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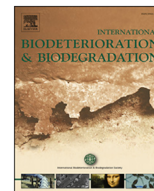

Laccase immobilized magnetic iron nanoparticles: Fabrication and its performance evaluation in chlorpyrifos degradation

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ABSTRACT

Chlorpyrifos degradation was studied using laccase immobilized on magnetic iron nanoparticles (CENPs). The magnetic iron nanoparticles (MNPs) prepared by co-precipitation method were characterized using Transmission electron microscopy (TEM), Scanning electron microscopy- Energy dispersive spectroscopy (SEM-EDS) and Thermogravimetric analysis (TGA). The size of the nanoparticles ranged between 10 and 15 nm. The MNPs were coated with chitosan, surface modified with carbodiimide (EDAC) immobilized with laccase enzymes. The chlorpyrifos degradation studies were performed in batch studies under constant shaking for a period of 12 h. Results of the study showed that laccase immobilized on magnetic iron nanoparticles were effective in degrading more than 99% chlorpyrifos in 12 h at pH 7 and 60 °C. In the overall degradation percentage, MNPs contributed to 32.3% of chlorpyrifos removal while ENPs resulted in 58.8% chlorpyrifos degradation. Immobilization of enzyme decreased the overall activity of the free enzyme. The CENPs showed 95% activity after five repeated washing and hence possess good reusability potential.

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1. Introduction

Chlorpyrifos [O, O-diethyl O-(3, 5, 6-trichlor-2-pyridyl) phosphorothioate] is a broad spectrum insecticide used widely, since 1965 for agricultural and household pest control (Singh et al., 2004). It has a potential of contaminating the site up to 24 km away from the point of use (Bhagobaty et al., 2007). The contamination of these pesticides results in deleterious ecological effects necessitating remediation. Among the different methods of chlorpyrifos degradation, microbial degradation is popularly recommended due to its eco-friendly nature and higher degradation potential (Subramanian et al., 1994; Briceño et al., 2012). Microbial enzymes are the one which plays a major role in pesticide detoxification and degradation. They are popularly studied since they showed higher efficiency within a short span of time compared to whole cells (Gao et al., 2012). Conversely free enzyme suffered from limitations of high production cost, stability issues, recovery and reusability problems (Zhang et al., 2013). Immobilization techniques were intensively studied to improve the limitations associated with free enzyme. Careful selection of the immobilization technique and carrier material for a particular enzyme will enable the enzyme to work in a broad spectrum of temperature and pH (Wang et al., 2006; Onderková et al., 2007; Shu et al., 2011; Moreno-Pirajàn and Giraldo, 2011). The recovery and reusability of the enzymes is also made possible. The advent of nanotechnology has opened up a new arena for the nanoparticles as a support system for enzyme immobilization (Wang et al., 2012, 2013). Properties such as small size, high surface area and large surface to volume ratio have grabbed the attention of the researchers in recent times (Dyal et al., 2003; Hu et al., 2009).

Magnetic iron nanoparticles have been widely studied for immobilization of enzymes and proteins due to their unique properties of superparamagnetism and easy separation in an applied magnetic field causing low mechanical stress to the enzymes (Masciangioli and Zhang, 2003; Kalkan et al., 2011; Jořenek and Zajoncová, 2015). The magnetic iron nanoparticles are functionalized using coupling agents such as glutaraldehyde, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) to covalently bind the enzymes. The functionalization of the nanoparticles improved the thermal and operational stability. Wang et al. (2013) and Kuo et al. (2012) reported that the enzymes immobilized in nanoparticles possessed the ability to retain its initial activity by 80–83% after 20 repeated cycles. So far several enzymes such as lipases, laccase, lactase, β-glucosidase,

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recombinant dehalogenase have been successfully immobilized onto the magnetic iron nanoparticles (Dyal et al., 2003; Johnson et al., 2008; Wang et al., 2013; Talbert and Goddard, 2013; Zhou et al., 2013). Among the different enzymes reported so far, laccases and hydrolases have potential application in pesticide degradation.

Laccases are blue copperoxidases enzymes found in fungi, certain bacteria, insects and plants. They have broad substrate specificity for compounds such as ortho and para-diphenols, phenolic acids, methoxy-substituted phenols, aromatic amines. They can oxidise more substrates in the presence of redox mediators such as HBT, guaicol etc. Moreover, the specificity of substrate by laccase is reported to vary from one organism to another (Atalla et al., 2013). Several studies have been reported so far to prove the ability of the laccase enzyme in organic pollutant degradation. Immobilized laccase enzyme was reported to degrade a wide range of dyes such as Direct Blue 78, Acid Blue 225, Reactive Red 195, Acid Blue 74, and Phenol Red (Wang et al., 2013; Jořenek and Zajoncová, 2015). In a study, Wang et al. (2013), reported around 80% colour reduction within 1 h of exposure to immobilized laccase enzyme. Likewise, laccase immobilized on nano-porous silica beads showed around 90% degradation of 2,4-dinitrophenol within 12 h of treatment. The enzymes were very stable and retained almost 85% of initial activity even after 30 days of treatment (Dehghanifard et al., 2013). Wang et al. (2012) reported two-fold increased phenol degradation with immobilized laccase enzyme. The enzyme retained 71.3% of its initial degradation activity even after 10 successive batch treatments. Rodríguez-Delgado et al. (2016) in his study degraded Diclofenac (50%), b-Naphthol (97%), 2,4 Dichlorophenol (71%) with 100 UL^{-1} of laccase cocktail within 8 h of incubation. Nguyen et al. (2014) studied the effect of *Trametes versicolor* (ATCC 7731) live fungus (biosorption & biodegradation) and its intracellular and extracellular enzyme extract (predominantly laccase) on the removal of 30 diverse trace organic contaminants (TrOC). Huifang et al. (2013), explored Laccase/Vanillin system for biodegradation of chlorpyrifos. Laccase used in the study was recombinant laccase obtained from *Pichia pastoris.*

