Horseradish Peroxidase Immobilized into Organogel-Silica Composite for Transformation of Chlorophenols to Biodegradable Organic Acids

Klorofenollerin Biyobozunur Organik Asitlere Dönüşümü İçin Organojel-Silika Kompozit Yaban Turpu Peroksidaz İmmobilizasyonu

Research Article

Rabel Soomro[1], Najma Memon[1*], M. Iqbal Bhanger[2], Adil Denizli[3]

[1]National Centre of Excellence in Analytical Chemistry, University of Sindh, Jamshoro, Sindh, Pakistan.

[2]H.E.J. Research Institute of Chemistry, University of Karachi, Karachi, Pakistan.

[3]Department of Chemistry, Biochemistry Division, Hacettepe University, Ankara, Turkey.

ABSTRACT

Phenol and its derivatives are considered detrimental to environment and need to be removed from environment. Enzymatic oxidation of chlorophenols is considered as viable option but leads to precipitate due to polymerization of phenolic compounds, which require additional step of flocculation towards safe discarding of contaminated waters. In present work, microemulsion organogel from cetyltrimethylammonium bromide reverse microemulsion and gelatin was prepared and used as immobilization matrix for horseradish peroxidase (HRP, E.C.1.11.1.7). The material was further hardened with silica to enhance aqueous solution stability. The composite material was studied for enzymatic kinetics and oxidation of chlorophenols and employed as catalyst in the presence of hydrogen peroxide to oxidize 2-chlorophenol (2-CP), 2,4-dichlorophenol (2,4 - DCP), and 2,4,6-trichlorophenol (2,4,6-TCP) in an aqueous media. It was worth noting that phenols get converted to organic acids rather than typical polymerized products. The protective effect of composite material was observed which render the polymerization reaction of phenols. Michaeles-Menten constant and activity of enzyme in free and immobilized system were evaluated using three modified Michaeles-Menten equations and progressive curve experiments, respectively. The immobilized HRP followed Michaeles-Menten kinetics with lower reaction velocity constant (V_{max}) and Michealis constant (K_m) values comparable to free HRP. Further, following parameters were optimized: contact time, pH, hydrogen peroxide concentration, enzyme dose and analyte concentration. Under different optimized condition, the oxidative removal of phenol and their derivatives reaches up to 95-99% in phosphate buffer.

Key Words

Chlorophenols, enzymatic oxidation, HRP; K_m Value, Michaeles-Menten Constant, oxidation reaction.

ÖZET

Fenol ve türevleri tehlikeli çevresel atıklardır ve bu nedenle çevreden uzaklaştırılmaları gerekir. Klorofenollerin enzimatik oksidasyonu uygun bir seçenek olarak düşünülür ama fenolik bileşiklerin polimerizasyonu nedeniyle çökmesine yol açar ve kontamine suların güvenli bir şekilde atılması yönünde flokülasyonun ek bir adımını gerektirir. Bu çalışmada, setiltrimetilamonyum bromür ters mikroemülsiyon ile mikroemülsiyon organojel ve jelatin hazırlandı ve yaban turpu peroksidazı (HRP, E.C.1.11.1.7) için immobilizasyon matriksi olarak kullanıldı. Bu malzeme sulu çözeltilerin kararlılığını artırmak için silika ile daha fazla sertleştirilmiştir. Bu kompozit malzeme enzimatik kinetiği ve klorofenollerin oksidasyonu için çalışılmış ve sulu ortamlarda 2-klorofenol (2-CP), 2,4-diklorofenol (2,4-DCP) ve 2,4,6-triklorofenolü (2,4,6-TCP) hidrojen peroksit varlığında okside etmek için katalizör olarak kullanılmıştır. Dikkat edilmesi gereken konu fenoller, tipik polimerize ürünler yerine organik asitlere dönüştürülmüştür. Kompozit malzemelerin koruyucu etkisi fenollerin polimerizasyon tepkimelerini açıklamak için incelenmiştir. Michaelis-Menten sabiti ve serbest ve immobilize sistemdeki enzimin aktivitesi sırasıyla üç modifiye Michaelis-Menten denklemleri ve ilerleme eğrisi deneyleri ile değerlendirilmiştir. İmmobilize HRP, düşük tepkime hız sabiti (V_{max}) ve Michaelis sabiti (K_m) değerlerini serbest HRP ile karşılaştırmak için izlenmiştir. Ayrıca, temas süresi, pH, hidrojen peroksit derişimi, enzim dozu ve analit derişimi parametreleri ile optimize edilmiştir. Farklı optimizasyon koşulları altında, fenollerin ve türevlerinin fosfat tamponunda oksidatif uzaklaştırılması % 95-99 arasında gerçekleştirilmiştir.

Anahtar Kelimeler

Klorofenoller, enzimatik oksidasyon; HRP, K_m değer, Michaelis-Menten sabiti, oksidasyon tepkimesi.

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Correspondence to: N. Memon, National Centre of Excellence in Analytical Chemistry, University of Sindh, Jamshoro, Pakistan.

Tel: +92 22 9213431 Fax: +92 22 9213429 E-Mail: najmamemon@gmail.com

INTRODUCTION

Phenols and its derivatives like chlorophenols are source of contamination to ground and shallow water bodies and has remained severe ecological concern. Moreover, US Environmental Protection Agency (EPA) has listed chlorophenols (CPs) as priority pollutants [1]. These hazardous phenols enter into water bodies through various sources including industrial, agricultural waste, burning of organic waste in the presence of chlorine or formed during pulp bleaching [2]. Noxious effect of these pollutants adversely affect digestive system, nervous and respiratory system and may eventually lead to fatal disease such as cancer and thyroid hormone imbalance. Based on the researches of International Program on Chemical Safety (IPCS), it is essential to find an operative method to remove these phenols from waste or polluted waters [3].

Treatment of chlorophenols containing wastewater by oxidation using ozone, hydrogen peroxide with Fe^{2+} as catalyst (Fenton's reagent) and other oxidizing agents as single or in combination are employed. Other treatment options include but are not limited to UV irradiation, electrochemical and galvanochemical oxidation, radiolysis, advanced oxidation and bioremediation with enzymes etc [3-6].

