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RESEARCH ARTICLE

Effect of operational factors on bioregeneration of binary phenol and 4-chlorophenol-loaded granular activated carbon using PVA-immobilized biomass cryogels

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Abstract The effects of dry biomass density in cryogel beads, shaking speed and initial concentration ratio of phenol to 4-chlorophenol (4-CP) on the bioregeneration efficiencies of binary phenol and 4-CP-loaded granular activated carbon (GAC) for phenol and 4-CP, respectively, were investigated under the simultaneous adsorption and biodegradation approach. The results revealed higher bioregeneration efficiencies of binary-loaded GAC for phenol and 4-CP at higher dry biomass density but moderate shaking speed. The optimum dry biomass density in cryogel beads and shaking speed for use in bioregeneration were found to be 0.01 g/mL and 250 rpm, respectively. With respect to the initial phenol to 4-CP concentration ratio, the bioregeneration efficiencies were lower under increasing phenol and 4-CP initial concentrations, respectively, with the effect being more conspicuous under increasing 4-CP concentration. Higher bioregeneration efficiencies were achieved with the use of immobilized rather than suspended biomasses.

Keywords Bioregeneration · Activated carbon · Phenol · 4-Chlorophenol binary adsorbate · Immobilized biomass

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Introduction

Phenolic compounds are persistent pollutants, and water pollution by phenolic compounds is a prominent environmental issue over the decades. Due to their extensive used in industries, phenolic compounds are widespread pollutants present in the environment. Wastewater containing phenolic compounds gives rise to serious discharge problems due to their poor biodegradability, high toxicity and long-term ecological damage (Bayramoglu et al. 2013; Putz et al. 2005). Activated carbons (ACs) have proven to be effective adsorbents to remove various contaminants in wastewater. The ability of ACs to adsorb vast amount of contaminants is due to high surface areas and porosities (Sze and Mckay 2012). However, the active sites of ACs will eventually be saturated after prolonged application. Since mere disposal of these spent ACs on landfill would be a waste of resources and would cause land pollutions, regeneration is the best option. Among various regeneration methods, bioregeneration which involves the use of microorganisms to induce more desorption of the adsorbed contaminants from the adsorbent followed by complete biodegradation of the contaminants is considered as the most eco-friendly method. In recent years, there were many reported studies on bioregeneration of adsorbents loaded with single contaminant (Aktas and e en 2007, 2009, 2010; Al-Amrani et al. 2012, 2013; Ng et al. 2009; Oh et al. 2011, 2013). In comparison, relatively few studies have been conducted on the bioregeneration of ACs loaded with binary contaminants. Among them, Aktas and e en (2009, 2010) reported that bioregeneration of AC loaded with non-growth substrates of 2-chlorophenol and 2-nitrophenol was possible in the presence of growth substrate (phenol). Lately, Oh et al. (2016) attempted to quantify the bioregeneration efficiency of binary phenol and 4-chlorophenol (4-CP)-loaded GAC based on the ratio between the sum of the amounts of phenol and 4-CP adsorbed on

bioregenerated GAC with that on fresh GAC. However, they were unable to determine the bioregeneration efficiencies of binary loaded GAC in terms of phenol and 4-CP, respectively.

Furthermore, almost all bioregeneration studies reported thus far employed suspended biomass with the associated problems of biofouling of the adsorbent surface and separation difficulty between the bioregenerated adsorbent and biomass. Toh et al. (2013) reported the use of polyvinyl alcohol (PVA) hydrogel beads encapsulating biomass and powdered activated carbon (PAC) for the bioregeneration of granular activated carbon (GAC) loaded with *o*-cresol and phenol, respectively. Although the use of PVA hydrogel-immobilized biomass beads in bioregeneration could address the problems associated with the use of suspended biomass as stated above, it suffers from poor bead stability and reusability (See et al. 2015).

In light of the above observations, the objectives of this study were to (i) develop the PVA-immobilized biomass cryogels to be used in the bioregeneration of binary phenol and 4-CP-loaded GAC, (ii) determine the bioregeneration efficiency of the binary-loaded GAC in terms of phenol and 4-CP as growth and non-growth substrates, respectively, and (iii) investigate the effects of operational factors, namely dry biomass density in cryogel beads, shaking speed and initial concentration ratio of phenol to 4-CP on the bioregeneration efficiency of binary-loaded GAC.

In this study, the PVA-immobilized biomass cryogel beads were prepared as they have proven to be of biotechnological interest and possess definite advantages when compared to other hydrogels which are commonly used for the same purposes (Lozinsky and Plieva 1998; Lozinsky et al. 2003). The advantages of cryogels include interconnected macropores that allow unhindered diffusion of solutes and have very high operational stability (Lozinsky and Plieva 1998). Bioregeneration of binary-loaded GAC was carried out under the simultaneous adsorption and biodegradation approach which simulates the reusability of GAC in systems such as the biological activated carbon and adsorbent-supplemented sequencing batch reactor (SBR).

Materials and methods

Adsorbent and chemicals

This research employed thermally activated coal-based GAC (Shirasagi X7100 H Dry) purchased from EnviroChemicals Ltd., Japan. The GAC was first sieved carefully into sizes of 10–20 mesh and kept in the oven overnight at 103 °C to remove moisture before transferring into a desiccator before use. A summary of the characteristics of GAC is given in Supplementary Materials I. Phenol and 4-CP (Merck) were of reagent grade with more than 98% purity whereas PVA (MW = 89,000–98,000 g/mol, >99% hydrolysed, Sigma brand) and other chemicals used were of analytical grade. All the chemicals were used without further purification.

Cultivation of biomass acclimated to both phenol and 4-CP

Initially, the seed of biomass was obtained from the activated sludge of a municipal wastewater treatment plant. The biomass acclimated to the binary phenolic compounds of phenol and 4-CP was cultured in a SBR with a working and exchange volumes of 12 and 8 L, respectively. The SBR was operated with FILL, REACT, SETTLE, DRAW and IDLE periods in the time ratio of 1:16:2:1:4 for a 24-h cycle time. The sludge age of biomass was controlled at 30 days. In each cycle, the SBR was initially fed with the base mix containing (in mg/L) sucrose (563), peptone (188), $FeCl_3 \cdot 6H_2O$ (49.4), $CaCl_2$ anhydrous (105), $MgSO_4$ (64.3), $NaHCO_3$ (465), $(NH_4)_2SO_4$ (557), KH_2PO_4 (84) and K_2HPO_4 (473). Sucrose and peptone were gradually replaced by both 4-CP and phenol as the substrates in increasing concentrations until the concentrations of both phenolic compounds attained 300 mg/L. A duration of 6 months was required to ensure that the binary acclimated biomass was ready for biodegradation and bioregeneration studies. Once the SBR had attained the steady state, the mixed liquor suspended solids (MLSS) concentration and the pH of mixed liquor were determined and found to be around 2500 mg/L and 7.4 ± 0.2, respectively.