So far, a limited number of studies have been reported on chlorpyrifos degradation using laccase enzyme. Thus, the objective of the present study was to elucidate the effect of laccase immobilized on magnetic iron nanoparticles on chlorpyrifos biodegradation. In addition to the influence of pH and temperature in chlorpyrifos biodegradation was also investigated. The efficiency of the process was assessed in terms of (i) chlorpyrifos concentration reduction (ii) enzyme kinetics and (iii) change in the activity of the enzyme on reusability studies.

2. Materials and methods

2.1. Materials

Chlorpyrifos of 99.7% purity and Laccase from *Trametes versicolor* (Specific activity $\geq$ 10 U mg^{-1}) were purchased from Sigma-aldrich. ABTS, EDAC and 99% n-hexane (Pesticide residue analysis) were purchased from MP Biomedicals, SRL chemicals and Loba Chemie, respectively.

2.2. Synthesis of magnetic iron nanoparticles

The magnetic iron nanoparticles were synthesized by co-precipitation method as per the protocol published by Ghandoor et al. (2012) with some modifications. The fine particles of ferric chloride and ferrous chloride were mixed in a ratio of 1:2 (Fe^{+2}: Fe^{+3}) with HCl (Hydrochloric acid). The co precipitated mixture was kept in an oven for 30 min at 80 °C. The mixture of iron particles was then poured onto a solution of boiling NaOH within ten seconds under constant and vigorous stirring. The black precipitated magnetite particles were separated from the solution with a magnet of ~3500 Gauss, washed with water and dried at 100 °C for 1 h and 30 min in oven.

2.3. Preparation of chitosan coated MNPs

The prepared magnetic nanoparticles (MNPs) were washed thrice with ethanol and coated with chitosan by reversed phase suspension technique. 200 mg iron nanoparticles were suspended in 50 ml mineral oil containing 0.5 ml Tween 80 in a round bottom flask. The suspension was sonicated for one hour for proper dispersion and to the mixture chitosan solution (1% w/v, 15.0 ml in 5.0% v/v acetic acid) was added. The Fe_3O_4-chitosan dispersion formed was once again sonicated for 60 min. After sonication, chitosan coated MNPs was mechanically stirred at 1000 rpm for 5 min and later suspended in glutaraldehyde solution (3.0 ml, 25% w/v in water) for functionalization of the particles. The suspension was magnetically stirred for 4 h at room temperature and separated using a permanent magnet (~3500 G). The chitosan coated MNPs (CNPs) obtained was repeatedly washed with acetone for five times and vacuum dried at 200 °C (Kalkan et al., 2011).

2.4. Laccase immobilization

The immobilization of laccase enzyme involves the surface activation of the chitosan coated nanoparticles using EDAC which is a carboxyl activating agent for the coupling of primary amines to yield amide bonds. Later, the laccase enzyme was immobilized onto the functionalized nanoparticles.

2.4.1. Surface activation of chitosan coated nanoparticles

The particles coated with chitosan were activated by EDAC (N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride) to facilitate immobilization of laccase enzyme by covalent binding (Kalkan et al., 2011). 50 mg of Fe_3O_4-CS nanoparticles was dispersed in 2.0 ml phosphate buffer [(PB), pH 6.0, 0.04M] in an ultrasonic bath at 10–15 °C for one hour. EDAC solution (0.5 ml, 2.5% w/v, in same buffer) was added to the dispersed mixture and sonicated for one hour. The mixture was than magnetically stirred at 1000 rpm, at 4 °C for 6 h and stored overnight in the refrigerator at same temperature. Finally, the EDAC activated chitosan coated MNP (CNPs-EDAC) was collected using ~3500 G magnet, washed repeatedly with 2.0 ml PB for five times and used for the immobilization of laccase enzyme.

2.4.2. Enzyme immobilization

50 mg of CNPs-EDAC was washed with 2.0 ml PB and immersed in 2.0 ml laccase solution (1.0 mg ml^{-1}, in PB, pH 6.0) and sonicated for 40 min. After sonication, the mixture was continuously stirred at 1000 rpm for 6 h at 4 °C using a magnetic stirrer. The sample was than stored overnight at 4 °C. After 24 h the magnetic nanoparticles were separated from the solution using a permanent magnet and washed repeatedly (5 times) with PB to ensure removal of unbound enzyme. The concentrations of the enzyme in the initial and washed solution were assessed by Bradford assay at 595 nm. The percentage of immobilization was calculated using Eq. (1) (Ramírez-Montoya et al., 2015).

$$\% \text{ immobilization} = \left(1 - \left(\frac{\text{Final protein concentration}}{\text{Initial protein concentration}}\right)\right) \times 100 \quad (1)$$

2.5. Characterization of the iron nanoparticles

The magnetite nanoparticles (MNPs), chitosan coated magnetic particles (CNPs) and laccase immobilized on nanoparticles (CENPs) were characterized for their size and morphology using TEM (Transmission Electron Microscopy) (Model TECNAI G^2 20S- TWIN). The thermal properties of MNPs and CNPs were obtained from the Thermo-gravimetric analysis (TGA). Samples (~10 mg) were heated from 30 to 580 °C at a heating rate of 10 °C min^{-1} in N_2 atmosphere. The covalent bonding of the enzymes on the particles coated with chitosan was confirmed using SEM-EDS (Scanning Electron Microscope-Energy Dispersive X-ray Spectroscopy) (Carl ZEISS, MERLIN compact). The superparamagnetic property of the magnetic iron nanoparticles and chitosan coated magnetic iron nanoparticles were determined using Vibrating Sample Magnetometer Model: Lakeshore VSM 7410.