Bioremediation of phenols and other organic pollutants using enzymes and other biological systems is considered eco friendly as compared to physicochemical process because bioremedial process produce nontoxic end products in most cases and yet these processes are efficient and scalable. Everyone knows that enzymes are biocatalyst that increase the rate of chemical reactions taking place inside living cells. The use of enzyme based techniques to remove organic compounds from aqueous solution was first proposed by Klibanov and colleagues and since then has been continuously improved [6].

Literature supports that among oxidoreductive enzymes, HRP has been used in many studies for removal of phenolic compounds from aqueous media. Because the enzyme is less costly to produce, inactivates less rapidly, or acts on a wider variety of substrates than other oxidoreductive enzymes. Consequently, the HRP-catalyzed procedure has prospective applications in the treatment of waters and wastewaters polluted with phenolic pollutants. However, the use of free enzymes shows some main drawbacks including the inconvenience of separating enzyme from reaction medium. Therefore, enzymes have been immobilized on natural and synthetic supports to overcome these drawbacks [6,7]. Enzymes are also studied in reverse micellar solutions, the activity of enzyme is reported to be dependent on various factors that govern the formation of droplet size of water in organic continuous phase. Reverse micellar solutions are microheterogeneous systems capable of solubilizing enzymes in water pools of in bulk organic phase and also considered mimic to biological systems. Enhanced enzymatic activity/superactivity in these solvents is demonstrated in many cases [8]. Anionic reverse micelles of AOT (sodium bis(2-ethylhexyl) sulfosuccinate) is extensively studied as compared to cationic or anionic surfactants [9].

Recently, a detailed study of cationic micellar solution for catalytic oxidation using HRP and later in another report, impact of nonionic surfactants blended with cationic surfactant is reported [10,11]. Stability and better relative activity of HRP in micellar systems as compared to aqueous solutions was observed. Inspite of enhanced activity of enzymes in reverse micellar solution, separation of products or removal of enzymatic material is challenging which limit its practical utility.

Organogels prepared from reverse microemulsions and using gelatin or lecithin as gelling materials are reported and have been used to immobilize enzymes [12,13]. Successful immobilization of lacasse in gelatin organogel of AOT was reported by Crecchio, few organic compounds were catalytically oxidized to demonstrate the potential of these materials for future applications [7]. Further, these oragnogels were hardened with metal-alkoxide to improve the mechanical and aqueous solution compatibility [14]. The advantage of using organogel as immobilizing medium is envisaged a better replacement of solubilized enzyme in aqueous or

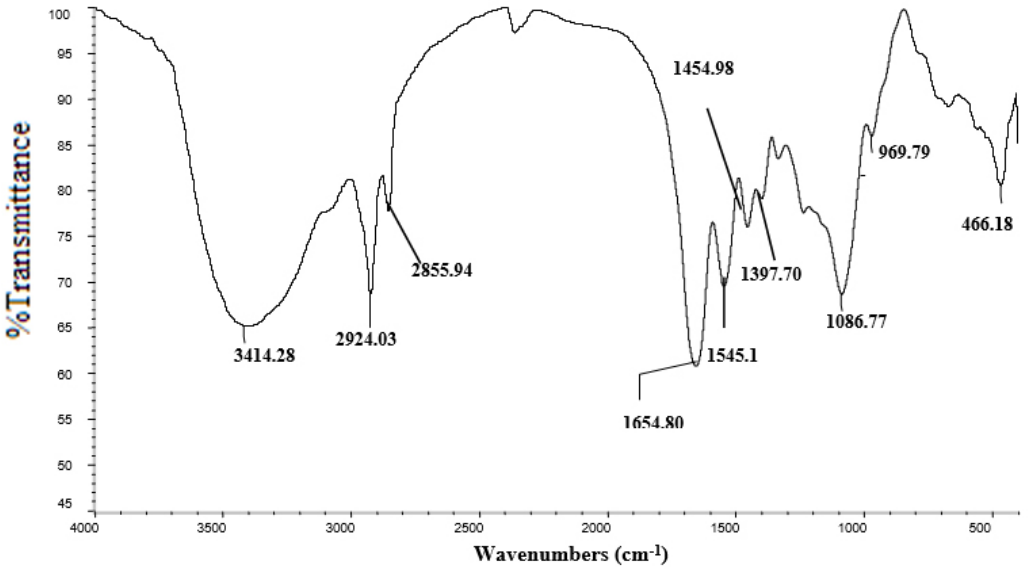

Figure 1. FT-IR spectrum of CTAB microemulsion based organogel-silica composite.

micellar solutions as separation of products from enzymatic may not require additional step.

In the present study, horseradish peroxidase was immobilized in gelatin organogel prepared from cetyltrimethylammonium bromide (cationic surfactant) reverse microemulsion and was hardened with silica. Kinetics of free and immobilized HRP was studied and compared. Further,2-chlorophenol(MCP),2,4-dichlorophenol (2,4-DCP) and 2,4,6-trichlorophenol (2,4,6-TCP) are oxidized simultaneously as model compounds from aqueous phase using enzyme immobilized into organogel-silica composite. Oxidation product formation and various parameters that may affect oxidation of chlorophenols were studied are also included.

RESULTS and DISCUSSION

Characterization Fourier-Transform Infrared Spectroscopy (FT-IR)

Figure 1 shows the FT-IR spectra of CTAB microemulsion based organogel-silica composite. The spectrum illustrates the binding of silica over the microemulsion-based organogel. FT-IR spectra of silica-gelatin was characterized by a large band around 3450 cm^{-1} corresponding to NH stretching vibration, two narrow bands at 2920 cm^{-1} and 2850 cm^{-1} attributed to CH_2 stretching vibrations. The presence of silica leads to a better resolution of the amide vibration area, showing two peaks at 1650 cm^{-1} (Amide I) and 1550 cm^{-1} (Amide II), indicating that the protein chain is in a folded conformation. The peaks at 1086 cm^{-1} are attributed to the triply degenerated stretching and bending vibration modes of the [SiO_4] tetrahedron at 1100 cm^{-1} and 470 cm^{-1}, the Si–OH vibration at 945 cm^{-1} [15]. The data suggested that silica-organogel composite was successfully prepared. Literature supports [16] that the polymerization of tetraethoxysilane (TEOS) inside the CTAB organogel leads to a formation of a material, which can be considered as a silica gelatin composite. This material (organogel + silicate) is completely different from the one obtained by the hardening process of hydrogel by silicate. In fact, in terms of stability, the cationic silica gelatin composite becomes almost temperature and solvent-independent upon addition of adequate amounts of the precursor (TEOS) [16].

