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Removal characteristics of phenol using horseradish peroxidase (HRP)-mediated polymerization in saturated porous media

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ABSTRACT

This paper reports experimental results, demonstrating the feasibility of horseradish peroxidase (HRP) and H_2O_2 to reduce phenol transport in saturated porous media. A laboratory-scale packed column reactor (ID: 4.1 cm, sand-bed height 12 cm) column was utilized to simulate injection of HRP and H_2O_2 into an aquifer contaminated with phenol. Effluent concentrations of phenol and polymerization products were monitored before and after enzyme addition under various experimental conditions (enzyme dose: 0–2 AU/mL, [ionic strength]: 5–100 mM, pH: 5–9). The concentration of phenol in the column effluent was found to decrease by nearly 90% in the presence of HRP (2 AU/mL) and H_2O_2 in the continuous flow system at pH 7 and ionic strength 20 mM. The influent phenol was converted in the system to insoluble precipitate, which deposited in pore spaces. The remains were discharged as soluble oligomers. About 8% of total pore volume in column system was decreased by deposition of polymer produced.

Keywords: Phenol; HRP; Polymerization; Soluble and insoluble polymer; Saturated porous media

1. Introduction

Phenol and its derivatives are the basic structural units for a wide variety of synthetic organics including many pesticides [1]. These chemicals have been introduced into the soil and water environment, through the application of pesticides and via manufacturing processes and waste disposal. Several phenolic compounds are toxic and can accumulate in the food chain. Because of their toxicity, phenolic compounds are restricted in many countries and require removal from wastewater before release into the environment [2].

Phenols can be oxidized by peroxidase and other enzymes to produce oligomeric products [3]. Horseradish peroxidase (HRP) has been proposed as an effective enzyme for the oxidative polymerization of phenols due to its stability, broad substrate specificity and its ability to operate at wide ranges of temperature and pH [3, 2]. HRP-mediated oxidative polymerization of phenol results in the formation of a variety of products. These reaction products consist of large molecular weight insoluble oligomers and low molecular weight soluble products [4, 5]. Phenol polymerization products generally have high molecular weight and tend to precipitate out of solution. These properties of the oligomers allow peroxidases to decontaminate water containing phenolic compounds to levels that are otherwise difficult to attain via conventional microbial degradation processes.

HRP mediates the oxidation of phenolic substrates via a three-step catalytic cycle. The enzymatic mechanisms for phenol oxidation by HRP are relatively well understood and have been mathematically modeled. The three-step catalytic cycle of phenol oxidation catalyzed by HRP can be described by the following chemical equations [6]:

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$$E + H_2O_2 \rightarrow E\text{-}I + H_2O \quad (1)$$

$$E\text{-}I + AH_2 \rightarrow E\text{-}II + AH\bullet \quad (2)$$

$$E\text{-}II + AH_2 \rightarrow E + AH\bullet \quad (3)$$

where E is the peroxidase enzyme, E-I and E-II are active enzyme intermediates, AH_2 is a reducing substrate such as phenol or aromatic amine, and AH • is a free radical product.

In this study, we evaluated the impact of pH, solution ionic strength and HRP concentration for the applicability of HRP mediated polymerization process in saturated porous media to remove phenol.

2. Method

This section describes the reagents, materials, equipment and methods employed to conduct the research.

2.1. Materials

Horseradish peroxidase (Type II, RZ 2.2, 181 activity units/mg), hydrogen peroxide (30%, w/w, 8.2 M) and phenol (>99%) were purchased from Sigma Chemicals (St. Louis, MO). Analytical grade potassium chloride, sodium chloride, potassium phosphate (mono and dibasic), acetic acid, methanol and scintillation cocktail (Fisher ScintiSafe, 50%) were obtained from Fisher Scientific (Pittsburg, PA). Uniformly ring-labeled ^{14}C-phenol (specific activity 40.1 mCi/mmol) was purchased from Sigma Chemicals. Working solutions of phenol were amended with ^{14}C-phenol for quantification of polymerization products generated after reaction with HRP and H_2O_2.

