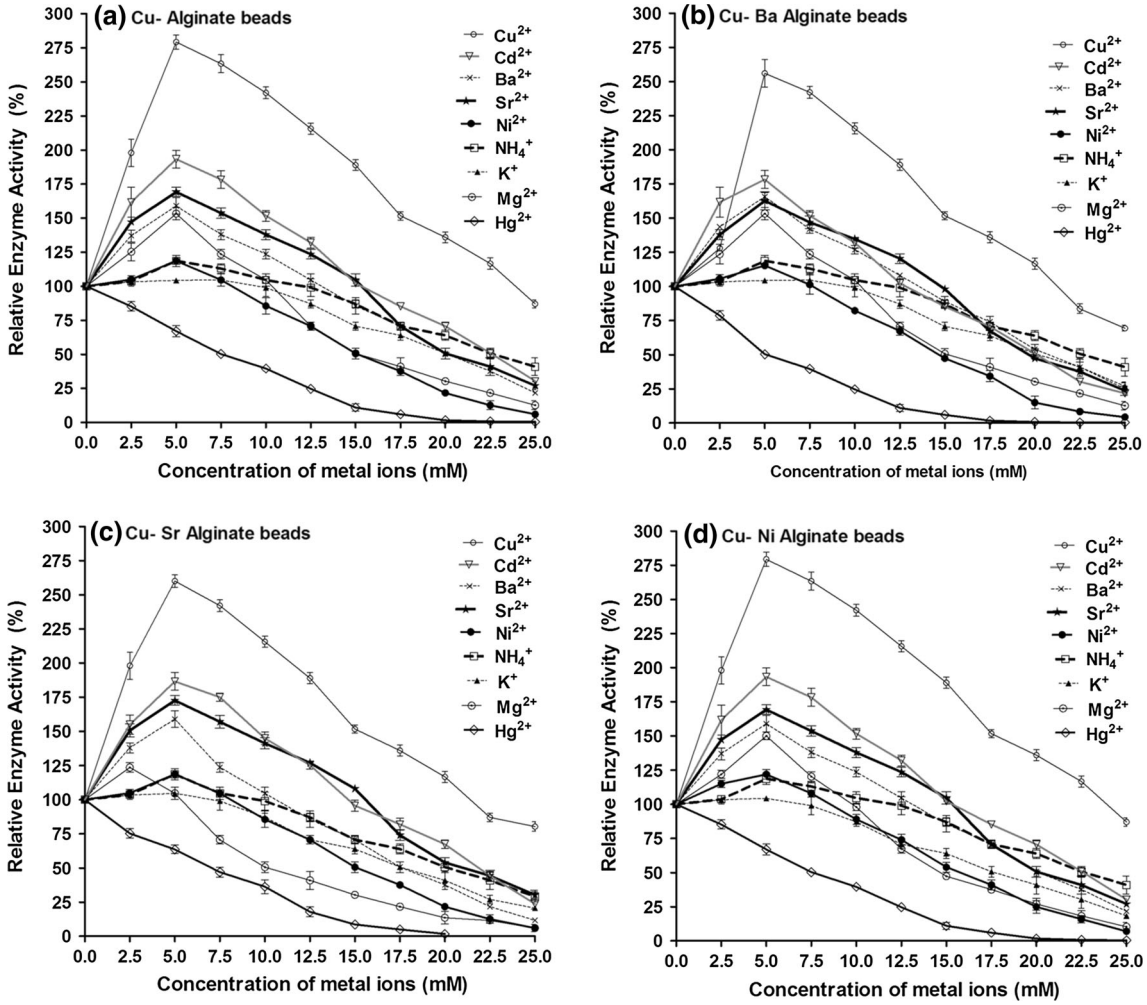

Fig. 3 Tolerance of immobilized laccase to metal ions. **a** Cu-alginate beads. **b** Cu–Ba alginate beads. **c** Cu–Sr alginate beads. **d** Cu–Ni alginate beads