Preparation of immobilized biomass in cryogel beads

Approximately 400 mL of mixed liquor was collected from the SBR at the end of the REACT period and allowed to settle. The settled biomass of about 40 mL in volume was then blended with the polymeric matrix with pre-dissolved 8 g of PVA and 1 g of sodium alginate to constitute 100 mL of mixture. Immobilized biomass beads of about 4–5 mm in diameter were formed when the mixture was extruded into saturated calcium chloride solution. The gel beads formed were allowed to mix gently for about 4 h for complete cross linking. Subsequently, the beads were washed with distilled water under aeration to remove excess unpolymerized chain molecules. In the preparation of cryogel beads, the formed beads were transferred carefully into an iron tray and frozen at 20 °C for 18 h followed by thawing at 4 °C for 6 h. The process of freezing and thawing was done in 2 cycles (each took 24 h) to improve the mechanical strength of the cryogel beads. After being aerated in nutrient solution for 12 h, cryogel beads of 0.01 g/mL in dry biomass density were ready to be used.

Adsorption studies

Single adsorbate system

A series of amber glass vessels containing 0.05 g of GAC with various concentrations of phenol and 4-CP, respectively, from 50 to 500 mg/L and in the presence of nutrients were shaken on an orbital shaker (IKA KS Basic 260) at 250 rpm. The contact time required for both phenol and 4-CP to attain the equilibrium was determined to be 24 h. At the end of the shaking period, the vessels were removed from the shaker, and the residual concentrations of phenol and 4-CP were determined using HPLC. The amounts of phenol and 4-CP adsorbed were calculated by employing the following equation:

$$Q_t = \frac{(C_0 - C_t)^* V}{m} \quad (1)$$

where C_0 and C_t are the concentrations initially and at time t, respectively, V is the volume of sample and m is the weight of GAC.

Binary adsorbate system

A series of amber glass reaction vessels each of which containing 0.05 g of fresh SAC and 50.0 mL of the binary solution of phenol and 4-CP binary at the phenol to 4-CP concentration (in mg/L) ratio of 100:100 in the presence of nutrients were shaken at 250 rpm for 24 h. At a specific time interval, a reaction vessel was removed from the shaker and the concentrations of phenol and 4-CP in the solution were determined, respectively, using HPLC. The amounts of phenol and 4-CP adsorbed were calculated, respectively, using Eq. (1).

The same procedure was repeated for binary solutions at the phenol to 4-CP concentration (in mg/L) ratios of 100:300, 200:300, 300:300, 300:200 and 300:100, respectively, in the presence of nutrients at the same GAC dosage.

Bioregeneration efficiencies of binary phenol- and 4-CP-loaded GAC

Determination

Batch studies were conducted to study the bioregeneration of GAC loaded with both phenol and 4-CP using immobilized and suspended biomasses, respectively, of about the same dried cell concentration. Exactly 0.05 g of GAC was weighed and placed in each of a series of amber glass reaction vessels. For biotic experiments, 50.0 mL of solution containing different concentration ratios of 4-CP to phenol in nutrients and 5 mL of cryogel beads of dry biomass density of 0.01 g/mL were then added into each of these reaction vessels. Subsequently, the reaction vessels were shaken at 250 rpm using an orbital shaker (IKA KS Basic 260) at 25 ± 1 °C. Concurrently, for each experiment, samples containing GAC and binary compounds of 4-CP and phenol (abiotic experiment) as well as binary compounds only (blank experiment) were shaken under the same conditions.

At an appropriate time interval, the reaction vessel was removed from the orbital shaker and the solution was analysed for the determination of the residual concentrations of phenol and 4-CP using HPLC. For immobilized biomass, the cryogels were easily separated from the bioregenerated GAC by means of physical separation while for suspended biomass, the bioregenerated GAC could easily be separated from the solution via filtration through a membrane filter. At the end of bioregeneration studies, phenol and 4-CP remaining on the bioregenerated GAC were Soxhlet extracted using methylene chloride as the solvent extractor. The bioregeneration efficiency (BE) of GAC in terms of phenol or 4-CP was then calculated using Eq. (2).

$$\mathrm{BE}(\%) = \frac{Q_f - Q_b}{Q_f} \times 100 \quad (2)$$

where Q_f and Q_b are the amounts of extractable phenol and 4-CP for fresh and bioregenerated GAC, respectively.

Effect of dry biomass density in cryogels

The bioregeneration efficiency of GAC loaded with both 4-CP and phenol was determined using PVA-immobilized biomass cryogel beads with dry biomass density varying from 0.00125 to 0.01 g/mL. For the experiment, 5 mL of the cryogel beads was shaken at 250 rpm with 0.05 g of GAC and 50.0 mL of a binary mixture consisting of 200 mg/L of phenol and 4-CP, respectively, in each of a series of reaction vessels at 25 ± 1 °C. At various time intervals, the residual concentrations of phenol and 4-CP in the supernatant and the adsorbed amounts of phenol and 4-CP on GAC were determined following the procedure described earlier. At the end of bioregeneration, the bioregeneration efficiency was determined using Eq. (2). In this study, the limit of dry biomass density in cryogel beads was found to be 0.01 g/mL. Further increase of the biomass density resulted in leaching of biomass into the bulk solution.

Effect of shaking speed

The bioregeneration efficiency of binary phenol and 4-CP-loaded GAC at a fixed phenol to 4-CP concentration (in mg/L) ratio of 200:200 was investigated at the shaking speeds of 150, 250 and 350 rpm, respectively, using the same binary solution at different dry biomass densities in cryogels. The optimum shaking speed and dry biomass density in the

cryogels, which were determined to be 250 rpm and 0.01 g/mL, respectively, were used for the rest of the bioregeneration studies.

Effect of initial concentration ratio of phenol to 4-CP

The effect of the initial concentration ratio of phenol to 4-CP on the bioregeneration of binary-loaded GAC was investigated at five different phenol to 4-CP concentration (in mg/L) ratios of 100:300, 200:300, 300:300, 300:200 and 300:100 using suspended biomass and PVA-immobilized biomass cryogels, respectively. Exactly 0.05 g of GAC was weighed and shaken with 50.0 mL of the binary solution of phenol and 4-CP at the desired concentration ratio and 5 mL of immobilized biomass cryogel beads in each of a series of reaction vessels at 25 ± 1 °C. At suitable time intervals, a vessel was removed from the shaker for the determination of the residual concentrations of phenol and 4-CP in the supernatant and the amounts of adsorbed phenol and 4-CP on GAC. The bioregeneration efficiencies of GAC in terms of phenol and 4-CP were calculated using Eq. (2).

Concurrently, studies on the effect of the initial concentration ratio of phenol to 4-CP on the bioregeneration of binary-loaded GAC using suspended biomass having the same biomass concentration as the cryogel beads were carried out following the procedure as described above.

All experiments were performed in duplicate, and the average values were taken and reported here. The maximum deviation observed was less than 2%.