2.6. Activity of enzyme

Enzymatic activity of both free and immobilized laccase enzyme were performed against ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] as a substrate at different pH (3,4,5,6) and temperature (20, 30, 40, 50, 60 °C) conditions using [0.1 M citrate/0.2M phosphate buffer] Citrate Phosphate Buffer. Addition of ABTS causes the formation of blue color due to oxidation of ABTS by laccase. The ABTS radical generated during oxidation is measured using a spectrophotometer at 414 nm.

2.7. Enzyme kinetics

The enzyme kinetics was investigated for both free and immobilized laccase using ABTS as a substrate with a concentration ranging from 0.01 mM to 0.25 mM at pH 5, 30 °C. Michaelis-Menten constant, K_m and V_{max} were identified using Lineweaver-Burk plots. V and [S] represent the rate and substrate concentration, respectively.

2.8. Degradation of chlorpyrifos by laccase immobilized on CNPs

To study the effect of CENPs on degradation of chlorpyrifos, 10 mg of CENPs contains ~7 μg laccase enzyme (protein concentration), added to Erlenmeyer flask containing 50 ml of 500 mg l^{-1} chlorpyrifos solution. The flasks are kept in the incubator shaker at 150 rpm for 12 h. After 3 h, 6 h and 12 h interval, 5 ml of sample was collected and extracted with hexane in the ratio of 1:1. The extracted samples were stored in amber colored chromatographic vials at 4 °C before analysis. Hexane stops the reaction between the enzyme and the pollutant. The degradation products and chlorpyrifos residue was quantitatively analyzed using GC-MS. The effect of pH (3, 7, 10) and temperature (20, 40, 60 °C) on chlorpyrifos degradation was also studied.

2.9. Analytical methods

The concentration of chlorpyrifos and the intermediate products of degradation in the reaction mixture are detected using Gas Chromatography- Mass Spectrometry (Make: Shimadzu OP 2010 Ultra). The GC-MS was equipped with RTxi-1ms capillary column (30 m × 1 mm x 0.1 μm) and splitless injection system. Helium gas at a flow rate of 1 ml min^{-1} was used as the carrier gas. The equipment was calibrated with standard chlorpyrifos solution for accurate quantification. All the assays and experiments were performed in triplicates. The data was statistically analyzed.

3. Results and discussion

3.1. Characterization of MNPs

Fig. 1 shows the TEM images of the bare magnetic nanoparticles, chitosan coated MNPs and enzyme immobilized MNPs. From the Figure, it was evident that the shape of the particles varied from irregular to quasi-spherical morphology. The magnetic iron nanoparticles appeared as black fine, loose and well separated particles with an average size of around 15 nm. Sonication and chitosan coating might have prevented the agglomeration of the magnetic nanoparticles resulting in high surface area and more reactivity (Reddy et al., 2012). In the defocused image of Fig. 1(c) appearance of white fringe of thickness ~1 nm can be attributed to the immobilization of enzyme over CNPs. Determination of shell thickness is difficult for the defocused particles as it causes disturbance. However, no such agglomeration was observed in the CENPs as evidenced by the formation of approximately equal size CENPs.

The composition of the particles was analyzed using SEM/EDS (Scanning Electron Microscopy- Energy Dispersive Spectroscopy). Figure (Provided in supplementary material) depicts the EDS spectra of the CNPs and CENPs particles. It was observed that the atomic percentage of the elements showed variations in both the spectra. Atomic carbon showed an increase from 38.7% to 68.7% in enzyme immobilized MNPs. Likewise, elements such as Fe and O showed reduced signals in CENPs. The presence of elemental sulphur in the spectra of enzyme immobilized magnetic iron particles confirmed the enzyme cross linking over the nanoparticles. Similar observations were also reported by Kalkan et al. (2011) who in his study confirmed the contribution of sulphur signals by the laccase enzymes. The absence of any other element in the spectra generated for the nanoparticles indicate its purity of preparation. The magnetic property and the thermal stability for the magnetic iron nanoparticles and chitosan coated magnetic iron nanoparticles were characterized using VSM and TGA analysis. The magnetic iron nanoparticles and chitosan coated magnetic iron nanoparticles showed saturation magnetization of 48 and 10 emu g^{-1}, respectively. A reduction in magnetic strength by 79% might be due to the surface modification of MNPs by chitosan biopolymer. The magnetic strength observed in the present study is less when compared to the results of Kalkan et al. (2011) and Mahdavi et al. (2013) that reported 2.5 times higher magnetic strength. The oxidation of magnetite particles into maghemite could be the possible reason for the reduction in the magnetic strength of the material (Mahdavi et al., 2013).