ions at lower concentrations with maximum activity at 5 mm concentration of metal ions. Cu^{2+} at 5 mM concentration induced laccase activity almost three times, and Cadmium also enhanced the activity almost two times. This was followed by Sr^{2+}, Ba^{2+} and Mg^{2+}. With increase in the concentration of metal ions, laccase activity reduced gradually, but retained 50–70 % of its activity even at 20 mM concentrations of all the metal ions tested except for Hg^{2+}. With further increase in the metal ion concentration above 20 mM, laccase activity decreased, but had detectable activity even at 25 mM. Copper and cadmium was also reported to increase the laccase activity in *P. ostreatus* (Baldrian et al. 2000). Baldrian has discussed the sensitivity of laccases toward heavy metal ions as well (Baldrian 2006). In agreement to this, we observed that Hg^{2+} inhibited laccase activity even at a very low concentration of 0.5 mM. In addition to monovalent salt, K^{+} didn't exert any significant effect on laccase activity. Recent study on purified laccase from *Ganoderma* sp. has shown maximum inhibition of laccase with $CoCl_2$ and $HgCl_2$ and stability in presence of $CaCl_2$, $MnSO_4$, $FeCl_3$, $ZnSO_4$ and $BaCl_2$ (0.5–3 mM) with a significant inhibition only at 10 mM of the metal ions (Sharma et al. 2013). However, the effect of metal ions on immobilized laccase was not reported earlier. This study for the first time demonstrates the effect of metal ions on immobilized laccase. The stability exhibited by the immobilized laccase toward high concentrations of vari-