Scanning Electron Microscope (SEM)

The material was characterized by scanning electron microscopy (SEM) before and after using it for oxidation of phenol in aqueous system. Figures 2a and 2b show the SEM morphology. These microphotographs indicate an open cell pore structure. The presence of strands of irregular shape is also evident. Microphotographs of the organogel-silica composite show the presence of particles connected with each other. As reported by Iler, [17] Mukkamala and Cheung [18] the connectivity between the spherical

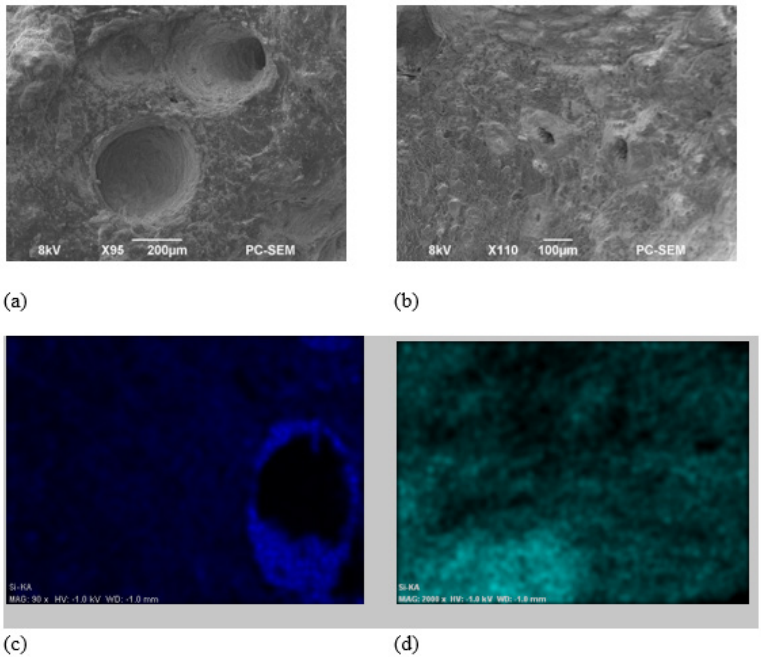

Figure 2. SEM micrograph of fresh organogel/silica composite (2a), SEM micrograph after treatment (2b), Si mapping of fresh gel (2c), blue color shows the distribution of silica over the surface, Si mapping of treated gel (2d), green color shows the distribution of silica over the surface.

particles can be attributed to the agglomeration effect of the surfactant (CTAB), through which, the silica particles are held together by micelles of the cationic surfactant. Figures 2a and 2b show porosity and large cavities in the composite morphology which is evident that the prepared material is capable to entrap the substrate. Distribution of silica in composite was observed by mapping silicon on the surface of the fresh microemulsion based organogel-silica composite before (Figure 2c) and after (Figure 2d) use. Silicon mapping of the composite shows that silica is well distributed over the surface, which maintains similar morphology even after use.

Kinetics of enzymatic oxidation using 2-chlorophenol

Based on the kinetic data obtained from the experiments mentioned earlier, modified forms of Michaelis-Menten ($v = (V_{max}.[S])/(K_m + [S])$) equation has been used to find out K_m and V_{max} values using 2-chlorophenol as substrate and are given in Table 1.

In the equations;
K_m=Micheles Menten Constant ($\mu mol.L^{-1}$)
V_{max}= reaction velocity ($\mu mol.L^{-1}\ min.t^{-1}$)
[S] = Concentration of substrate at time t ($mmol.L^{-1}$)

Following modified forms of Michaelis-Menten equation are used in this work;

The regression analysis of three forms of linearized equations for free and immobilized enzyme indicated that quality of fit was quite

$$\frac{1}{v} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}} \quad \text{Lineweaver-Burk equation} \quad (1)$$

$$\frac{[S]}{v} = \frac{1}{V_{max}} \times [S] + \frac{K_m}{V_{max}} \quad \text{Hoftsee Equation} \quad (2)$$

$$v = K_m \times \frac{v}{[S]} + V_{max} \quad \text{Eadie Equation} \quad (3)$$

good, with R^2 values ranging 0.888-0.995. The three forms of equation shows comparable results (Table 1) other than the Lineweaver-Burk plot which provided higher V_{max} and K_m values for immobilized enzyme. Since reliability of other two plots is demonstrated over Lineweaver-Burk [19] therefore, results obtained from two other equations were taken for comparison of free and immobilized HRP.

Table 1 shows that K_m Values range from 2.03 to 2.3 $mmol.L^{-1}$ for immobilized and free enzyme respectively. Value obtained with Lineweaver Burk equation in case immobilized enzyme is significantly higher and was treated as odd. Close values of K_m for free and immobilized

Table 1. Kinetic parameters for enzymatic oxidation of 2-chlorophenol with free and immobilized HRP.

Calculation Method		Lineweaver-Burk (1/S vs 1/*v*)	Hofstee (S vs S/V)	Eadie (V vs V/S)
Vmax ($\mu mol.L^{-1}\ min^{-1}$)	Free	1.53	1.52	1.20
	Imm	0.875	0.217	0.20
Km ($mmol.L^{-1}$)	Free	2.30	2.32	2.12
	Imm	4.68	2.02	2.03
Regression Coefficient	Free	0.959	0.889	0.954
	Imm	0.932	0.999	0.995

forms suggest that the HRP does not experience conformational changes after immobilization; therefore, the affinity of substrate with immobilized HRP is not different from that of free HRP. The V_{max} values for immobilized and free HRP are 0.2 and 1.2/1.5, respectively which is nearly six times lower and may be due to the alterations of the microenvironment upon immobilization onto organogel and then hardening with silica or due to lower diffusibility of substrate towards enzyme located in the organogel-silica hardened matrix [20,21].