A glass column (41 mm internal diameter, 110 mm length) was fabricated in Kansas State University's glass blowing laboratory (Fig. 1a). Teflon end fittings and tubes (1.14 mm) were purchased from Fisher Scientific. Ottawa sand (20–30 mesh) was used as the granular media and was purchased from Fisher Scientific. A peristaltic pump (Bulcher, Model 426-2000) was used to introduce the solution into the column. An automated fraction collector (ISCO, Model Foxy Jr.) was used to collect samples from the column effluent.

A high pressure liquid chromatograph (HPLC) system and a liquid scintillation counter (Beckman, Model 6500) were used for quantification of phenol and the total ^{14}C-activity in aqueous solution as disintegrations per minute (dpm).

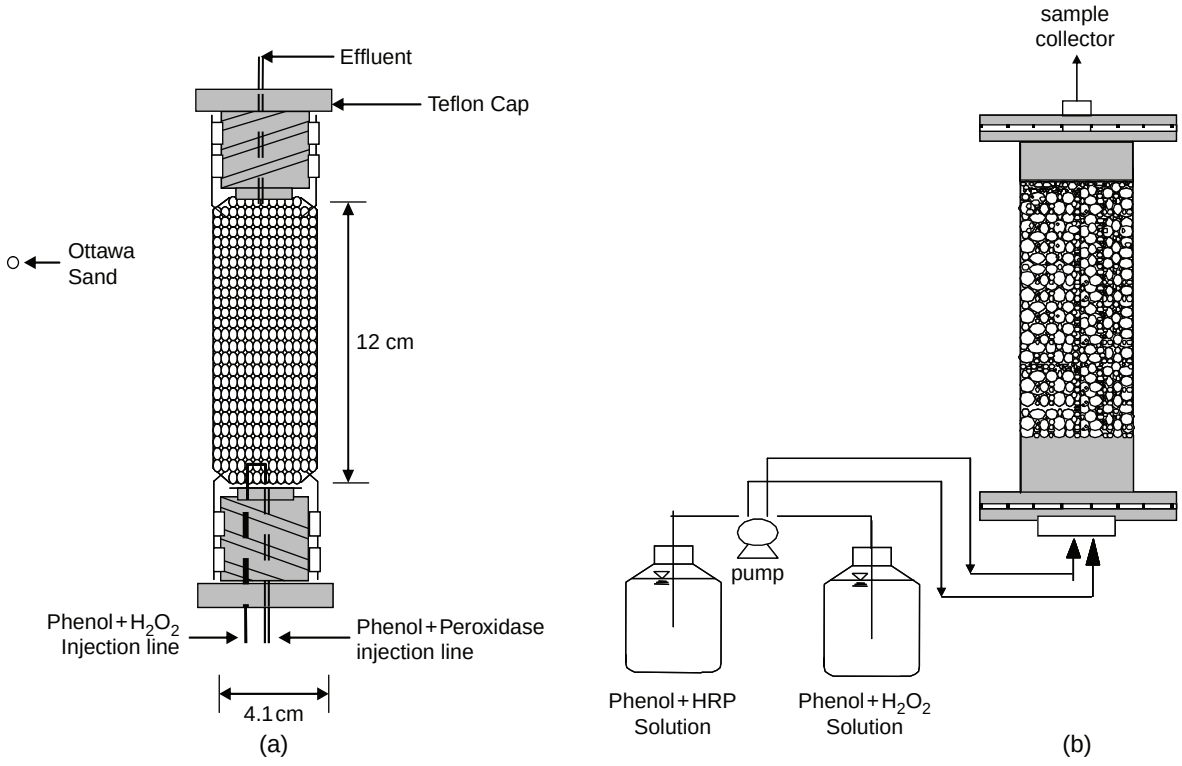

Fig. 1. Schematic diagram of upflow column system used to conduct the experiment.

2.2. Experimental method

The experimental set up for studying phenol polymerization in porous media is illustrated in Fig. 1b. Ottawa sand was packed as uniformly as possible in a glass column. The packed column was saturated and flushed with 20 pore volumes of distilled-deionized water. Experimental conditions were summarized in Tables 1 and 2. A tracer test using KCl as a nonreactive tracer was conducted to determine the hydrodynamic properties of the packed column.