The thermal stability of the MNPs and CNPs calculated via the percent weight loss of a compound in TGA analyzer. From that, it is clearly observed that the chitosan mixture began to lose weight at 30 °C. Conversely, the MNPs showed a gradual weight loss accounting to total loss of 7% at 580 °C. According to Mahdavi et al. (2013), moisture and Fe_3O_4 conversion to FeO might have been the possible reason for the loss in weight. Apparently the CNPs in comparison to MNPs showed a maximum total weight loss of 35% at 410 °C. The CNPs began to lose weight at a temperature above 200 °C. At high temperatures, the bonds in the chitosan molecules could have been destroyed resulting in high weight loss percent. The CNPs showed no further reduction in weight at a temperature

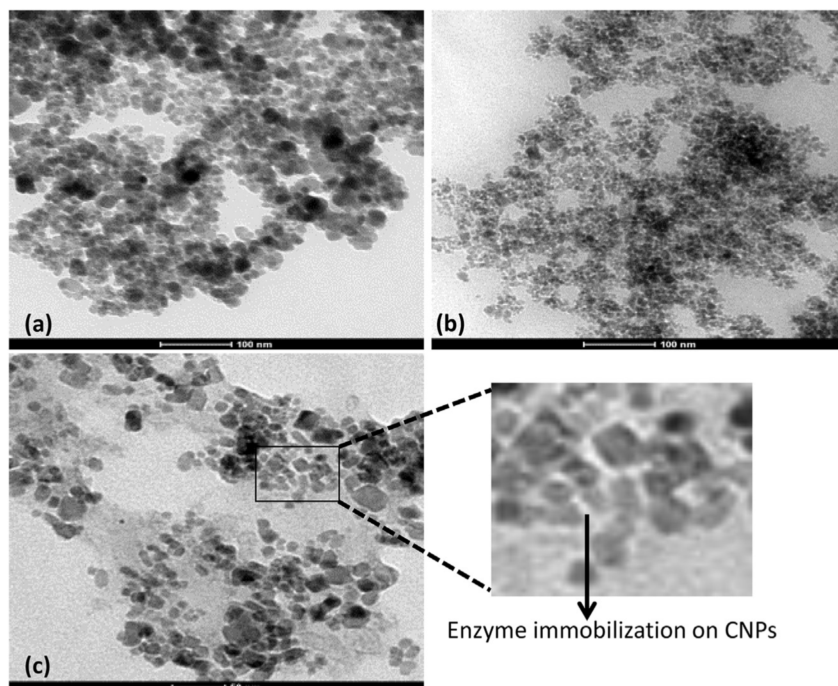

Fig. 1. TEM micrographs of (a) magnetic iron nanoparticles (MNPs) (b) Chitosan coated magnetic iron nanoparticles (CNPs) and (c) Laccase enzyme immobilized magnetic iron nanoparticles (CENPs).

above 500 °C. Probably, the chitosan molecules and the enzymes would have been degraded completely at 500 °C leaving behind iron as residue. As per the experimental calculations chitosan contributed to 90% of the total weight of CNPs.

3.2. Immobilization efficiency

Bradford assay was used to assess the immobilization efficiency of the enzyme onto chitosan coated nanoparticles. The immobilization efficiency achieved for 50 mg nanoparticles treated with 2 ml of 1 mg ml^{-1} laccase enzyme was in the range of 65–70%. The loss of enzyme decreased with increase in number of washings. The maximum release of unbound enzyme (16.1 μg ml^{-1}) was observed during the first wash. The immobilization efficiency achieved in the present study is lower as compared to the earlier reports (Kalkan et al., 2011; Jořenek and Zajoncová, 2015).

3.3. Effect of pH and temperature on free and immobilized enzymatic activity

pH and temperature plays an important role in determining the rate of the enzyme assisted reactions. Fig. 2 depicts the effect of pH and temperature on the enzyme activity of free and immobilized laccase. The enzymatic activity of the free and immobilized laccase was measured at pH (4–6) and temperature (20 °C–60 °C). Maximum activity of the free enzymes was observed at pH 4 and 40 °C. A further increase in pH reduced the activity of the free enzymes with the lowest activity of 4.55 U mg^{-1} recorded at pH 5 and 60 °C.

The free laccase activity decreased when pH increases from 4 to 6. However, a change in trend was observed for the immobilized enzyme. Maximum specific activity of immobilized enzyme around 15.55 U mg^{-1} was recorded at pH 5 and 40 °C. Similar to pH, temperature variations also showed influence on the enzyme specific activity. The specific activity of both free and immobilized enzymes increased with increase in temperature up to 40 °C after