Scanning electron microscopic analysis

The surface morphology of fresh and bioregenerated GAC prepared using suspended biomass and immobilized biomass cryogels, respectively, was investigated using SEM (Leo Supra 50 VP). The bioregenerated GAC sample was collected at various time intervals (2 and 12 h for suspended biomass and 2, 12 and 24 h for immobilized biomass) during the bioregeneration process. All samples were gold-coated, and the specimen surface was non-destructively bombarded by a finely focused beam of electrons to produce the SEM images which showed mainly the outer surface details. Various magnifications of the images were captured.

Analytical methods

The concentrations of phenol and 4-CP in the bulk solution were determined by HPLC/UV (Prostar, Varian) using a C_{18} column as the stationary phase and a mixture of methanol and H_2O (50:50, in vol.) as the mobile phase. The flow rate was maintained at 1.0 mL/min and the wavelength of 280 nm was used. For the determination of bioregeneration efficiencies, the adsorbed amounts of phenol and 4-CP on the bioregenerated GAC were determined by using a gas chromatograph equipped with FID utilizing a HP-5 column (length 30 m, I.D. 0.32 mm, film thickness 0.25 μm). Helium was used as the carrier gas, and the flow rate was set at 20 cm/s. The inlet and detector temperatures were both set at 250 °C. The oven temperature was held at 100 °C for 1 min and subsequently increased to 160 °C at 10 °C/min. Other quantitative determinations such as OD_{600} and MLSS concentration were based on the Standard Methods (APHA 1998).

Results and discussion

Adsorption studies

Single adsorbate system

Two adsorption isotherm models were considered, namely Langmuir and Freundlich isotherms. The Langmuir isotherm model was employed to describe the adsorption equilibrium for activated carbon and is shown in Eq. 3.

$$Q_t = \frac{Q_m K_L C_t}{1 + K_L C_t} \tag{3}$$

where Q_m (mg/g) is the Langmuir maximum monolayer capacity, C_t (mg/L) and Q_t (mg/g) are the residual adsorbate concentration in the bulk solution and the amount of adsorbed adsorbate per unit weight of adsorbent at equilibrium, respectively, and K_L (L/mg) is the Langmuir constant related to the affinity of the binding sites.

Meanwhile, the Freundlich isotherm model is an empirical relationship between the concentrations of a solute on the surface on an adsorbent to the concentration of the solute in the liquid with which it is in contact. The model is commonly used to describe the adsorption characteristics for heterogeneous surfaces. The Freundlich model is shown as (Freundlich 1906)

$$Q_t = K_F C_t^{1/n} \tag{4}$$

The batch adsorption experiments were conducted by determining the equilibrium concentrations of phenol and 4-CP, respectively, at various initial concentrations ranging from 50 to 600 mg/L. The adsorption equilibrium data were fitted to the Langmuir and Freundlich isotherms by non-linear regression using Matlab version 7.8.0 (Fig. 1). The Langmuir and Freundlich isotherm parameters are tabulated in Table 1. The values of the coefficient of determination (R^2) for equilibrium data of phenol and 4-CP onto both GACs fitted well to both Langmuir and Freundlich isotherms with both having the R^2 values of more than 0.98. These show that phenol and 4-CP

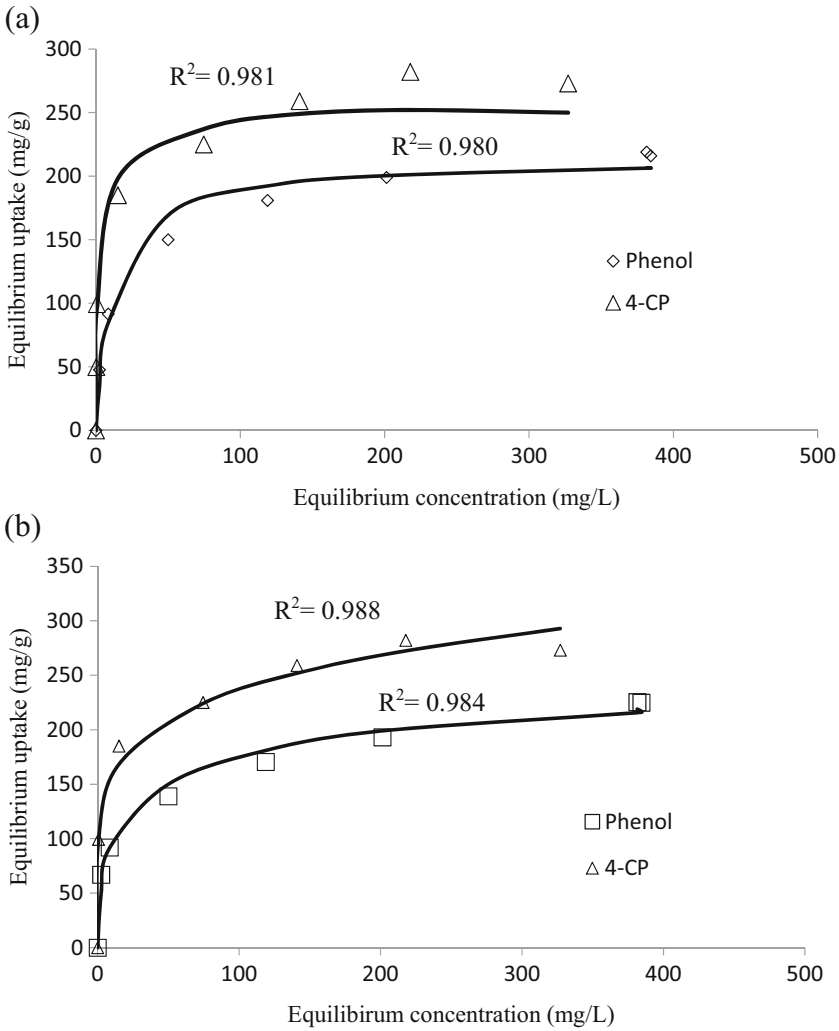

Fig. 1 **a** Langmuir and **b** Freundlich isotherms for phenol and 4-CP adsorption onto GAC. The *straight lines* and *symbols* denote the calculated and experimentally fitted data, respectively

molecules would adsorb on either the homogeneous or heterogeneous active sites at a given time. For kinetic modelling of bioregeneration process, the Q_m value of the Langmuir isotherm was employed because the values were based on the values at equilibrium.

The Q_m values generated for phenol and 4-CP based on Eq. 3 were 213 and 251 mg/g, respectively. The results are consistent with the solubility trend whereby 4-CP is less soluble than phenol and tends to be more readily adsorbed on GAC. The higher K_L values for 4-CP (1.00 L/mg) as compared to phenol (0.077 L/mg) suggests that 4-CP has a higher affinity for adsorption onto GACs than phenol, and the results are in agreement with the K_F values which is an indicator for the adsorption capacity for phenol and 4-CP onto GACs. Meanwhile, the values for $1/n$ were 0.24 and 0.18 for phenol and 4-CP, respectively, and this indicates that the adsorption of phenol and 4-CP onto GACs are favourable.