The tracer test consisted of pumping 1.5 pore volumes of KCl solution through the column before flushing it with two pore volumes of distilled-deionized water. Effluent samples were analyzed directly using an in-line conductivity meter. The results were plotted as relative concentration (C/C_0; effluent concentration divided by influent concentration) versus the number of pore volume (discharge volume divided by water retention volume of column). All experiments were performed at room temperature (20 ± 2°C).

Table 1
Properties of porous material.

Granular media	Properties
ρ (g/cm^3)	2.65
ρ_b (g/cm^3)	1.64
ε	0.38

ρ: solid density; ρ_b: bulk density; ε: porosity.

Table 2
Parameters in column experiments.

Parameter	Condition	Q (mL/min)	v (cm/min)	D (cm^2/min)
Enzyme dose (AU/mL)	0.5	1.45	0.1096	0.0101
	1	1.48	0.1120	0.0152
	2	1.45	0.1096	0.0100
	5	1.32	0.0997	0.0081
pH	7	1.45	0.1096	0.0100
	9	1.32	0.0997	0.0140
Ionic strength (mM)	5	1.32	0.0997	0.0127
	20	1.45	0.1096	0.0100
	10	1.32	0.0997	0.0142

Q: flow rate; *v*: pore water velocity; D: dispersion coefficient.

Table 3
Experimental conditions evaluated in column experiments.

Experimental parameter	Value
Influent phenol concentration	500 μM
Influent H_2O_2 concentration	500 μM
Influent HRP concentrations	0.5, 1.0 and 2.0 AU/mL
Solution ionic strengths	5, 20, and 100 mM
Solution pHs	5, 7, 9

The glass column was packed with 215 g of Ottawa sand and operated with flow in the upward direction. The column consisted of two closely spaced inlet lines. One line delivered a solution consisting of enzyme and phenol in a buffer solution. The other line delivered a solution containing H_2O_2 and phenol in an identical buffer solution. The two solutions contained enzyme and H_2O_2 at twice their target "in-column" concentrations, which were achieved when the two flows merged immediately after entering the column. Two separate lines were used for solution delivery to initiate polymerization within the saturated porous media. The buffer pH was controlled by utilizing appropriate ratios of KH_2PO_4 and K_2HPO_4 in solution, while the buffer ionic strength was adjusted using NaCl.

The column study consisted of the following sequence: (i) injection of a conservative tracer (KCl, 250 mg/L); (ii) washing of column with deionized water; (iii) injection of phenol to saturate column with phenol solution; (iv) injection of HRP and H_2O_2 with phenol to facilitate in-situ polymerization of phenol in the porous media; (v) washing of column with deionized water; (vi) injection of tracer; and (vii) washing of column with deionized water. The in-situ polymerization of phenol was evaluated for the combinations of experimental conditions summarized in Table 3.

3. Results and discussions

This section presents results of experiments evaluating peroxidase-mediated phenol removal and accumulation of polymerization products in saturated porous media. The results of nonreactive tracer tests before and after polymerization are also presented and discussed. The impact of solution pH, ionic strength and HRP dose on phenol polymerization in the column system are discussed.

3.1. Tracer test

The breakthrough curve obtained for the nonreactive tracer (KCl) is shown in Fig. 2. The tracer appeared in the effluent after approximately 0.8 pore volumes had passed through the column reactor. As illustrated in Fig. 2, the behavior of tracer and phenol in saturated porous media were nearly identical. The relative concentration (C/C_0) reached a value of 1 at approximately 1.2 pore volumes. When the mobile phase in the column was replaced with distilled-deionized water, the relative