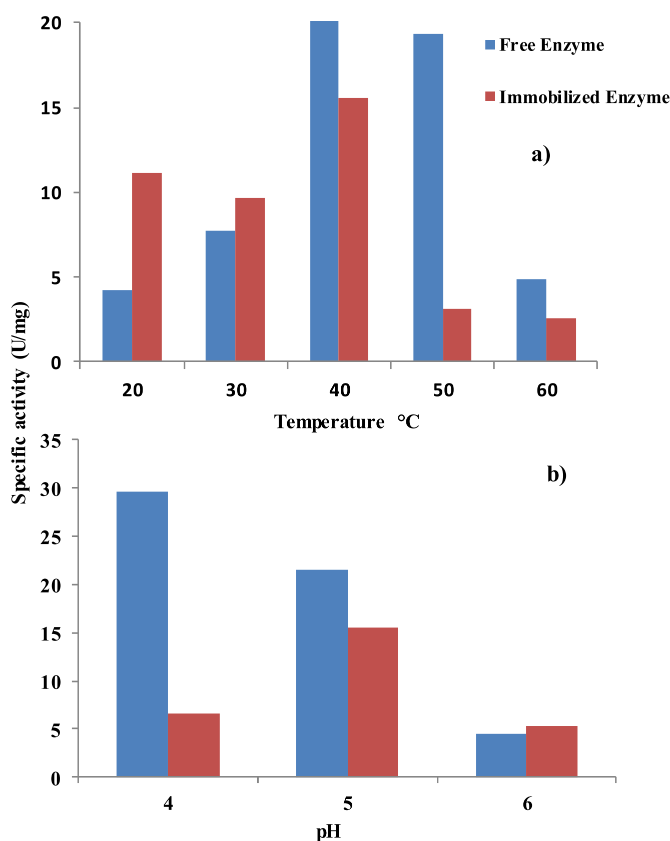

Fig. 2. Effect of (a) temperature and (b) pH on laccase activity.

which it began to drop with the lowest value recorded at 60 °C. From the results it can be concluded that immobilization increased

the stability of the enzyme to alterations in pH and temperature. The shift in pH might be due to the variations in the conformation of the enzyme during covalent bond formation leading to changes in the microenvironment of the enzyme (Chiou and Wu, 2004). Our study on effect of temperature correlates with the studies of Lante et al. (2000) and Kalkan et al. (2011). Kunamneni et al. (2008) reported that the occurrence of chemical transformation in the enzymes during temperature variations might be the reason for the shift in enzyme activity of free and immobilized enzymes. In addition, to the support material might have caused hindrances and diffusion limitations for the enzymes which could have also been the reason for the changes in the free and immobilized enzymes (Ramírez-Montoya et al., 2015). The maximum activity range of immobilized enzyme than the free enzyme indicates its resistance to the alkaline changes occurred in the medium (Quiroga et al., 2011). Madhaviand Lele (2009), Stoilova et al. (2010), Kalkan et al. (2011) and Jořenek and Zajoncová (2015) also reported laccase activity at pH 3–4 for free enzyme so it can be found in our study that the free enzyme activity decreased with increase in pH.

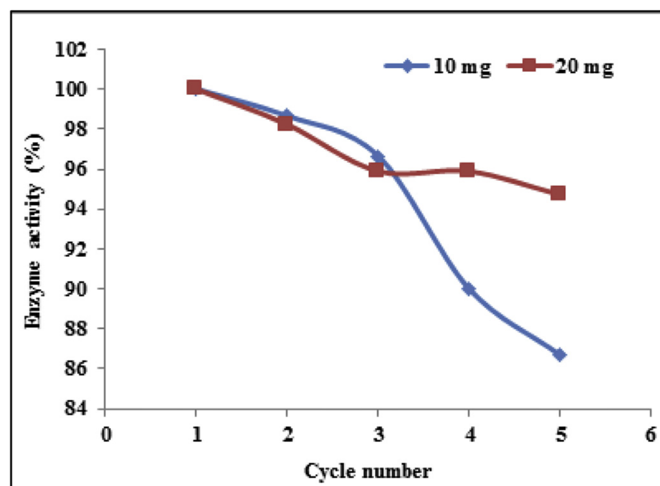

Fig. 3. Reusability of laccase enzyme immobilized magnetic iron nanoparticles.

3.4. Enzyme kinetics

The enzyme kinetics of free and immobilized enzyme was studied with ABTS at pH 5, 30 °C. ABTS is used by the free and immobilized enzyme as a substrate for catalysis to study the reaction kinetics. The K_m and V_{max} values were estimated using Lineweaver-Burk Plot. The K_m for free and immobilized laccase was 0.25 and 0.58, respectively. K_m indicates the binding affinity of the enzyme. Higher K_m indicates low affinity of the substrate to the enzyme. An increase in K_m was observed for immobilized enzyme indicating the fact that the immobilized laccase just requires a small amount of substrate to activate the enzyme. (Jiang et al., 2005; Georgieva et al., 2008). V_{max} determines the maximum rate at which the reaction proceeds. The rate of the reaction (V_{max}) was 0.96 mM min^{-1} and 0.25 mM min^{-1} for free and immobilized enzyme, respectively. The reaction rate was decreased by almost 4 times for the immobilized enzyme. Georgieva et al. (2008) reported that the changes in enzyme conformation, steric hindrances and the protein partitioning effects might be the reason for decreased affinity and reaction rate in immobilized enzymes. The results of the present study are comparable to Kalkan et al. (2011), Jořenek and Zajoncová (2015) who observed similar trend of immobilized enzyme showing higher K_m than the free enzyme.

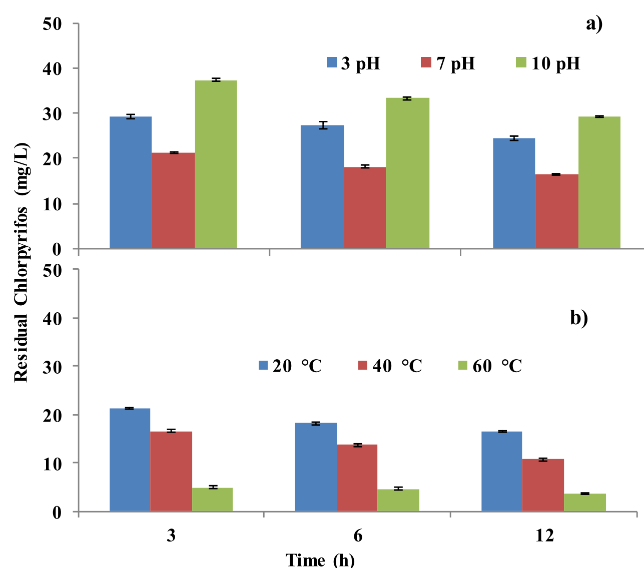

Fig. 4. Effect of pH (a) and temperature (b) on chlorpyrifos degradation.