Binary adsorbate system

The competitive abiotic adsorption studies were carried out at different concentration ratios of phenol to 4-CP to determine the respective amount adsorbed on GAC. Figure 2 shows the removal of phenol and 4-CP from the binary solutions of

Table 1 Langmuir and Freundlich isotherm parameters for the adsorption of phenol and 4-CP onto GAC

Adsorbate	Langmuir			Freundlich		
	Q_m (mg/g)	K_L (L/mg)	R^2	K_F (mg/g)	$1/n$ (L/g)$^{1/n}$	R^2
Phenol	213	0.077	0.981	54.2	0.24	0.988
4-CP	251	0.999	0.980	103	0.18	0.984

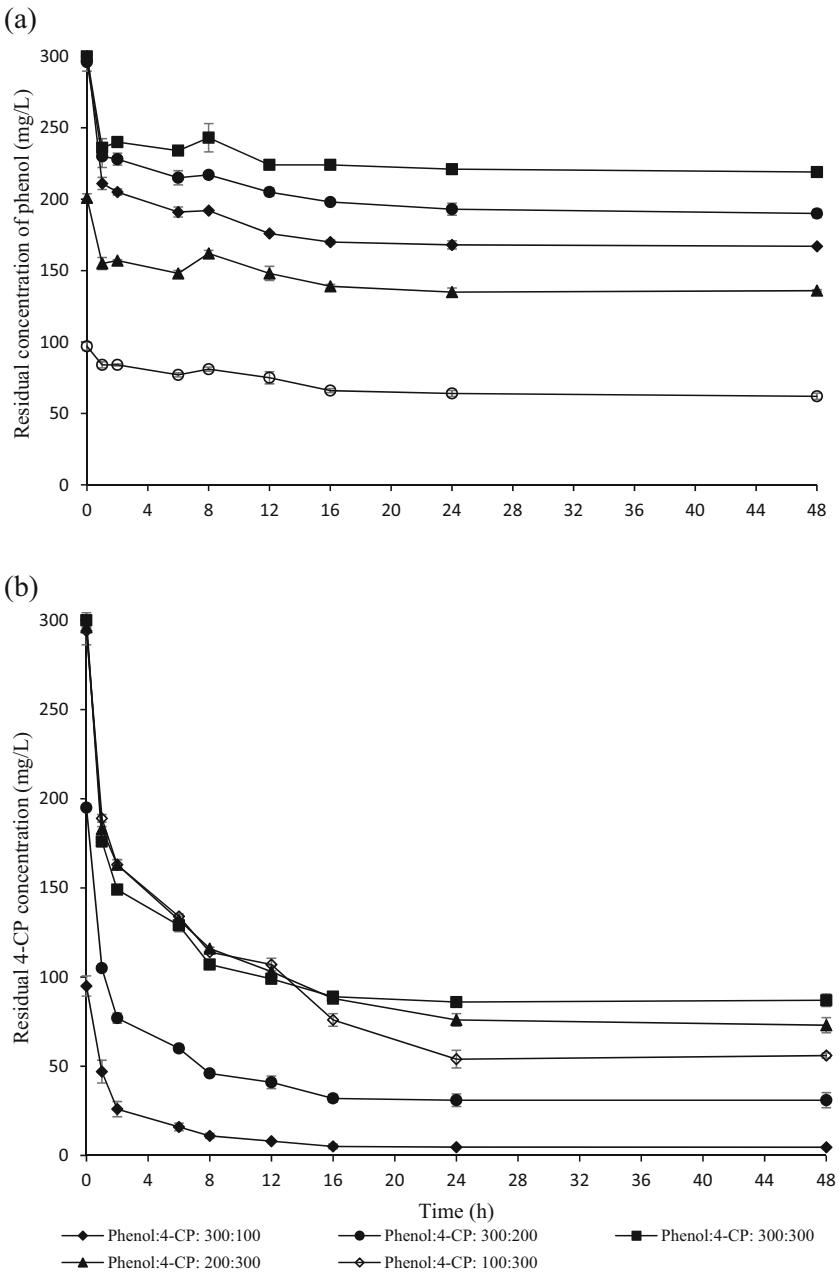

Fig. 2 Time courses of residual **a** phenol and **b** 4-CP concentrations at different initial phenol to 4-CP concentration ratios in abiotic binary adsorption studies

phenol and 4-CP that were shaken with GAC of 1.0 g/L dosage at the phenol to 4-CP concentration (in mg/L) ratios of 300:100, 300:200, 300:300, 200:300 and 100:300, respectively, and the amounts of phenol and 4-CP adsorbed are shown in Table 2. The reason for investigating the concentration ratios up to 300 to 300 mg/L only was because the binary acclimated activated sludge was adapted to only 300 mg/L of both phenol and 4-CP. It was observed that irrespective of any concentration ratio, the percentage of 4-CP removal was always greater than that of phenol. The higher sorption capacity of 4-CP over phenol could be attributed to the polarity of the chloride group attached to 4-CP as suggested by Garcia-Mendieta et al. (2003). The presence of chloride as the electron withdrawing group will enhance the π-π interactions by reducing the electron density of the aromatic ring thus rendering higher adsorption capacity (Li et al. 2010).

From Table 2, it could be observed that the percentage of removal for phenol and 4-CP decreased as the phenol to 4-CP concentration (in mg/L) ratio changed from 300:100 to 300:200 and to 300:300, respectively. This could be attributed to the toxicity effect of 4-CP exerted on the performance of the biomass (Oh et al. 2011). However, the slight increase in the percentage removal of both phenol and 4-CP as the phenol to 4-CP concentration (in mg/L) ratio varied from 300:300 to 200:300 and to 100:300 could be due to the better biodegradability of phenol as compared to 4-CP (Aktaş and Çeçen

Table 2 Amounts of phenol and 4-CP adsorbed onto GAC at different initial concentration ratios of phenol to 4-CP in abiotic binary adsorption studies

Phenol/4-CP	q_{phenol} (mg/g)	$q_{4\text{-}CP}$ (mg/g)	% phenol removed	% 4-CP removed
300:100	136 ± 2	90 ± 0	45 ± 1	95 ± 0
300:200	102 ± 4	164 ± 1	34 ± 2	84 ± 0
300:300	79 ± 3	213 ± 2	26 ± 2	71 ± 1
200:300	64 ± 2	222 ± 2	32 ± 1	75 ± 2
100:300	33 ± 2	239 ± 1	34 ± 2	81 ± 1

2009). The highest removal was obtained for both phenol and 4-CP when the 4-CP concentration was the lowest, i.e. 300:100.

Bioregeneration of GAC loaded with binary adsorbates of phenol and 4-CP

Under the simultaneous adsorption and biodegradation approach, the effects of dry biomass density in cryogel beads and shaking speed on the bioregeneration of GAC loaded with both phenol and 4-CP were investigated using immobilized biomass whereas the effect of the initial concentration ratio of phenol to 4-CP was studied using both suspended and immobilized biomasses of the same concentration.