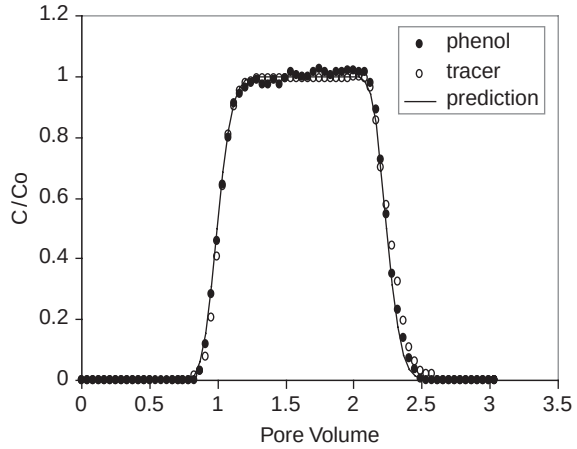

Fig. 2. Transport behavior of tracer and phenol through the packed column reactor. Influent phenol concentration = 500 μM, solution pH = 7, solution ionic strength = 20 mM.

concentration of the tracer in the effluent dropped to zero within one pore volume.

The symmetrical breakthrough curve of the tracer indicated the uniformity of the porous material in the column. The sand column appeared to have been packed uniformly since the tracer demonstrated an ideal transport behavior in the column.

The phenol curve appears to coincide with that of the nonreactive tracer. Phenol does not experience any retardation during flow through the saturated porous media. The breakthrough curves also verified that the tracer was conserved since more than 98% of both tracer and phenol injected were recovered in the effluent. The column packing in all other experiments had similar hydrodynamic properties since all breakthrough curves had very similar shapes and retention times.

3.2. Effect of enzyme dose

In general, phenol removal using HRP-mediated oxidative coupling reaction is observed to increase with increasing enzyme dose [7]. However, since an inefficient use of the enzyme in enzyme-mediated treatment can result in high operational costs, the process should be optimized before deployment. Based on preliminary experiment results, up to 98% of phenol was removed using 2 AU/mL of HRP in batch reaction tests. Therefore, the enzyme doses selected for the column experiments were 0.5, 1.0, and 2.0 AU/mL.

The results of phenol polymerization and transport in continuous flow, saturated porous media for various enzyme doses are illustrated in Fig. 3. The effluent phenol concentration sharply increased after 0.8 pore volume. Since phenol was continuously supplied to the column,