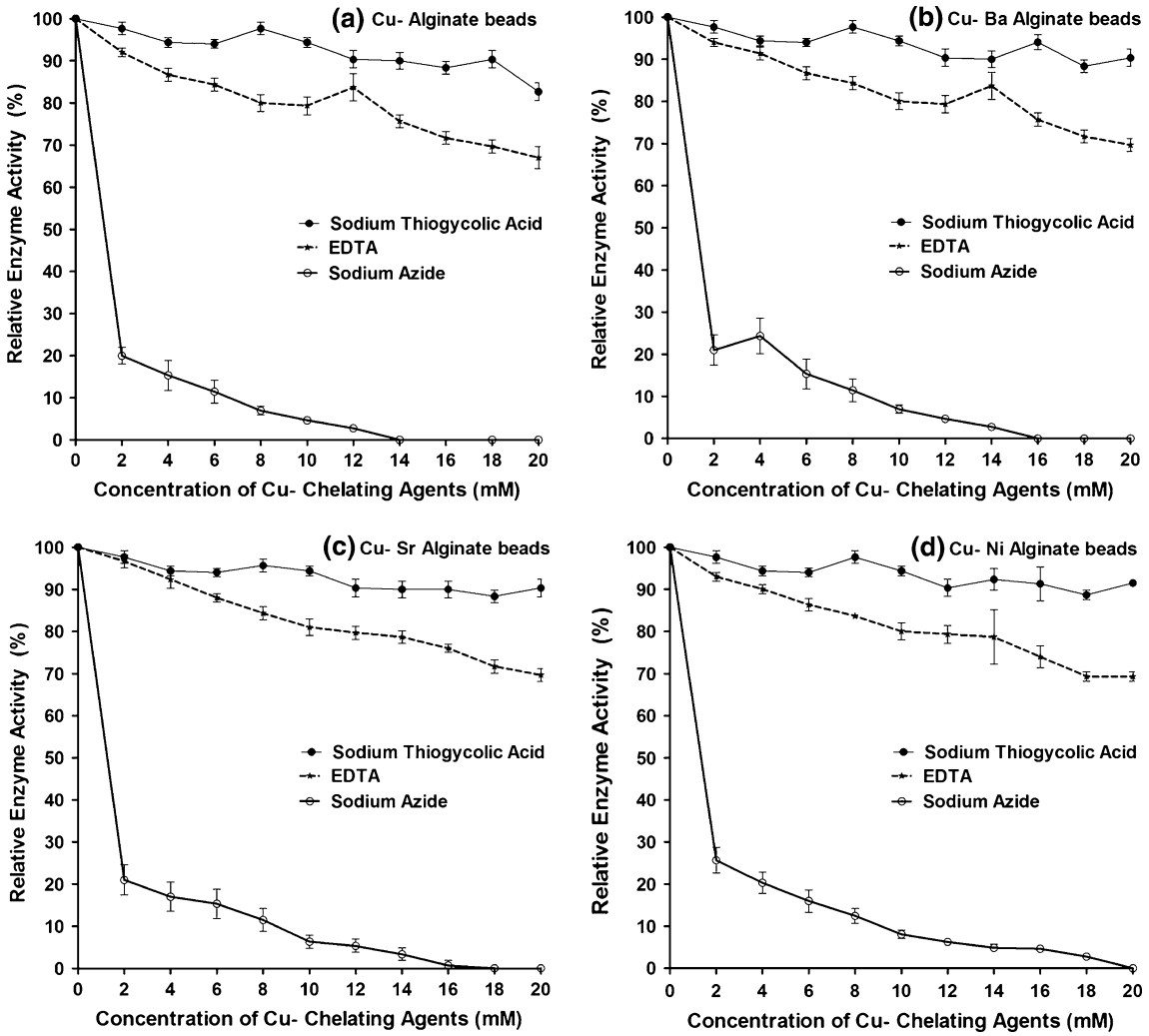

Fig. 4 Tolerance of immobilized laccase to Cu-chelators. **a** Cu-alginate beads. **b** Cu–Ba alginate beads. **c** Cu–Sr alginate beads. **d** Cu–Ni alginate beads

ous metal ions can be utilized in treating metal contaminated industrial effluents.

Effect of copper chelating agents

Laccase is a copper-dependent protein, and the presence of copper chelating agents in treatment plants usually poses 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. As shown in the Fig. 4, 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. On the other hand, sodium azide inhibited 80 % of the initial activity of all the beads even at a very low concentration of 0. 5 mM. The slight decrease in the tolerance by Cu-alginate beads to the chelating agent in comparison with the combination beads may be due to the effect of copper chelating agents on the physical stability of the Cu-alginate beads (Teerapatsakul et al. 2008). The tolerance exhibited by the immobilized laccase toward high concentrations of EDTA and sodium thioglycolic acid signifies its tolerance to some copper chelators which may be considered as an advantage in treating the industrial effluents contaminated with such chemicals.