Effect of dry biomass density in cryogel beads

The time courses of the residual phenol and 4-CP concentrations during the bioregeneration process using immobilized biomass with the dry biomass density varying from 0.00125 to 0.01 g/mL are shown in Fig. 3a, b, respectively. It was observed that, in contrast to the abiotic runs, complete

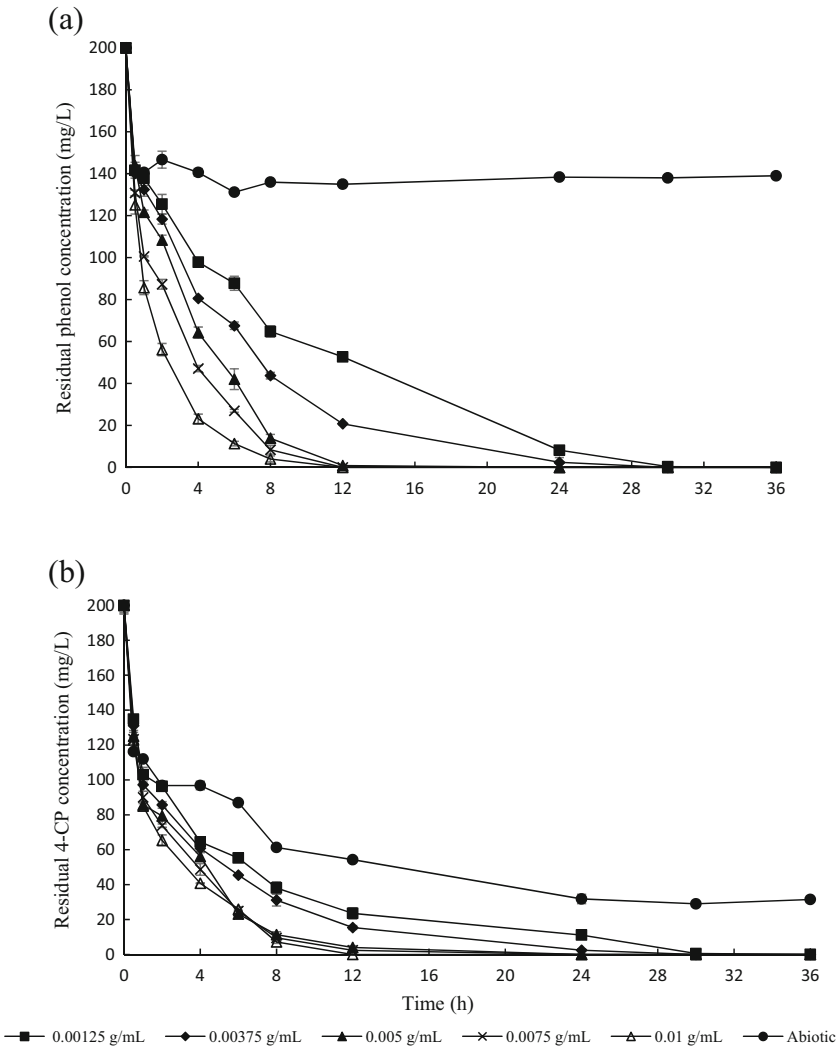

Fig. 3 Time courses of residual **a** phenol and **b** 4-CP concentrations during bioregeneration of binary phenol and 4-CP-loaded GAC using PVA-immobilized biomass cryogels of different dry biomass densities at shaking speed of 250 rpm and initial phenol to 4-CP concentration (in mg/L) ratio of 200:200

removal of phenol and 4-CP was achieved in the biotic runs. This was due to the rapid adsorption of phenol and 4-CP onto GAC during bioregeneration initially thus reducing the toxicity of phenol and 4-CP in the bulk solution. With respect to the dry biomass density, it was observed that the time taken for complete phenol and 4-CP removal decreased from 36 to 12 h when the dry biomass density in the cryogel beads was increased from 0.00125 to 0.01 g/mL. Therefore, higher dry biomass density in cryogel beads would facilitate faster removal of phenol and 4-CP.

The bioregeneration efficiency was calculated using Eq. (2), and the mean bioregeneration efficiencies of binary-loaded GAC in terms of phenol and 4-CP, respectively, using cryogel beads of different dry biomass densities and shaking speeds at the initial phenol to 4-CP concentration (mg/L) ratio of 200:200 are shown in Table 3. It was observed that at the shaking speed of 250 rpm, the bioregeneration efficiency of GAC in terms of phenol increased moderately from 52 ± 4 to 75 ± 4% whereas that in terms of 4-CP increased more drastically from 16 ± 1 to 70 ± 3% when the dry biomass density in cryogel beads was increased from 0.00125 to 0.01 g/mL. A plausible reason for the mean bioregeneration efficiency of loaded GAC for 4-CP being lower than for phenol at lower dry biomass densities of 0.00125 and 0.00375 g/mL would be the occurrence of greater extent of oxidative polymerization for 4-CP than for phenol (Aktaş and Çeçen 2007) at lower dry biomass density. Chloro- and methyl-substituted phenols have been demonstrated to undergo oxidative polymerization on the carbon surface in the presence of molecular oxygen resulting in irreversible adsorption (Grant and King 1990; Vidic et al. 1993). A similar observation of lower bioregeneration efficiencies for chlorophenol than for phenol using suspended biomass was reported by other researchers on binary loaded systems (Aktaş and Çeçen 2007; Ha et al. 2001; Vinitnantharat et al. 2001). However, at higher dry biomass densities of 0.005 to 0.01 g/mL, the bioregeneration efficiencies for phenol and 4-CP were found to be comparable. This was likely due to the desorbed 4-CP being degraded more rapidly at higher dry biomass density thus shortening the contact period between the GAC surface with 4-CP in the solution leading to a reduction in the extent of oxidative polymerization and adsorption irreversibility. In essence, both the bioregeneration efficiencies of binary-loaded GAC in terms of phenol and 4-CP could be enhanced by increasing the dry biomass density in cryogel beads.