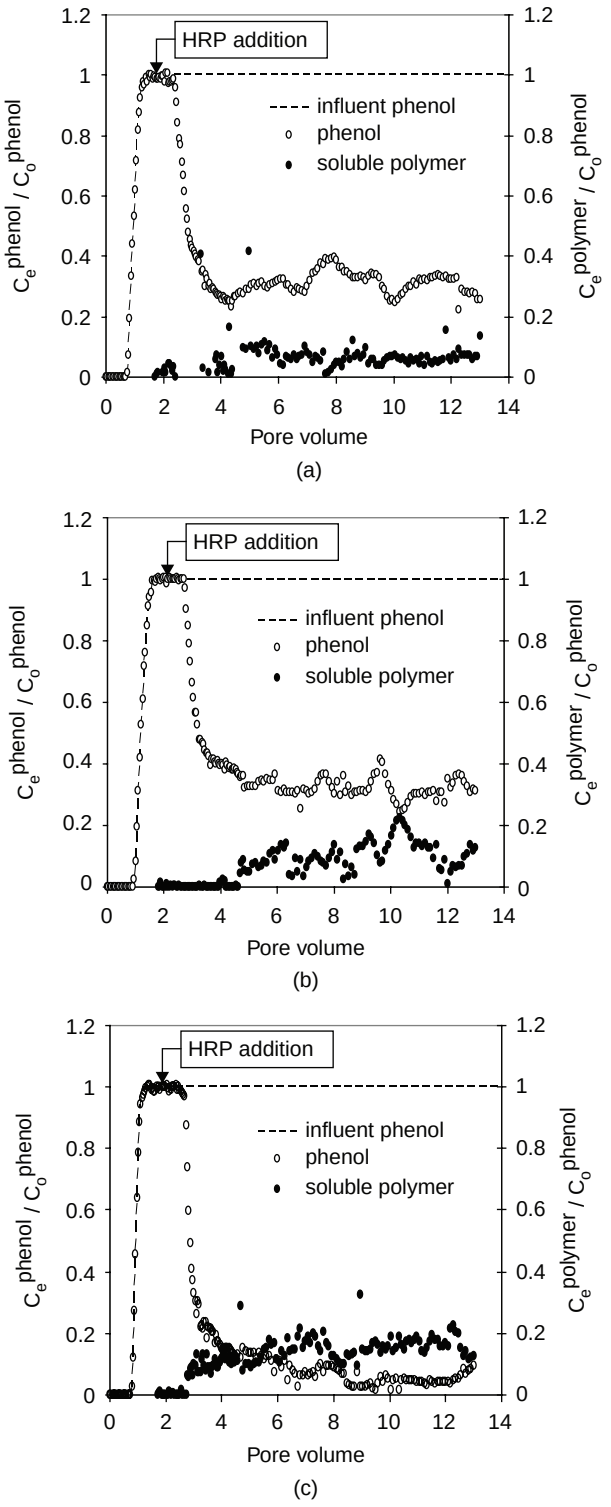

Fig. 3. Performance of the packed column reactor with respect to phenol removal and soluble polymer generation due to polymerization reaction. Influent phenol concentration = 500 μM, solution pH = 7.0, solution ionic strength = 20 mM, H_2O_2 concentration = 500 μM and HRP dose = (a) 0.5, (b) 1.0 and (c) 2.0 AU/mL.

the relative phenol concentration ($C_e^{phenol}/C_0^{phenol} = 1$) was expected to be maintained under saturated conditions when no HRP was added (as indicated by the dashed line). HRP and H_2O_2 were introduced into the column inlet once the column was saturated with phenol. Phenol polymerization reaction in the porous column was expected to occur as soon as HRP and H_2O_2 were added.

Injection of 0.5 or 1 AU/mL of HRP (Fig. 3a and b, respectively) into the column, resulted in a 70% reduction in the effluent phenol concentration. The effect of 2 AU/mL HRP dose is illustrated in Fig. 3c. Approximately 0.8 pore volumes after HRP addition was initiated, the phenol breakthrough curve (open circle) sharply dropped to a relative phenol concentration less than 0.1, indicating 90% removal of the phenol in the flowing solution. Soluble polymer was also observed in the flow exiting the column at approximately 0.8 pore volumes after HRP addition. The relative concentration of soluble polymer (secondary y-axis in Fig. 3) increased and steadied at a value of 0.2 indicating that 20% of the influent phenol was converted to soluble polymers that were not retained in the column. Phenol concentration decreased and steadied at ~10% of the initial phenol concentration after about 4 pore volumes of HRP addition.

The relative concentration of soluble polymer in the effluent was found to increase with HRP dose. The fraction of influent phenol exiting the column as soluble polymer was about 0.05, 0.1, and 0.2 at HRP doses of 0.5, 1 and 2 AU/mL, respectively. This trend was attributed to greater phenol polymerization at higher HRP dose.

The mass balance of phenol for the column system experiments was represented in Table 4. This shows the mass of influent and effluent phenol as well as the soluble and insoluble polymerization products generated as a consequence of HRP and H_2O_2 injection. The insoluble polymer consisted of the polymer accumulated in the sand column and was calculated by subtracting the mass of soluble polymer and effluent phenol from the total phenol injected. In the case of 2 AU/mL HRP dose, 65.5% of the injected phenol was found to accumulate in the column as insoluble polymer while 59.3% and 51.1% of the influent phenol was retained in the porous media at HRP doses of 1 and 0.5 AU/mL. More insoluble polymers were produced at higher enzyme dose due to more complete polymerization of the influent phenol. The deposition of phenolic oligomers in the column was expected to result in the modification of hydraulic properties of the porous media.

Table 4
Mass balance with enzyme dose.