The efficiency factor can be calculated from the maximum reaction rates of the immobilized enzyme over that of free counterpart = V_i/V_f, where V_i and V_f are rates for immobilized and free enzyme, respectively. From this calculation, HRP-immobilized onto silica-hardened organogel provides efficiency factor of 0.16.

The ratio of V_{max}/K_m defines the measure of catalytic efficiency of an enzyme-substrate pair. In this study catalytic efficiency was 9.9×10^{-5} and 56×10^{-5} for immobilized and free HRP. Our findings follow similar trend for V_{max} as reported for immobilized enzymes where as K_m values obtained here does not follow the trends as in earlier reports for immobilized enzymes which shows that immobilization of HRP into organogel-silica composite experience diffusion limitation whereas enzyme active sites/conformation is not affected.

Activity assay of free and immobilized HRP

Enzyme activity is expressed as moles of substrate converted per unit time or rate multiplied by reaction volume. Enzyme activity is dependent on conditions, which should be specified. Activity of enzyme may be evaluated different procedures; we have used progress curve experiments. In these experiments, the kinetic parameters are determined from expressions for the species concentrations as a function of time.

Figure 3 shows the reaction rates (slope values); catalyzed amount of chlorophenol per minute using same amount of HRP (200 μg) in total reaction volume of 6 mL for free and immobilized forms. At all concentrations, free HRP has nearly five times higher activity as compared to immobilized HRP in organogel-silica hardened material. These findings are comparable with literature for immobilized enzymes and is due to diffusion limitations of substrate for immobilized enzymes [20]

Optimization of different parameters for oxidation of chlorophenols

It is well-known that Horseradish peroxidase catalyzes H_2O_2-mediated oxidation of substrates with different functional attribution, including phenols [22] where it is customary to observe decrease in chlorophenol concentration while studying the oxidative removal/degradation of chlorophenols. We used liquid chromatography to determine phenol concentrations in aqueous solutions before and after enzymatic oxidation.

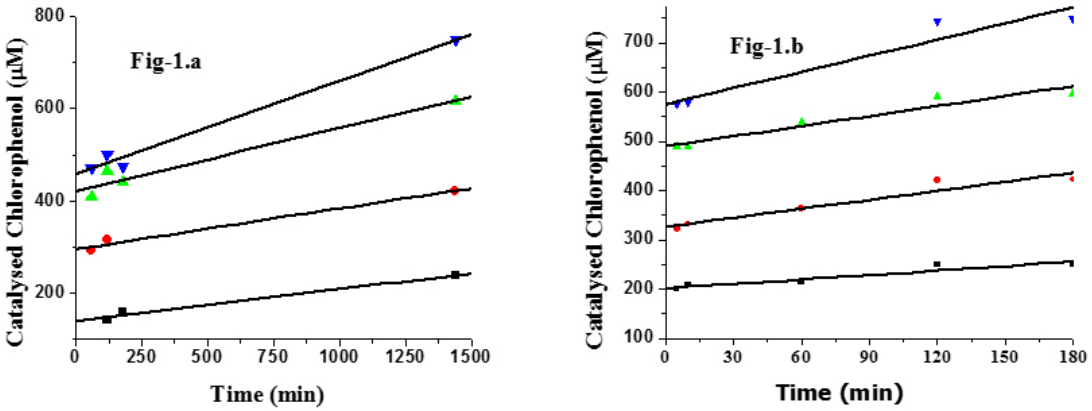

Figure 3. Progress curve plot using oxidation of 2-chlorophenol using 200 µg of HRP immobilized in silica hardened-organogel (1.a) and free HRP 200 µg (1.b) at various substrate concentration; 972 µ mol.l^{-1} (■), 777 µ mol.l^{-1} (●), 583 µ mol.l^{-1} (▲) and 388 µ mol.l^{-1} (▼).

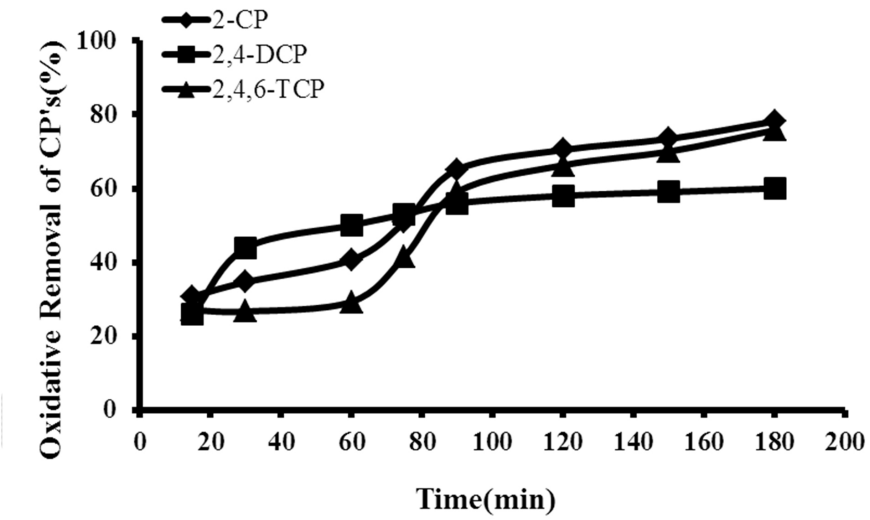

Figure 4. Contact time profile for the oxidative removal of chlorophenols.

Similar approach was adopted to observe the effect of various parameters on oxidative removal of chlorophenol. Details on the effect of various chemical parameters is given in following sections.

Contact Time

To study the optimal reaction time for the oxidation of chlorophenol, mixture of chlorophenol (2-chlorophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol) were prepared in deionized water. To study the optimal reaction time for the oxidation of chlorophenols, organogel-silica composite (150 mg) was added to the solution containing 100 mg L^{-1} chlorophenol mixture, 1 mmol L^{-1} hydrogen peroxide at room temperature in a final volume of 6 mL of phosphate buffer (0.05 mol L^{-1} of pH 7.5). The organogel composite was allowed to remain in contact with the phenol solutions up to 180 min. The total enzyme concentration was 200 µg in composite material. After each 15 minutes of time interval, 20 µL of filtered sample was injected to HPLC column and the remaining amount of filtrate was added back to the reaction vessel in order to maintain the volume of sample. Results are shown in Figure 4 which indicates the oxidation of 2-chlorophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol reaches up to 78%, 65%, 76% respectively in 3 hours. It was also observed that with the increase of time the percent removal of chlorophenols also increases and 3 hours of contact time were optimized for the total removal of chlorophenols from aqueous phase.