Table 3 Bioregeneration efficiencies of binary phenol- and 4-CP-loaded GAC in terms of phenol and 4-CP, respectively, at different dry biomass densities in cryogel beads and shaking speeds

Dry biomass density (g/mL)	Shaking speed (rpm)	Final amount extracted from GAC (mg/g)		Bioregeneration efficiency (%)	
		Phenol	4-CP	Phenol	4-CP
0.00125	150	33 ± 5	150 ± 3	46 ± 6	10 ± 5
	250	29 ± 2	140 ± 1	52 ± 4	16 ± 1
	350	32 ± 3	139 ± 1	48 ± 5	17 ± 1
0.00375	150	31 ± 4	125 ± 6	49 ± 5	25 ± 5
	250	27 ± 2	108 ± 5	56 ± 4	35 ± 3
	350	29 ± 3	113 ± 4	52 ± 5	32 ± 2
0.005	150	28 ± 2	87 ± 2	54 ± 3	48 ± 1
	250	22 ± 4	65 ± 3	64 ± 7	61 ± 2
	350	25 ± 2	91 ± 3	59 ± 3	46 ± 2
0.0075	150	25 ± 2	67 ± 6	59 ± 3	60 ± 6
	250	19 ± 2	48 ± 4	69 ± 3	71 ± 3
	350	23 ± 1	69 ± 2	62 ± 2	59 ± 2
0.01	150	19 ± 3	64 ± 2	69 ± 5	62 ± 2
	250	15 ± 2	50 ± 4	75 ± 4	70 ± 3
	350	17 ± 3	70 ± 3	72 ± 5	58 ± 2

Effect of shaking speed

Figure 4 shows the time courses of the residual concentrations of phenol and 4-CP at the shaking speeds of 150, 250 and 350 rpm, respectively, for the dry biomass density varying from 0.00125 to 0.01 g/mL. It was observed that irrespective of the dry biomass density employed, the removal of phenol and 4-CP was the slowest at the shaking speed of 150 rpm. Table 3 shows that the highest bioregeneration efficiencies of binary adsorbate-loaded GAC in terms of phenol and 4-CP were achieved at the shaking speed of 250 rpm. At a lower shaking speed of 150 rpm, it is envisaged that incomplete mixing among the cryogel beads, binary solution and GAC hindered the occurrence of the adsorption, desorption and biodegradation during the bioregeneration process. On the other hand, higher shaking speed at 350 rpm would create excessive sheer force resulting in a reduction of the cellulose productivity in the bacterium cells (Gusek et al. 1991). Apparently, the employment of a moderate shaking speed of 250 rpm provided adequate mixing of the key components and oxygen availability for biodegradation thus enhancing the bioregeneration efficiency.

Effect of initial concentration ratio of phenol to 4-CP

Figure 5 shows the time courses of residual phenol and 4-CP concentrations during the bioregeneration of binary phenol and 4-CP-loaded GAC at the initial phenol to 4-CP concentration (in mg/L) ratios of 300:100, 300:200 and 300:300, respectively, at the GAC dosage of 1 g/L using suspended biomass whereas Fig. 6 shows the time courses of residual phenol and 4-CP concentrations using immobilized biomass, respectively. The results clearly show that the rates of phenol and 4-CP removal were faster using suspended biomass than immobilized biomass. This could be explained by the fact that the immobilized biomass, unlike the suspended biomass, was not in direct contact with the binary adsorbates due to the presence of the immobilization matrix.

For phenol removal, comparison of the time courses of residual phenol concentration (Figs. 5a and 6a) shows that

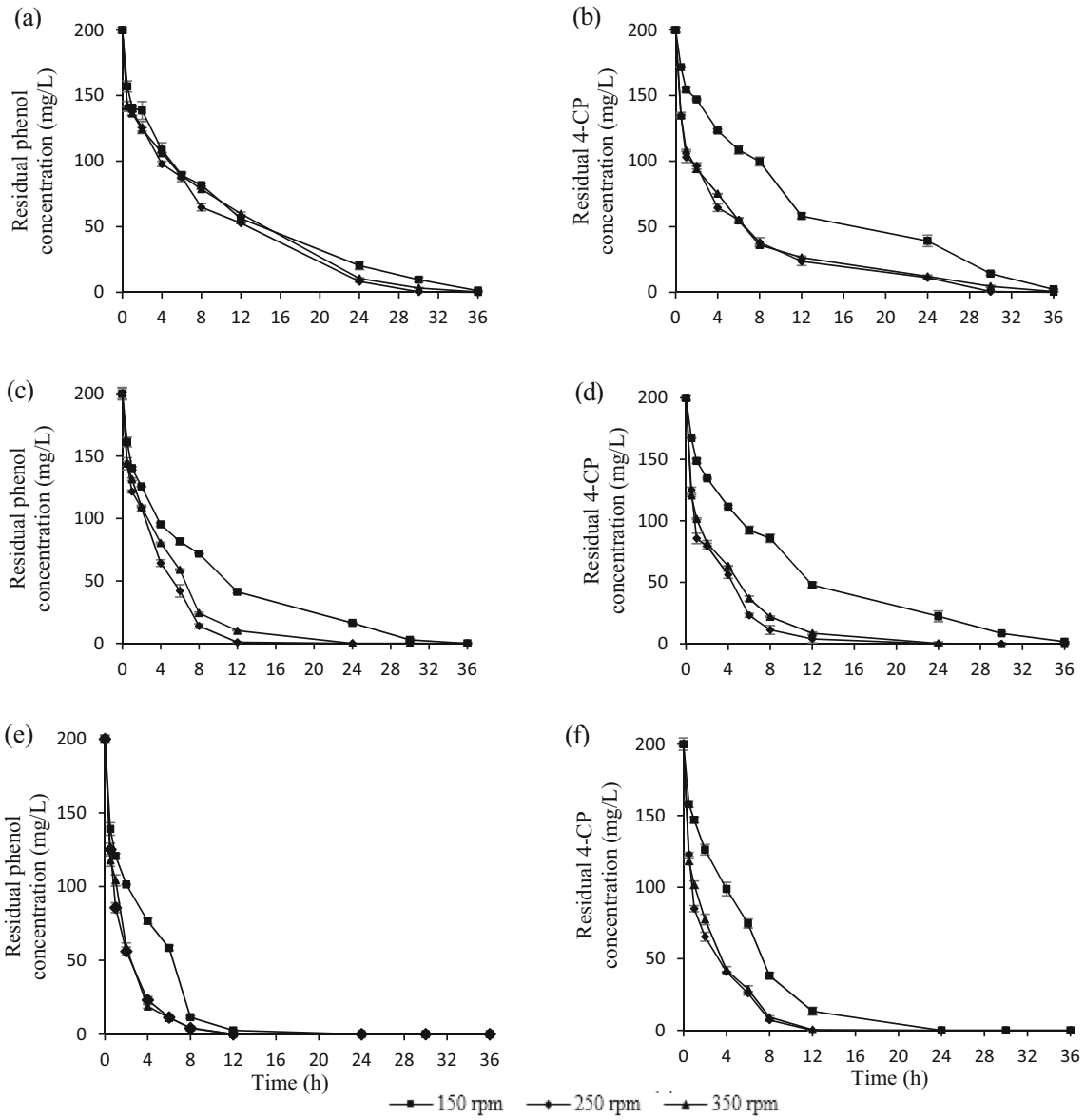

Fig. 4 Time courses of residual phenol and 4-CP concentrations during bioregeneration using cryogels with dry biomass density of 0.00125 (**a** and **b**), 0.005 (**c** and **d**) and 0.01 g/mL (**e** and **f**) at various shaking speeds

the rate of phenol removal was almost the same at the initial concentration ratios of 300:100 and 300:200, but the rate was the slowest at a higher 4-CP concentration of 300 mg/L using both suspended and immobilized biomasses. This could be attributed to the toxicity effect of 4-CP at higher concentration on the bioactivity of biomass (Oh et al. 2011) and higher residual phenol concentration at the concentration ratio of 300:300 based on the results of the abiotic adsorption studies (Fig. 2a). Likewise, for 4-CP removal, the rate of removal was the slowest at the concentration ratio of 300:300 (Figs. 5 and 6b) due to the relatively higher residual 4-CP concentration as shown in the abiotic adsorption studies (Fig. 2b).