HRP dose (AU/ mL)	Total phenol in (μmol)	Total phenol out (μmol)	Total soluble polymer out (μmol as phenol)	Total insoluble polymer in column (μmol as phenol)
0.5	337.5	136.7	28.3	172.5
1.0	342.4	119.9	19.32	203.2
2.0	342.4	61.1	57.23	224.1

3.3. Effect of solution pH

Several researchers have reported the importance of solution pH for enzyme-mediated polymerization [8–10]. Solution pH may affect the catalytic efficiency of the enzymatic reaction system. The pH can also affect the speciation of the weakly acidic phenol molecules.

The phenol removal and polymer production data at pH 7.0 was illustrated in Fig. 4b. Figure 4a and c show the transport and removal of phenol at pHs 5.0 and 9.0. The concentration of soluble polymer in the effluent was highest at pH 7, followed by pH 5 and pH 9 (Table 5). It is likely that the solution pH affected the configuration of oligomers produced.

Table 5 illustrates that the insoluble polymer deposited in the column was highest at pH 7 and lowest at pH 9. In the case of pH 7.0, 65.5% of the injected phenol was found to accumulate in the column as insoluble polymer while 51.7% and 48.2% of the influent phenol was retained in the porous media at pH 5.0 and pH 9.0. In Ref. [11] reported that precipitation of coupling products increased significantly as solution pH decreased in batch polymerization reaction tests, especially in the range from pH 5 to pH 3 [11]. They reported total phenol conversion did not vary with pH and postulated that a fraction of the phenolic sites on the products may be dissociable around neutral pH [12] and protonation of proton-disassociated sites reduced the ionic character of the products and increased their tendency to precipitate when solution pH dropped. In these experiments, phenol removal was highest at pH 7.0, which is the optimum pH for the HRP-mediated polymerization reaction. Removal efficiency of phenol was higher at pH 5.0 compared to pH 9.0. These results agree with the observations of Ref. [11].

3.4. Effect of solution ionic strength

Fig. 5 illustrates the impact of solution ionic strength on phenol polymerization and polymer production in the continuous flow packed column. Three different ionic strengths (5, 20 and 100 mM) were selected to observe the behavior of phenol, soluble polymer and in soluble polymer. The highest phenol removal was observed at an ionic strength of 20 mM. In the case of