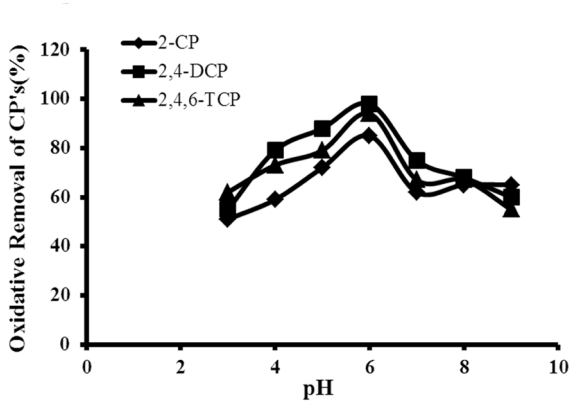

Figure 5. pH profile for the oxidative removal of chlorophenols. During treatment concentration of chlorophenols 100mg.L^{-1}.

Effect of pH

Literature reports that the optimum working pH value of free HRP for phenolic compounds was from 6 to 9.23 To find out the optimal pH for maximum conversion of the chlorophenols substrates by immobilized HRP a series of experiments were performed by varying pH of chlorophenol solution from 3-9 using 1 M HCl/ NaOH at room temperature. To examine the optimal pH for the oxidation of chlorophenols, 150 mg of organogel-silica composite, 1 mmol.L^{-1} hydrogen peroxide was added to the solution containing 100 mg.L^{-1} chlorophenol mixture. The final volume of the sample was makeup with phosphate buffer (0.05 mol.L^{-1} of pH 7.5).

Course of oxidation as affected by pH of reaction medium is shown in Figure 5. Chlorophenols; 2-CP, 2,4-DCP, 2,4,6-TCP get oxidized at relatively higher extent when pH of reaction medium is slightly acidic (pH 6) whereas outside these pH ranges (either acidic or basic), oxidation reaction is comparatively slower. Similar results are obtained by other researchers for enzymatic oxidation of phenol and chlorophenols, [23,24] that conversion is less when the pH of the solution is above 7. This also correlates well with the HRP enzyme activity which is more active around pH 7.

Effect of peroxidase gel dosage

Literature reports that enzymes are the biological catalyst and the amount of catalyzing enzyme affects the reaction [25] and biocatalyst has a finite lifetime as well. The conversion of chlorophenols is found to be dependent on the contact time; usually removal of chlorophenols is dependent on the amount of catalyst added. To study the effect of enzyme concentration on chlorophenol removal, five different enzyme immobilized gel dose were used to compare the efficiency of immobilized enzyme. The chlorophenol and hydrogen peroxide concentration along with the physical condition of reaction remained unchanged (chlorophenol mixture concentration 100 mg.L^{-1}, pH = 6.0).

The addition of different gel dose to a solution of chlorophenol allowed the oxidation of 2-chlorophenol to be increased from 78-80.99%, 2,4-dichlorophenol 65-90.69% and 2,4,6-trichlorophenol from 76-84%.

Figure 6 shows that the increase in amount of enzyme, increases the rate of oxidation for all three chlorophenols up to a certain point so we can say that reaction rate depends linearly on amount of gel dosage containing peroxidase enzyme. It was also observed that 150 mg is the optimized gel dose for the removal of 100 mg.L^{-1} chlorophenol from aqueous phase.

Effect of Oxidant Concentration

In this series of experiments, a progressive reduction in the peak height of the chlorophenol was observed at different oxidant (H_2O_2) concentration 0.3 to 2.5 mmol.L^{-1} at a fixed concentration of chlorophenol mixture solution (100 mg.L^{-1}).

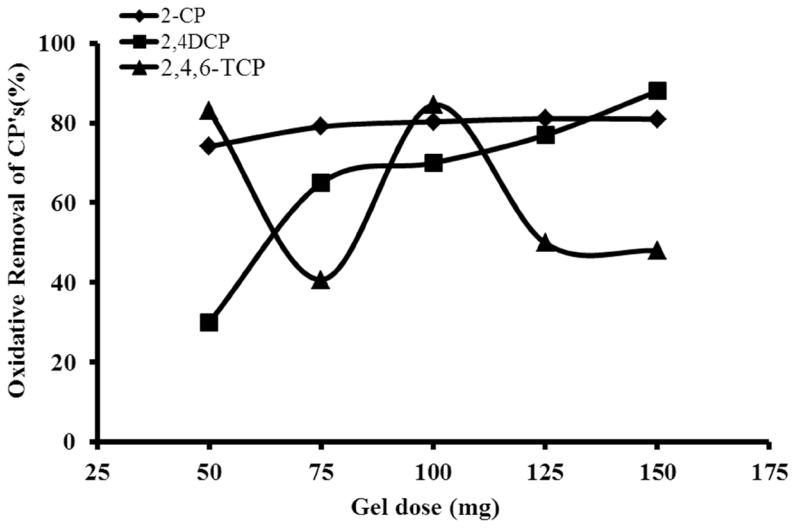

Figure 6. Effect of gel dose on oxidative removal of chlorophenols.