In the cases of the effect of the initial phenol to 4-CP concentration (in mg/L) ratio with fixed 4-CP but varying phenol concentrations, namely 300:300, 200:300 and 100:300 on the bioregeneration of the binary adsorbate-loaded GAC at 1 g/L GAC dosage using suspended and immobilized biomasses, Figs. 5c and 6c show the time courses of residual phenol concentration during bioregeneration whereas Figs. 5d and 6d show the time courses of residual 4-CP concentration. It was observed that the time taken for the removal of both phenol and 4-CP during bioregeneration was faster using suspended biomass than immobilized biomass, a result in agreement with what was observed earlier. Figures 5c and 6c show that the time required for phenol removal was the longest due to higher residual phenol concentration at the phenol concentration of 300 mg/L as shown in the abiotic adsorption studies (Fig. 2a). For 4-CP removal, Figs. 5d and 6d show that

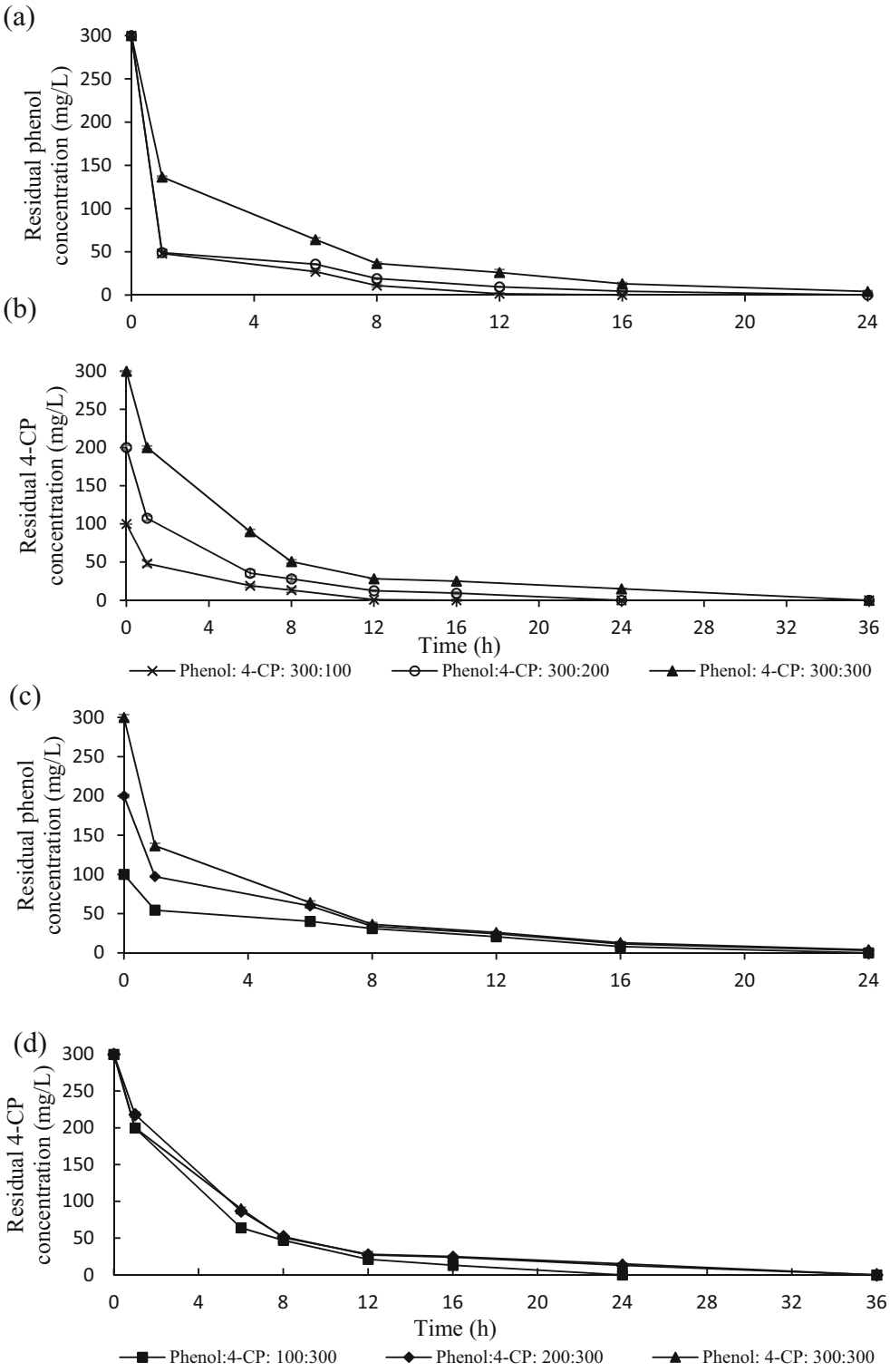

Fig. 5 Time courses of residual concentrations of phenol (**a**, **c**) and 4-CP (**b**, **d**) during the bioregeneration of binary phenol- and 4-CP-loaded GAC using suspended biomass at different initial phenol to 4-CP concentration ratios

the rate of 4-CP removal was almost the same irrespective of the varying phenol concentration. It was therefore evident that 4-CP removal during bioregeneration was only dependent on the initial 4-CP concentration.

Table 4 shows the bioregeneration efficiencies of binary phenol and 4-CP-loaded GAC for phenol and 4-CP, respectively, at a different concentration ratio of phenol to 4-CP using suspended and immobilized biomasses. It was observed that, irrespective of the forms of biomass used, the bioregeneration efficiency for phenol was always higher than for 4-CP. This could be explained by a higher binding affinity, K_L, for 4-CP in comparison to phenol, which contributed to