3.5. Reusability of immobilized enzyme

The reusability of immobilized laccase enzyme was assessed with ABTS as substrate. The experiments were performed at two different particle loading rates 10 and 20 mg. Fig. 3 depicts the changes in enzyme activity during reusability studies. From the figure, it was observed that at a loading rate of 10 mg, the enzyme activity dropped gradually after each cycle and at the end of 5th cycle it dropped to 87%. Similar trend was also observed at 20 mg laccase immobilized on magnetic nanoparticles. However, after the 3rd cycle, the enzyme activity became stable and the activity of the fourth and fifth cycle remained closer to 95%. The results were comparable to that of Kalkan et al. (2011) where the immobilized enzyme was observed to retain its activity upto 95% after five batch of usage.

3.6. Chlorpyrifos degradation studies

Fig. 4 depicts the effect of pH and temperature on degradation of chlorpyrifos using enzyme immobilized nanoparticles. From the Fig. 4(a), it is clearly evident that a change in pH influenced chlorpyrifos degradation with maximum chlorpyrifos degradation of 99% achieved at pH 7. Subsequent to pH 7, better results were obtained at pH 3 showing a removal efficiency of 94.1%. Similar to pH, influence of temperature on degradation was assessed at pH 7 as shown in Fig. 4(b). At 20 °C and 40 °C, the chlorpyrifos degradation was observed to be 95.6% and 99.1%, respectively. The highest removal efficiency was achieved at 60 °C and 12 h reaction time leaving a residual chlorpyrifos of 3.53 mg l^{-1}. In control samples, the residual chlorpyrifos ranges from 493.7 mg l^{-1} to 497.5 mg l^{-1} for pH 3–10 and from 492.2 mg l^{-1} to 497.3 mg $l^{-1}$for temperature 20–60 °C. Henceforth, on the basis of the preliminary studies, pH 7 and temperature 60 °C was considered optimum for chlorpyrifos degradation. Figure (provided in supplementary data) shows the degradation of chlorpyrifos at optimized pH 7 and temperature 60 °C. The degradation efficiency achieved by CENPs ranged between 97.9 and 99% for chlorpyrifos concentration of 250–750 mg l^{-1}. The residual concentration at time zero for 250, 500 and 750 mg l^{-1} was 247.35 ± 1.1, 501.56 ± 0.95 and 746.92 ± 1.2 mg l^{-1}, respectively. The results of the present study

were observed to be better than that reported by Reddy et al. (2012). In this study, chitosan coated nanoparticles resulted in 100% degradation of 20 mg l^{-1} chlorpyrifos while at 50 mg l^{-1} a marked decrease in degradation (68%) was observed. In a Laccase/mediator system, chlorpyrifos was degraded over 70% after 24 h where vanillin was used as a mediator (Huifang et al., 2013).

The Chlorpyrifos degradation products were identified by gas chromatography-mass spectrometry (GC-MS) analysis. Two metabolites namely 2,4-bis(1,1 dimethylethyl) phenol and 1,2 benzenedicarboxylic acid, bis(2-methyl propyl) ester were observed in the extract. The mass of the metabolites was confirmed through NIST library. On the basis of the degradation products obtained, a degradation pathway was proposed (provided in supplementary data). The metabolite 2,4-bis(1,1 dimethylethyl) phenol has been previously reported by Fulekar and Geetha (2008) and Fulekar (2012) in a study conducted for chlorpyrifos degradation. Alkylation of 3,5,6-trichloro-2-pyridinol (TCP) or diethylphosphorothioate leads to the formation of 2,4-bis(1,1 dimethylethyl) phenol. To the best of our knowledge, the metabolite 1,2 benzenedicarboxylic acid, bis(2-methyl propyl) ester has not been reported in previous studies. 1,2 benzenedicarboxylic acid, bis(2-methyl propyl) ester is a precursor of 1,2 benzenedicarboxylic acid and is formed from the cleavage of pyridine ring. Bumpus et al. (1993), in his study reported that fungal species *Phanerochaete chrysosporium* cleaved pyridinyl ring during chlorpyrifos mineralization. There are well established research findings on the ability of white-rot fungi to degrade a wide range of xenobiotic compounds (Kuhad et al., 1997; Singh et al., 1999; Singh and Kuhad, 1999, 2000).

4. Conclusions

It can be concluded that the magnetic iron nanoparticles acted as an efficient carrier material for immobilization of laccase enzyme. The CENPs remained stable throughout the degradation process and showed almost 99% chlorpyrifos degradation within 12 h of incubation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibiod.2017.01.007.