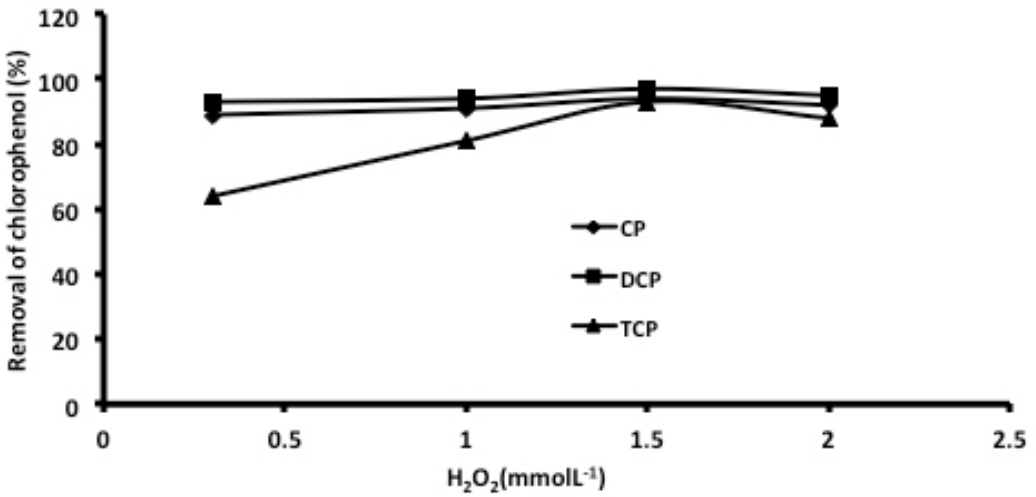

Figure 7. Effect of oxidant concentration on oxidative removal of chlorophenol.

Figure 7 shows the percent removal of 2-CP and 2, 4-DCP increase with the increase in H_2O_2 concentration, while in case of 2,4,6-TCP the percent removal of chlorophenols show irregular increased up to 2.5 mmol.L^{-1} therefore for TCP 1.5 mmol.L^{-1} selected as the maximum H_2O_2 concentration for further studies. Literature reports that an increase in the increase in the hydrogen peroxide concentration consequences in increased availability of the HOO- ion in solution, thereby increasing the rate of oxidation of the phenolic compounds [26] which was in agreement with our study.

Effect of Substrate Concentration

Phenolic pollutant concentration had been reported in the range of trace quantities to hundreds of milligrams to per liter [27]. Different initial concentrations of phenol were examined to the catalytic performance of immobilized HRP from low to high pollution concentration. The amounts of immobilized enzyme and H_2O_2 in all experiment were fixed at 150 mg and 1.5 mmol.L^{-1}, respectively and the total volume of reaction solution was 6 mL. Reaction was allowed to go to completion by providing a reaction period in excess of 3 hours. The application of both oxidant

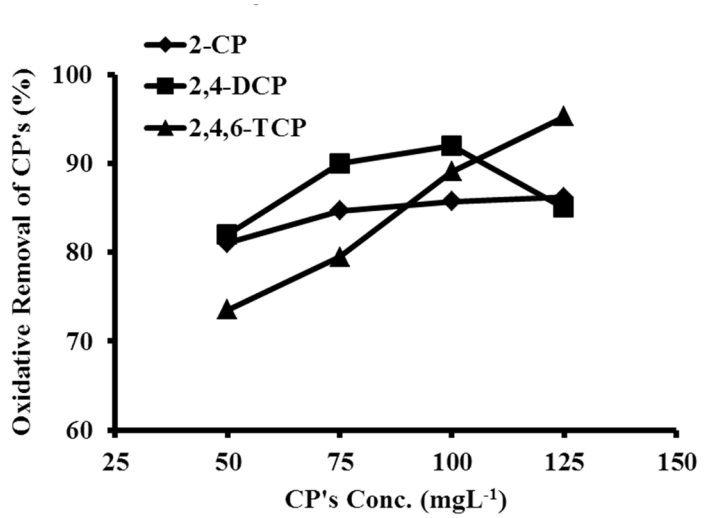

Figure 8. Effect of analyte concentration on the oxidative removal chlorophenol.

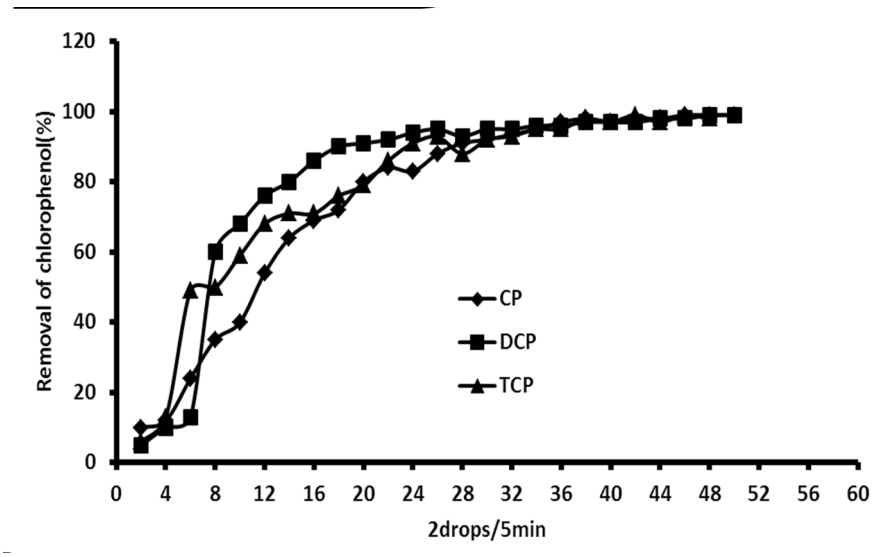

Figure 9. Effect of drop wise addition of H_2O_2 on the oxidative removal chlorophenol.

and immobilized gel enhances the removal percent and hence it is verified that high percent oxidation of chlorophenol achieved at high concentration of chlorophenol.

Figure 8 shows that immobilized gel containing 200 µg of enzyme and 1.5 mmol.L^{-1} H_2O2 oxidizes chlorophenol mixture up to 95% into harmless products.

Dropwise addition of oxidant (H_2O_2)

In order to understand the effect of oxidant in the reaction, the oxidant of optimal concentration i.e. 1.5 mmol.L^{-1} was added drop wise into the sample. Reaction was carried out in 25 mL beaker by taking 100 mg.L^{-1} of chlorophenol solution and 150 mg of enzyme immobilized CTAB based organogel-silica composite at room temperature and final volume was made upto 6 mL by adding phosphate buffer (50 mmol.L^{-1}, pH 6). Experiment was conducted at 25°C and results are shown in Figure 9.

It was observed that initially dropwise addition shows poor effect upto 8 drops per 20 minutes However, in terms of effectiveness, the

Table 2. Optimized parameters for the removal of chlorophenols.