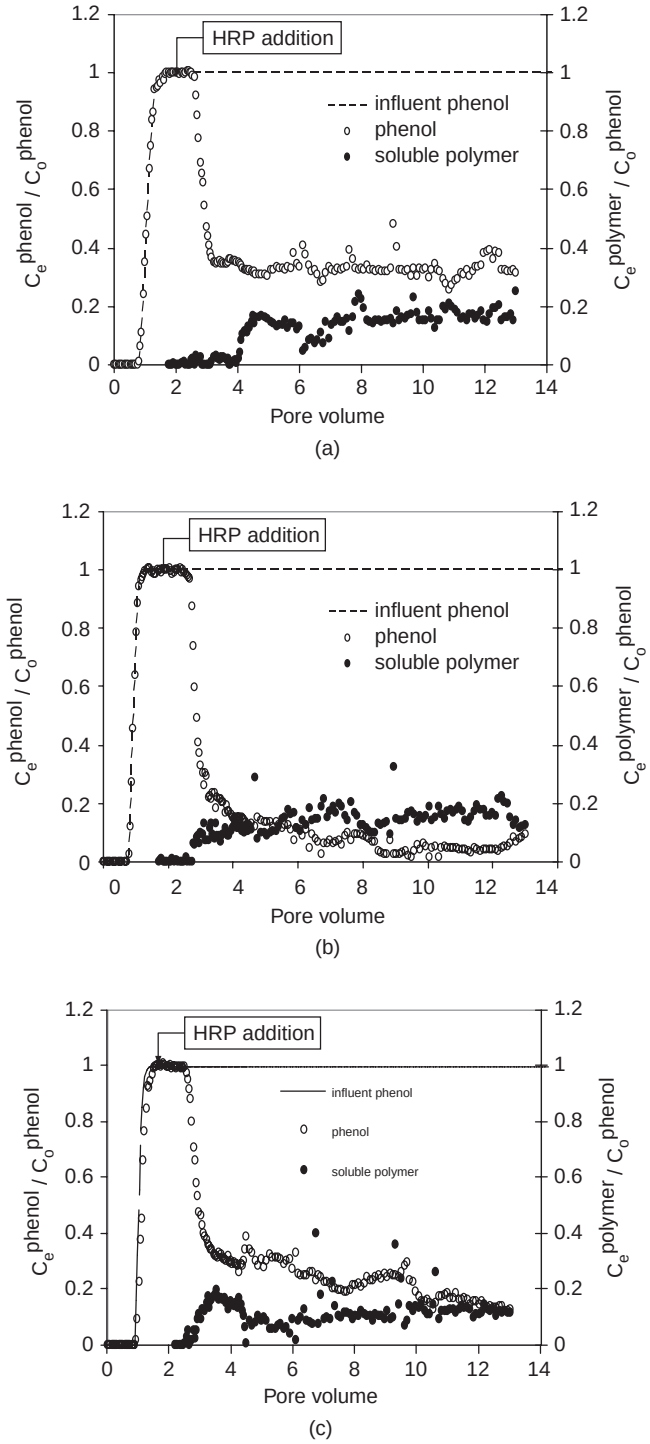

Fig. 4. Effect of pH on performance of the packed column reactor with respect to phenol removal and soluble polymer generation due to polymerization reaction: (a) pH 5; (b) pH 7; (c) pH 9. Influent phenol concentration = 500 μM, HRP dose = 2.0 AU/mL, solution ionic strength = 20 mM and H_2O_2 concentration = 500 μM .

Table 5
Mass balance with pH.

Solution pH	Total phenol in (μmol)	Total phenol out (μmol)	Total soluble polymer out (μmol as phenol)	Total insoluble polymer in column (μmol as phenol)
5	347.5	133.3	34.48	179.7
7	342.4	61.1	57.23	224.1
9	347.5	133.2	45.9	167.4

5 mM ionic strength, phenol removal was reduced to ~70% (Fig. 5a) and effluent phenol concentration continued to decrease with time (volume). The soluble concentration exiting the column was lowest at the 100 mM ionic strength (Table 6), most likely due to a combination of configurational changes in the polymeric macromolecules and the "salting out" effect at the high ionic strength solution [13]. In Ref. [11] observed similar results when they increased solution ionic strength in batch polymerization of phenol using HRP. They found that phenol conversion itself was not sensitive to salt addition. It is evident that certain dissolved coupling products become more "precipitable" as background ion concentrations increase.

Deposition of insoluble polymer was significantly higher at 20 and 100 mM ionic strength solution than at 5 mM ionic strength. The retardation factors for both 20 mM and 100 mM are similar, which is consistent with the mass balance results. These results indicate that high ionic strength positively affects polymer deposition (Table 6). About 8% of pore volume was decreased using 100 mM of ionic strength. The decrease of pore volume was increased with ionic strength as shown in Table 6. However, higher than 20 mM of ionic strength does not significantly affected the deposition of polymer in porous media.

4. Conclusions

This study investigated the impact of polymerization reaction condition in continuous flow-saturated porous media. Effluent concentrations of phenol and polymerization products were monitored before and after enzyme addition under various experimental conditions (enzyme dose: 0–2 AU/mL, [ionic strength]: 5–100 mM, pH: 5–9). Results for these experiments showed that phenol entering a packed column under simulated aquifer conditions was removed from the aqueous phase by injecting HRP and H_2O_2 into the flow. Removal of phenol increased with HRP dose. More than 90% of the influent phenol was removed after about 1.5 pore volume from injection of 2.0 AU/mL of HRP