References

Atalla, M.M., Zeinab, H.K., Eman, R.H., Amani, A.Y., Abeer, A.A., 2013. Characterization and kinetic properties of the purified *Trematosphaeria mangrovei* laccase enzyme. Saudi J. Biol. Sci. 20, 373–381.
Bhagobaty, R.K., Joshi, S.R., Malik, A., 2007. Microbial degradation of organophosphorous pesticide: chlorpyrifos (mini-review). Internet J. Microbiol. 4, 1–12.
Briceño, G., Fuentes, M.S., Palma, G., Jorquera, M.A., Amoroso, M.J., Diez, M.C., 2012. Chlorpyrifos biodegradation and 3,5,6-trichloro-2-pyridinol production by actinobacteria isolated from soil. Int. Biodeterior. Biodegr. 73, 1–7.
Bumpus, J.A., Kakkar, S.N., Coleman, R.D., 1993. Fungal degradation of organophosphorus insecticides. Appl. Biochem. Biotechnol. 39/40, 715–726.
Chiou, S.H., Wu, W.T., 2004. Immobilization of *Candida rugosa* lipase on chitosan with activation of the hydroxyl groups. Biomaterials 25, 197–204.
Dehghanifard, E., Jonidi Jafari, A., Rezaei Kalantary, R., Mahvi, A.H., Faramarzi, M.A., Esrafili, A., 2013. Biodegradation of 2,4-dinitrophenol with laccase immobilized on nano-porous silica beads. Iran. J. Environ. Health Sci. Eng. 10, 1–9.
Dyal, A., Loos, K.M., Noto, M., Chang, S.W., Spagnoli, C., Shafi, K.V.P.M., Ulman, A., Cowman, M., Gross, R.A., 2003. Activity of *Candida rugosa* lipase immobilized on γ-Fe_2O_3 magnetic nanoparticles. J. Am. Chem. Soc. 125, 1684–1685.
Fulekar, M.H., 2012. Bioremediation technology: recent advances. Springer Publishers, The Netherlands, ISBN 978-90-481-3678-0, pp. 147–150 (e book).
Fulekar, M.H., Geetha, M., 2008. Bioremediation of Chlorpyrifos by *Pseudomonas aeruginosa* using scale up technique. J. App. Biosci. 12, 657–660.
Gao, Y., Chen, S., Hu, M., Hu, Q., Luo, J., Li, Y., 2012. Purification and Characterization of a novel chlorpyrifos hydrolase from *Cladosporium cladosporioides* Hu-01. PLoS One 7, 1–8.
Georgieva, S., Godjevargova, T., Portaccio, M., Lepore, M., Mita, D.G., 2008. Advantages in using non-isothermal bioreactors in bioremediation of water polluted by phenol by means of immobilized laccase from *Rhus vernicifera*. J. Mol. Catal. B Enzyme 55, 177–184.
Ghandoor, H.El, Zidan, H.M., Khalil, M.M.H., Ismail, M.I.M., 2012. Synthesis and some physical properties of magnetite (Fe_3O_4) nanoparticles. Int. J. Electrochem. Sci. 7, 5734–5745.
Hu, B., Pan, J., Yu, H.L., Liu, J.W., Xu, J.H., 2009. Immobilization of *Serratia marcescens* lipase onto amino-functionalized magnetic nanoparticles for repeated use in enzymatic synthesis of Diltiazem intermediate. Process Biochem. 44, 1019–1024.
Huifang, Xie, Li, Qi, Wang, M., Zhao, L., 2013. Production of a recombinant laccase from *Pichia pastoris* and biodegradation of chlorpyrifos in a Laccase/Vanillin system. J. Microbiol. Biotechnol. 23, 864–871.
Jiang, D.S., Long, S.Y., Huang, J., Xiao, H.Y., Zhou, J.Y., 2005. Immobilization of *Pycnoporus sanguineus* laccase on magnetic chitosan microspheres. Biochem. Eng. J. 25, 15–23.
Johnson, A.K., Zawadzka, A.M., Lee, A.D., Crawford, R.L., Paszczynski, A.J., 2008. Novel method for immobilization of enzymes to magnetic nanoparticles. J. Nanopart. Res. 10, 1009–1025.
Jořenek, M., Zajoncová, L., 2015. Immobilization of Laccase on magnetic carriers and its use in decolorization of dyes. Chem. Biochem. Eng. Q. 29, 457–466.
Kalkan, N.A., Aksoy, S., Aksoy, E.A., Hasirci, N., 2011. Preparation of chitosan-coated magnetite nanoparticles and application for immobilization of laccase. J. Appl. Polym. Sci. 123, 707–716.
Kuhad, R.C., Singh, A., Erickson, K.-E.L., 1997. Microorganisms and enzymes involved in the degradation of plant fibre cell walls. Adv. Biochem. Eng. Biotechnol. 57, 45–125.
Kunamneni, A., Ghazi, I., Camarero, S., Ballesteros, A., Plou, F.J., Alcalde, M., 2008. Decolorization of synthetic dyes by laccase immobilized on epoxy-activated carriers. Process Biochem. 43, 169–178.
Kuo, C.H., Liu, Y.C., Chang, C.M.J., Chen, J.H., Chang, C., Shieh, C.J., 2012. Optimum conditions for lipase immobilization on chitosan-coated Fe_3O_4 nanoparticles. Carbohydr. Res. 87, 2538–2545.
Lante, A., Crapisi, A., Krastanov, A., Spettoli, P., 2000. Biodegradation of phenols by laccase immobilised in a membrane reactor. Process Biochem. 36, 51–58.
Mahdavi, M., Ahmad, M.B., Haron, J. Md, Namvar, F., Nadi, B., Rahman, M.Z., Amin, J., 2013. Synthesis, surface modification and characterisation of biocompatible magnetic iron oxide nanoparticles for biomedical applications. Molecules 18, 7533–7548.
Madhavi, V., Lele, S.S., 2009. Laccase: properties and applications. Bioresources 4, 1694–1717.
Masciangioli, T., Zhang, W., 2003. Environmental nanotechnologies at nanosacle. Environ. Sci. Technol. 37, 102A–108A.
Moreno-Pirajàn, J.C., Giraldo, L., 2011. Study of immobilized *Candida rugosa* lipase for biodiesel fuel production from palm oil by flow microcalorimetry. Arab. J. Chem. 4, 55–62.
Nguyen, L.N., Hai, F.I., Yang, S., Kang, J., Leusch, F.D.L., Roddick, F., Price, W.E., Nghiem, L.D., 2014. Removal of pharmaceuticals, steroid hormones, phytoestrogens, UV-filters, industrial chemicals and pesticides by *Trametes versicolor*: role of biosorption and biodegradation. Int. Biodeterior. Biodegr. 88, 169–175.
Onderková, Z., Bryjak, J., Polakovič, M., 2007. Properties of fructosyltransferase from *Aureobasidium pullulans* immobilized on an acrylic carrier. Chem. Pap. 61, 359–363.
Quiroga, E., Illanes, C.O., Ochoa, N.A., Barberis, S., 2011. Performance improvement of araujiain, a cystein phytoprotease, by immobilization within calcium alginate beads. Process Biochem. 46 (4), 1029–1034.
Ramírez-Montoya, L.A., Hernández-Montoya, V., Montes-Morán, M.A., Jáuregui-Rincón, J., Cervantes, F.J., 2015. Decolorization of dyes with different molecular properties using free and immobilized laccases from *Trametes versicolor*. J. Mol. Liq. 212, 30–37.
Reddy, A.V.B., Reddy, K.G., Madhavi, V., Madhavi, G., 2012. Degradation of chlorpyrifos in aqueous solutions with chitosan-stabilized Fe^0 nanoaparticles. Int. J. Sci. Inn. Dis. 2, 106–112.
Rodríguez-Delgado, M., Orona-Navar, C., García-Morales, R., Hernandez-Luna, C., Parra, R., Mahlknecht, J., Ornelas-Soto, N., 2016. Biotransformation kinetics of pharmaceutical and industrial micropollutants in groundwaters by a laccase cocktail from *Pycnoporus sanguineus* CS43 fungi. Int. Biodeterior. Biodegr. 108, 34–41.
Shu, C., Cai, J., Huang, L., Zhu, X., Xu, Z., 2011. Biocatalytic production of ethyl butyrate from butyric acid with immobilized *Candida rugosa* lipase on cotton cloth. J. Mol. Catal. B Enzym 72, 139–144.
Singh, B.K., Kuhad, R.C., 1999. Biodegradation of lindane by the white-rot fungus *Trametes hirsutus*. Lett. Appl. Microbiol. 28, 238–241.