Parameters	Studied Range	Selected value
pH	3-9	6
Reaction Time (h)	1-24	3
Analyte Concentration ($mg.L^{-1}$)	25^{-1}50	100
Oxidant (H_2O_2) concentration ($mol.L^{-1}$)	$3\text{-}2.5x10^{-3}$	1.5×10^{-3}
Enzyme immobilized organogel (Dose mg)	25-150	150

best results were obtained at 30drops per 75 minutes and beyond. And it was concluded that the maximum removal of chlorophenol reaches in 2 hours by the dropwise addition of oxidant.

Optimized Parameter: optimized parameters for oxidative removal of three chlorophenols are shown in Table 2. Briefly, chlorophenol mixture having same concentration i.e 100 $mg.L^{-1}$ at pH 6.0, oxidant concentration 1.5 $mmol.L^{-1}$ and ezyme gel dose 150 mg having 200 μg immobilized HRP was held at 25ºC.

Transformation Products of chlorophenols catalyzed by HRP

Literature reports 28 that the HRP is one of the most intensively studied peroxidases. This enzyme is comparable to LiP of the white rot fungus T. multicolor. The catalytic cycle in both enzymes, LiP and HRP, is the same. Even with small differences in the protein structure, HRP ought to be able to catalyze the degradation of chlorophenols with hydrogen peroxide. HRP catalyze the phenolic substrates by producing HO* free radical (in the presence of H_2O_2), which produce phenoxy radicals resulting in phenolic polymers, dimmers and trimers [28-29]. Other short chain organic acids such as tartaric acid (TA), oxalic acid (OA), maleic acid (MaA) and glycolic acid (GA). Tartaric acid along with other two acids, malic acid and succinic acid, are possibly formed from either oxidation of muconic acid or maleic acid [27].

No polymer precipitate was observed in the conditions used for oxidation of chlorophenols when immobilized enzyme was employed, however, under similar condition solution turn brown and then precipitate appears using free enzyme. This shows that in immobilized HRP system, reaction does not follow conventional free radical polymerization.

Oxidation of chlorophenols was monitored by RPLC (reverse phase liquid chromatography) (for details, see experimental section), which shows an additional peak appeared before the chlorophenols peak. Elution of compound in RPLC at earlier retention times indicates the polar nature of molecules, which may be suspected as organic acid in this case. This was further confirmed by running several organic acids such as tartaric, oxalic, maleic, glycolic, succinic, muconic and maleic acids. The relation between retention time and identity of these acids in HPLC system was established.

Three peaks in the chromatogram are labeled for respective phenols whereas peak at 4.7 minutes shows degradation compound for chlorophenols (Figure 10). The inset shows the UV spectra obtained for degradation peak which correspond to muconic acid, however the peak shape is somewhat different. The data indicates the formation of muconic acid however it require verification through other identification techniques.

The change in product formation in free and immobilized HRP may be explained on the possible protective effect of gelatin or whole organogel-silica system. Similar protective effect

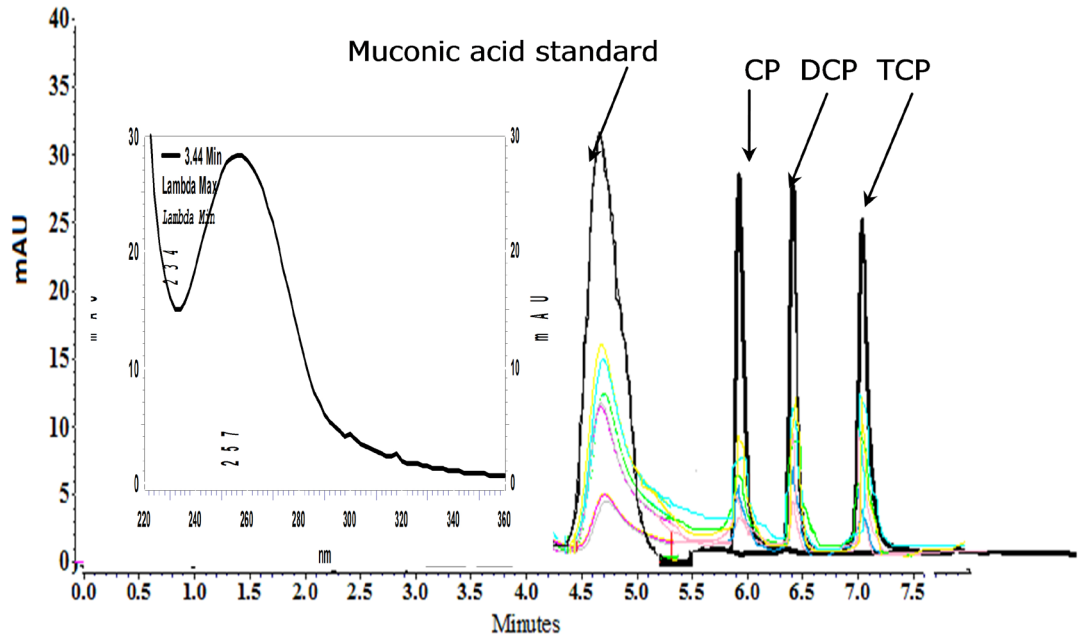

Figure 10. Chromatogram showing the three chlorophenols and their degradation peak. Inset shows the UV spectra obtained by diode array detector for degradation peak.

is reported by gelatin, ethyleneglycol and some geosorbents [24,29-31].

Effect of geosorbents on the product formation and reduction inactivation of HRP catalyzed oxidation has been studied in detail by Huang et. al [24]. Cross-coupling is dominated mechanism as compared to self-coupling of phenolic radicals in the presence of geosorbents which offer effective protection of HRP from inactivation. Out of two types of geosorbents studied, one having high humic substance (Chelsea SOM) was efficient as compared to the other one. Hydrophilic groups on Chelsea SOM were thought to be responsible for better binding efficiency of cross-coupling. Similar chemistries may be expected for organogel-silica material in this study. However, more elaborative studies are needed to confirm the findings.

From the above study, it is concluded that organogel composite is capable of holding peroxidase and effective oxidation of chlorophenols in aqueous phase at optimized pH 6. Using 150 mg composite material and 1 mmol.L^{-1} hydrogen peroxide, chlorophenols were oxidized up to 95% and converted into biodegradable organic acids. Protective effect of organogel-silica composite may be responsible for rendering the reaction towards the formation of polymers. Enzymatic treatment for the oxidation of chlorophenols using peroxidase immobilized into silica-organogel composite leads to one step removal for safe discarding of wastewaters contaminated with phenols. . Kinetic data showed the HRP immobilized in organogel-silica composite has slow kinetics but enzyme conformation is not affected.