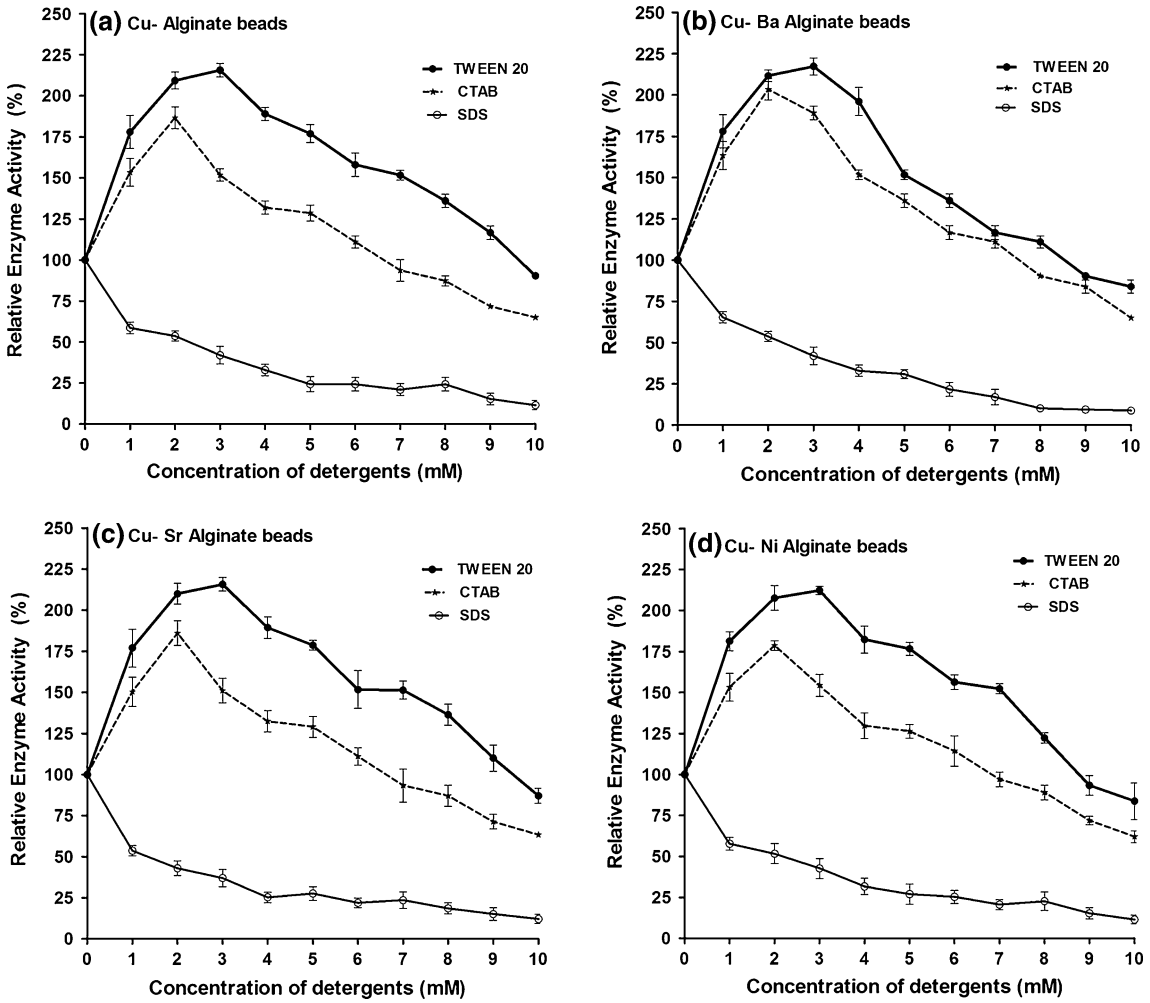

Fig. 5 Tolerance of immobilized laccase to detergents. **a** Cu-alginate beads. **b** Cu–Ba alginate beads. **c** Cu–Sr alginate beads. **d** Cu–Ni alginate beads

Effect of detergents

Since most detergents are known to denature enzymes, their presence in treatment plants usually poses a threat to the biotreatment processes. So, the effect of detergents on the activity of immobilized laccase was checked. As shown in Fig. 5, all tested beads expressed almost a similar pattern of activity with Ba–Cu alginate exhibiting somewhat higher activity than the rest. CTAB ($t = 7.327$ $df = 10$, P value $= 0.0001$) and Tween 20 ($t = 8.201$ $df = 10$, P value $= 0.0001$) significantly increased the activity of Ba–Cu alginate beads compared to other detergents tested. A very recent study by Sharma et al. (2013) has shown moderate stability of purified laccase to surfactant, Triton X-100 and all tweens (except Tween 20), SDS and CTAB at 5 and 10 mM concentration. Immobilized beads showed tolerance to all detergents analyzed except SDS even at a high concentration of 10 mM. Interestingly, CTAB and Tween 20 at a concentration of 2 and 3 mM, respectively, enhanced the activity of immobilized laccase to its double and triple fold, respectively. The effect of detergents on immobilized laccase was not reported earlier. In contrast to earlier studies reported on free enzymes (Laufer et al. 2006; Sharma et al. 2013), SDS decreased the enzymatic activity of immobilized laccase at concentrations higher than 2 mM. This study for the first time demonstrates the effect of detergents on immobilized laccase. The stability exhibited by the immobilized laccase toward surfactants can be utilized in treating effluents contaminated with detergents.

In conclusion, immobilization conditions of laccase were optimized, and the sensitivity of immobilized laccase from *T. viride* Pers NFCCI-2745 to heavy metals, detergent and copper chelating agents was analyzed. The study was mainly undertaken because of the threat raised by the presence of heavy metals, detergents and metal chelating

agents in the industrial effluents which act as non-competitive inhibitors/denaturing agents for several enzymes. In the present study, we found that the detergents, metal ions and Cu-chelators did not significantly alter either the physical stability of the beads or its enzymatic activity. In addition, lower concentration of these agents significantly enhanced the activity of immobilized laccase. The concentrations and combinations of cations influenced the activity of immobilized enzyme and its yield. The immobilized Cu–Ba alginate enzyme showed comparatively higher activity compared to the Cu-alginate enzyme. Most of the metal ions tested enhanced laccase activity at lower concentration. Immobilized laccase retained more than 70 % of its activity even in the presence of 20 mM copper chelating agents like EDTA and sodium thioglycolic acid. 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. Cu–Ba and Cu–Ni alginate enzyme exhibited comparatively higher tolerance than Cu-alginate beads to EDTA and sodium thioglycolate. Tween 20 and CTAB significantly increased the activity of all the beads. These properties of the immobilized laccase may be 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.

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Conflict of interest There are no conflicts of interest in the opinion given in the manuscript.

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