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Biodegradation of *o*-cresol by polyvinyl alcohol–alginate immobilized mixed culture

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ABSTRACT

The performance of polyvinyl alcohol (PVA)–alginate immobilized biomass in the biodegradation of *o*-cresol was evaluated and compared with that of the suspended biomass. The effects of initial *o*-cresol concentration, initial pH, and immobilized biomass bead size on the biodegradation of *o*-cresol were investigated. Compared with the *o*-cresol biodegradation rate by suspended biomass, the biodegradation rate by immobilized biomass was barely affected at higher initial *o*-cresol concentration and extreme pH conditions suggesting that the immobilized biomass was protected by the PVA–alginate matrix. The reusability of the immobilized biomass beads up to three cycles of use could be achieved, but only at relatively lower initial *o*-cresol concentration.

Keywords: Biodegradation; *o*-Cresol; Immobilization; Polyvinyl alcohol; Biomass

1. Introduction

Cresols are often employed in the production of pesticides, epoxy resins, dyes, disinfectants, and pharmaceuticals. They are found not only in leachates from creosote sites but also in a wide range of industrial effluents [1]. Owing to widespread usage, efficient treatment technologies are required to reduce the cresol concentration in wastewater to an acceptable level. Many treatment techniques, including biodegradation and adsorption have been employed in recent years to reduce the cresol concentration in wastewater [2–4]. Yet, biological treatment has so far proved to be the most promising and economical method for the removal of *o*-cresol from wastewater [5]. Studies have shown that the suspended cells are severely inhibited by cresol isomers of concentration up to 1,000 mg/L. Among the cresol isomers, *o*-cresol is more toxic to microbial communities in activated sludge and pure culture than *m*- or *p*-cresol [6,7]. Owing to the high toxicity of *o*-cresol to micro-organisms, its presence in aqueous media is stringently limited by regulations.

The treatment of xenobiotic compounds using immobilized biomass, including encapsulated and biofilm immobilization is receiving increasing research interest [8–12]. The application of biomass immobilization technique has several merits including protection of the biomass from direct exposure of high initial substrate concentrations, ease of separation, and potential reutilization of the biomass [13,14]. Till date, the information on the degradation of *o*-cresol by immobilized

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biomass is limited. Hence, the objective of this study is to evaluate the effects of various operational factors, including the initial *o*-cresol concentration, the pH of the medium, as well as the bead size on the biodegradation of *o*-cresol by mixed culture immobilized in polyvinyl alcohol (PVA)–alginate matrix.

2. Materials and methods

2.1. Chemicals

The *o*-cresol, polyvinyl-alcohol (PVA ~ Mw = 89,000–98,000 g/mol), and sodium alginate were purchased from Merck and Sigma–Aldrich, respectively and used without further purification.

2.2. Culturing of the o-cresol–acclimated mixed culture

A plexiglass sequencing batch reactor (SBR) with a working volume of 10 L was employed to culture the *o*-cresol–acclimated mixed culture. The seeds of the mixed culture were obtained from a local municipal sewage treatment plant. The SBR was operated with Fill, React (aerobic + anoxic), Settle, Draw and Idle periods in the ratio of 2:(9 + 3):1:1:8 for a cycle time of 24 h. In each cycle, 7 L of the synthetic wastewater containing 800 mg/L *o*-cresol as the sole substrate and the nutrients with the following compositions (mg/L): $(NH_4)_2SO_4$ (212), KH_2PO_4 (32), K_2HPO_4 (180), $MgSO_4$ (49), $NaHCO_3$ (525), $FeCl_3{\cdot}6H_2O$ (18.8), and $CaCl_2$ (40) was introduced into the reactor during the Fill period, while the same volume of the treated effluent was removed during the Draw period. The sludge age was controlled for 20 days by wasting an appropriate amount of mixed liquor at the end of the React period in each cycle. The wasted mixed liquor was collected from the reactor for the biomass immobilization studies. When the SBR had attained the steady state condition, the mixed liquor suspended solids (MLSS) concentration was found to be around 5,000 mg/L.