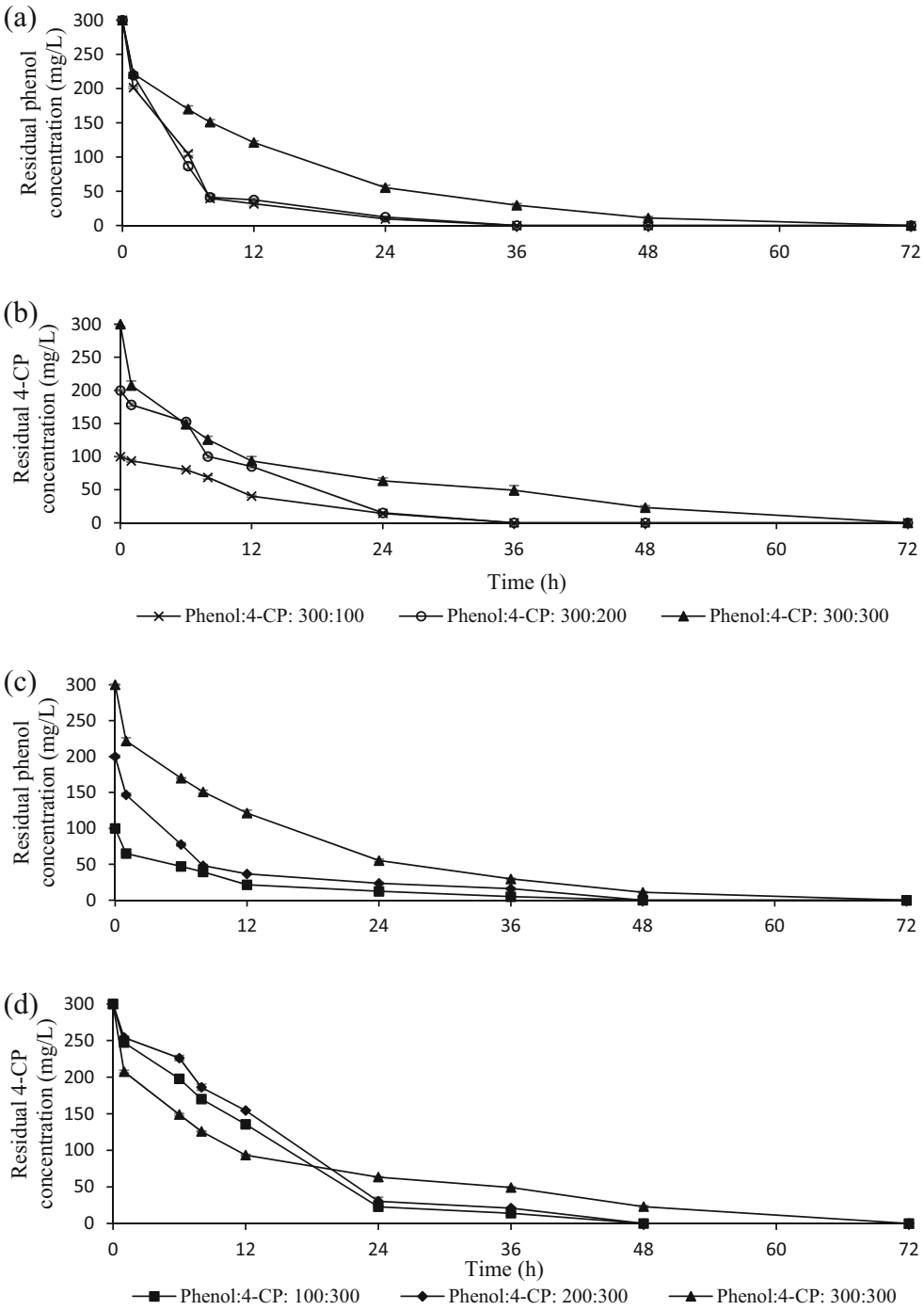

Fig. 6 Time courses of residual concentrations of phenol (**a**, **c**) and 4-CP (**b**, **d**) during the bioregeneration of binary phenol- and 4-CP-loaded GAC using immobilized biomass at different initial phenol to 4-CP concentration ratios

higher degree of irreversible adsorption. Higher bioregeneration efficiencies of binary phenol and 4-CP-loaded GAC if immobilized biomass rather than suspended biomass was used could be explained by the obstruction of GAC pores in the presence of biomass slime matrix that could reduce the adsorption capacities when suspended biomass was used (Aktaş and Çeçen 2009). Comparison of the bioregeneration efficiencies of binary-loaded GAC at different initial phenol to 4-CP concentration ratios in Table 4 shows that the lowest bioregeneration efficiencies of GAC for both phenol and 4-CP were attained at the phenol to 4-CP concentration ratio of 300:300. This result is hardly surprising in view of the observation that the contact period between phenol and 4-CP with GAC surface was the longest (see Figs. 5 and 6) resulting in higher degree of irreversibility. Overall, it was observed that the bioregeneration efficiencies became lower under increasing phenol and 4-CP initial concentrations, respectively, with the effect more conspicuous under increasing 4-CP concentration.

Table 4 Bioregeneration efficiencies of binary loaded GAC in terms of phenol and 4-CP using suspended and PVA-immobilized biomasses, respectively, at different initial phenol to 4-CP concentration ratios

Initial phenol concentration (mg/L)	Initial 4-CP concentration (mg/L)	Final amount of extracted (mg/g)				Bioregeneration efficiencies (%)			
		Suspended biomass		Immobilized biomass		Suspended biomass		Immobilized biomass	
		Phenol	4-CP	Phenol	4-CP	Phenol	4-CP	Phenol	4-CP
300	100	26 ± 3	23 ± 4	21 ± 2	20 ± 1	81 ± 2	75 ± 5	85 ± 2	78 ± 1
300	200	24 ± 2	57 ± 2	19 ± 3	45 ± 3	77 ± 2	64 ± 1	82 ± 3	72 ± 2
300	300	19 ± 1	91 ± 5	14 ± 2	74 ± 4	70 ± 2	55 ± 3	78 ± 3	63 ± 2
200	300	18 ± 2	84 ± 4	12 ± 1	70 ± 2	72 ± 3	58 ± 2	82 ± 2	65 ± 1
100	300	8 ± 1	76 ± 4	6 ± 0	63 ± 4	77 ± 3	67 ± 2	83 ± 0	73 ± 2

SEM morphology

Comparison was made between the SEM images of fresh GAC and bioregenerated GAC to investigate the probable presence of attached biomass (Wang and Loh 2000). From the SEM images, the colonization of the cocci and filamentous microorganism in the pores of GAC was observed at the early stages of bioregeneration (see Supplementary Material IIb). At 12 h of bioregeneration, GAC exposed to suspended biomass was found to have greater degree of microorganism colonization which eventually clogged up the GAC pores (see Supplementary Material IIc). However, colonization by microorganisms was not observed when immobilized biomass was employed in the bioregeneration process (see Supplementary Material IId). This provides strong evidence that the immobilized biomass can retain the biomass in the polymeric gel matrix thus preventing the attachment of biomass onto the GAC surface and pores. This allows the bioregenerated GAC to be used for further adsorption in multiple cycles.

Conclusions

PVA-immobilized biomass cryogels were prepared for the bioregeneration of binary phenol and 4-CP-loaded GAC under the simultaneous adsorption and biodegradation approach. The optimum dry biomass density in cryogel beads and shaking speed for use in bioregeneration were found to be 0.01 g/mL and 250 rpm, respectively. Studies on the effect of the initial phenol to 4-CP concentration ratio on bioregeneration showed that the efficiencies for phenol and 4-CP became lower under increasing phenol and 4-CP initial concentrations, respectively, with the effect being more conspicuous under increasing 4-CP concentration. Higher bioregeneration efficiencies were achieved with the use of immobilized rather than suspended biomasses.

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