Experimental

Chemical and reagents

Peroxidase (donor; hydrogen peroxide oxidoreductase; E.C.1.11.1.7) Type II from horseradish (200 purpurogallin units/mg solid), 2-chlorophenol (Alfa Aesar, A Jhonson Matthey Company), 2,4-dichlorophenol (Sigma, USA), 2,4,6-Trichlorophenol (TCI, USA) were purchased and used without further purification. Methanol and all other chemicals were of analytical grade and were purchased from Merck AG (Darmstadt, Germany).The HPLC studies were made on an Agilent 1200 (Agilent Technologies, USA) HPLC system equipped with a single pump, a UV- detector and a column. The UV detection wavelength was

283 nm. A Gemini-NX C18 column (250 mm_ 4.6 mm, 5 mm) was used for separation. The mobile phase consisted of 60% methanol formic acid mixed solution (0.05% formic acid) and the flow rate was 1.0 mL min^{-1}, and the injection volume was 20 μL. Samples were filtered through a 0.45 mm syringe filter (Millipore) before injection.

Enzyme immobilization into silica-hardened organoge

Preparation of organogel-silica composite containing immobilized enzyme is reported earlier by our group.[25] Preparation of organogels and silica hardening is two step process, where first organogels are prepared and enzyme is added into it subsequently hardening with silica is then carried out. Solutions that need to be prepared in specific compositions are as follows: (1) Microemulsion was prepared in composition of; 100 mmol.L^{-1} CTAB, water/CTAB mole ratio (W0) of 5 and pentanol/CTAB mole ratio (W0) of 4.9 where hexane was used as organic solvent. (2) The stock solution of peroxidase enzyme (HRP) was prepared by dissolving 2 mg of enzyme in 2 mL of 50 mmol.L^{-1} potassium phosphate buffer of pH 7.5.

The CTAB organogel was prepared by taking 1.71 mL of water and 7.3 mL of microemulsion (CTAB/water/pentanol/hexane) in a 30 mL beaker. Then 1.0 g of gelatin was added to the solution. The solution was stirred for 20 min at 45ºC in water bath and then allowed to cool to 25ºC under continuous stirring. A 1.5 mL of solution containing horseradish peroxidase (HRP) enzyme was also added to the above mixture while cooling process. The mixture was left standing at 25ºC without stirring till the gel like phase was observed. To this gelled material 5.6 mL of pure TEOS was added maintaining 25ºC which results in r- value of 5.55 where r- is water/TEOS ratio in mixture of organogel and TEOS. 16 The resulting mixture was subsequently stirred rigorously, cooled to 0 ºC and left for 5 minutes. The white solid mass of CTAB microemulsion–silica gelatin composite was allowed to stand in 20 mL of hexane for three days at 20ºC to take up ethanol produced in conversion of TEOS to silicic acid. Successively the solid was completely dried and placed in water for five hours to allow the hydration and complete formation of silica. The peroxidase containing gel was then transferred in a petri dish, and cut into small pieces/chunks. Finally, the solvent was removed by evaporation which resulted in solid material having enzyme entrapped in new matrix and ready for use.

Enzymatic oxidation procedure and determination of chlorophenols

The HRP catalyzed reaction for chlorophenol oxidation was carried out in a 25 mL beaker. The total reaction volume was 6 mL. Reagents were added in the following order: mixture of chlorophenol solution (each 100 mg.L^{-1}), 1.5 mM H_2O_2, and 150 mg organogel material in phosphate buffer (pH 6.5). The concentrations of chlorophenols and H_2O_2 correspond to concentrations in final solution. Reaction was initiated by the addition of H_2O_2. For the time course study, 200 μL aliquot were withdrawn at a particular time intervals and injected to the HPLC column. The results were calculated by observing the change in peak height of the treated chlorophenol sample peak. All the parameters were optimized at room temperature. All the samples were run in a triplicate and each sample filtered through 0.45 μm filters (Millipore, Bedford, MA).

Kinetic Study

Enzymatic oxidation of chlorophenols is controlled by substrate and enzyme concentration. Therefore the kinetic parameters such as maximum reaction velocity (V_{max}) and Michealis constant (K_m) were calculated by varying the 2-chlorophenol substrate concentration from 50-150 mg.L^{-1} by keeping enzyme dose and H_2O_2 concentration constant at 200 μg and 1.5 mmol.L^{-1}, respectively. Total volume of reaction mixture was 6 mL. Appropriate amount of enzyme-organogel silica hardened material was used that correspond to 200 μg of enzyme.

Free enzyme experiments were carried out as follows; In a vial, different concentration (50, 75, 100, 125) mg.L^{-1} of chlorophenol were taken and hydrogen peroxide was added to bring final concentration at 1 mmol.L^{-1} whereas the total volume was made up to 6 mL by 50 mmol.L^{-1} phosphate buffer (pH 6.5). After adding

Hydrogen peroxide, immediately 200 μL of HRP (1 mg/mL in phosphate buffer; pH 6.5) was added and the reaction time was studied individually for each concentration. All the samples were run in triplicate form.

The $V_{max,}$ and K_m value were calculated using 2-chlorophenol as substrate from linear regression analysis for Lineweaver-Burk plot, Hoftsee and Eadie plots [32,33]. These parameters were calculated by using the Microsoft Excel (2007) Program.

Characterization Techniques

The FT-IR spectral study was performed using FT-IR Nicolet 5700 (Thermo Electron Corporation, USA). KBr disks in the range of 4000-400 cm^{-1} were used as a blank to the sample. Sample pellets made from a 1% KBr (by weight) to the silica hardened CTAB gel powder and were used for recording the spectra of the silica hardened CTAB based gel. For the SEM analysis a chunk of fresh and treated (used) gel were placed on an aluminum stub and coated with carbon black (20 nm thickness). The micrographs were taken at an acceleration voltage of 3 kV in a JEOL, JSM-820 scanning electron microscope.

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