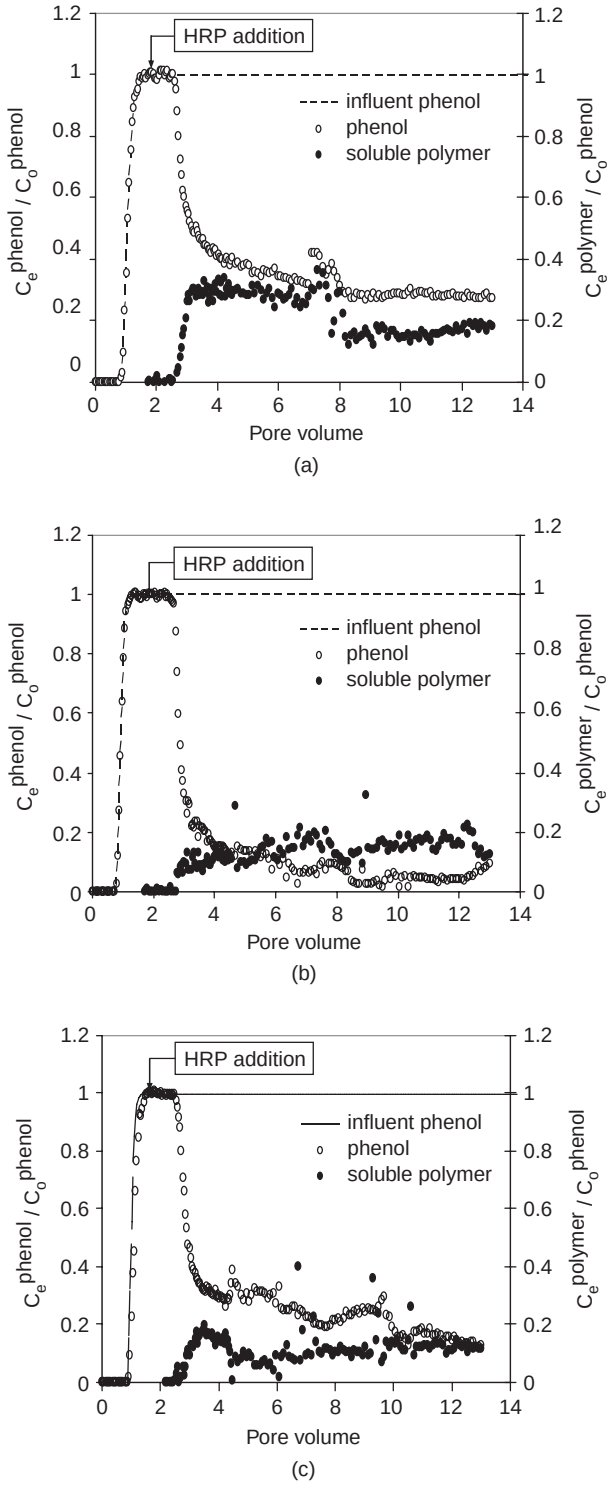

Fig. 5. Effect of ionic strength on performance of the packed column reactor with respect to phenol removal and soluble polymer generation due to polymerization reaction: (a) solution ionic strength = 5 mM; (b) solution ionic strength = 20 mM; (c) solution ionic strength = 100 mM. Influent phenol concentration = 500 μM, solution pH = 7.0, HRP dose = 2.0 AU/mL, and H_2O_2 concentration = 500 μM.

Table 6
Mass balance with ionic strength.

Ionic strength (mM)	Total phenol in (μmol)	Total phenol out (μmol)	Total soluble polymer out (μmol as phenol)	Total insoluble polymer in column (μmol as phenol)
5	347.5	129.2	73.5	144.8
20	342.4	61.1	57.2	224.1
100	347.5	99.2	37.3	211.1

dose. HRP-mediated phenol removal in continuous flow-saturated porous media was accompanied by the generation of soluble and insoluble oligomeric products. Soluble reaction products and insoluble products increased with HRP dose. While about 20% of the influent phenol exited the column as soluble polymerization products, nearly 62.6% of the total influent phenol was retained in the porous media as precipitated products.

Phenol removal and the production of oligomeric products under the action of HRP and H_2O_2 was affected by the enzyme dose, solution pH and solution ionic strength. The concentration of soluble reaction products exiting the column was lowest at the 100 mM ionic strength. Deposition of insoluble polymer was significantly higher at 20 and 100 mM ionic strength solution than at 5 mM ionic strength. Optimum polymer deposition occurred at pH 7. The amount of soluble polymer produced was also highest at pH 7, followed by pH 5 and pH 9.

Acknowledgement

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