Singh, B.K., Kuhad, R.C., 2000. Degradation of the pesticide lindane by white-rot fungi *Cyathus bulleri* and *Phanerochaete sordida*. Pest Manag. Sci. 56, 142–146.
Singh, B.K., Kuhad, R.C., Singh, A., Lal, R., Triapthi, K.K., 1999. Biochemical and molecular basis of pesticide degradation by microorganisms. Crit. Rev. Biotechnol. 19, 197–225.
Singh, B.K., Walker, A., Morgan, J.A., Wright, D.J., 2004. Biodegradation of chlorpyrifos by *Enterobacter* Strain B-14 and its use in bioremediation of contaminated soils. Appl. Environ. Microbiol. 70, 4855–4863.
Stoilova, I., Krastanov, A., Stanchev, V., 2010. Properties of crude laccase from *Trametes versicolor* produced by solid-substrate fermentation. Adv. Biosci. Biotechnol. 1, 208–215.
Subramanian, G., Sekar, S., Sampoornam, S., 1994. Biodegradation and utilization of organophosphorus pesticides by cyanobacteria. Int. Biodeterior. Biodegr. 33 (2), 129–143.
Talbert, J.N., Goddard, J.M., 2013. Characterization of lactase-conjugated magnetic nanoparticles. Process Biochem. 48, 656–662.
Wang, F., Hu, Y., Guo, C., Huang, W., Liu, C.Z., 2012. Enhanced phenol degradation in coking wastewater by immobilized laccase on magnetic mesoporous silica nanoparticles in a magnetically stabilized fluidized bed. Bioresour. Technol. 110, 120–124.
Wang, H., Zhang, W., Zhao, J., Xu, L., Zhou, C., Chang, L., Wang, L., 2013. Rapid decolorization of phenolic azo dyes by immobilized laccase with Fe_3O_4/SiO_2 nanoparticles as support. Ind. Eng. Chem. Res. 52, 4401–4407.
Wang, T., Li, H., Nie, K., Tan, T., 2006. Immobilization of lipase on epoxy activated (1–3)-α-D-glucan isolated from *Penicillium chrysongenum*. Biosci. Biotechnol. Biochem. 70, 2883–2888.
Zhang, D.H., Yuwen, L.X., Peng, L.J., 2013. Review article parameters affecting the performance of immobilized enzyme. J. Chem. 2013, 1–7.
Zhou, Y., Pan, S., Wei, X., Wang, L., Liu, Y., 2013. Immobilization of β-glucosidase onto magnetic nanoparticles and evaluation of the enzymatic properties. Bioresources 8, 2605–2619.