2.3. Biomass immobilization

The acclimated mixed culture was employed as inocula for the biomass immobilization. The mixed culture collected from the SBR was washed repeatedly with distilled water to remove soluble components and centrifuged at 2,500 rpm for 5 min. The centrifuged sludge was then mixed with 2% (w/v) sodium alginate and 10% (w/v) PVA to form a biomass-polymeric solution. The entrapment procedure follows the method described by other researchers [15,16] with some modifications. For the solidification process, the biopolymeric solution was added drop wise into 6% (w/v) calcium chloride solution using a peristaltic pump through a silicon tube fitted with syringe (for 3 mm) and glass droppers (for 4, 5, and 6 mm) of various dimensions to obtain the immobilized biomass beads of different diameter sizes. The beads in the Ca^{2+} solution were incubated at room temperature (~28°C) for 1 h to ensure complete calcification. After that, they were then immersed in 6% (w/v) boric acid solution for another 1 h to allow cross-linking with the PVA. The cross-linked immobilized biomass beads were washed several times with distilled water to remove excess Ca^{2+} ions and boric acid and were stored at 4°C before use.

2.4. Biodegradation of o-cresol by suspended and PVA–alginate immobilized biomass

Batch biodegradation experiments were carried out to evaluate the effect of initial *o*-cresol concentration on the biodegradation of *o*-cresol by suspended and PVA–alginate immobilized biomass, respectively. The batch experiment was conducted by agitating 50 mL of solution containing 300 mg/L of acclimated biomass (suspended or immobilized), nutrients, and different concentrations of *o*-cresol (100–800 mg/L) in 140-mL amber glass reaction vessel at 250 rpm using an orbital shaker. The nutrient solution had the following composition (in mg/L): $(NH_4)_2SO_4$ (212), KH_2PO_4 (32), H_3BO_3 (25), $MgSO_4$ (49), $NaHCO_3$ (525), $FeCl_3{\cdot}6H_2O$ (18.8), and $CaCl_2$ (40). The samples were drawn at regular intervals and analyzed for the concentration of *o*-cresol in the bulk solution. Abiotic experiment was done to evaluate possible *o*-cresol removal by volatilization or other processes. It was found that the removal of *o*-cresol by Abiotic processes can be neglected. All the experiments were conducted in triplicate.

Similar procedure was adopted for the effects of pH medium (from pH 2 to 11), bead size (3, 4, 5, and 6 mm), and biomass concentration in PVA–alginate gel (from 0.5 to 2.5 g wet wt/100 mL gel), respectively on the degradation rate of *o*-cresol. The initial pH of the medium was adjusted by using either 0.01 M HCl or 0.01 M NaOH.

The reusability of the immobilized biomass beads was investigated by repeatedly using the same beads in the biodegradation of *o*-cresol for a number of cycles. After the first cycle of use as described above, the beads were washed with distilled water and transferred into 50 mL solution containing 100 mg/L of *o*-cresol as the sole carbon and nutrients for the subsequent cycle of use. The sampling of the sample was carried out at suitable time intervals. Only three

cycles of use were accomplished as the beads disintegrated after the third cycle of use. The reusability experiment was repeated for the initial *o*-cresol concentrations of 400 and 800 mg/L.

2.5. Morphological observation

For optical analysis, the PVA carrier and PVA–alginate immobilized biomass beads were rinsed with distilled water and examined under the optical microscope (OLYMPUS SZX16, Camera DP72).

For scanning electron microscope (SEM) analysis, the PVA carrier and PVA–alginate immobilized biomass beads were rinsed with distilled water and cut into two halves with a sterile scalpel to obtain the cross-sectional area of the beads. The beads were then exposed to 2% (w/v) osmium tetroxide for 2 h. After that, both the beads were freeze-dried under nitrogen gas atmosphere (−210°C) for another 10 h. The dried beads were coated with gold and examined using a scanning electron microscope (LEO SUPRA 50 VP, FE-SEM).

2.6. Analytical methods

The determinations of *o*-cresol and MLSS concentrations were based on the Standard Methods [17]. The 4-aminoantipyrene method was modified and employed to determine the concentrations of *o*-cresol spectrophotometrically using a UV–Vis spectrophotometer (Thermo Spectronic Heλ10Sα) at λ_{max} of 510 nm. The main route of biodegradation of *o*-cresol was identified to be the one via 3-methylcatechol which further degraded to yield, probably, 2-hydroxy-3-methylmuconic semi-aldehyde with yellow color [18,19]. In this study, the UV–Visible spectra obtained during the biodegradation of *o*-cresol at various time intervals revealed only two peaks, one at 270 nm which had been identified as *o*-cresol and the other, a yellow compound at 385 nm which was probably 2-hydroxy-3-methylmuconic semi-aldehyde. This result indicates that the lifetime of the intermediate phenolic compound of 3-methylcatechol would be relatively too short to interfere with the analysis of *o*-cresol. Even if 3-methylcatechol was present, the absorption peak would appear at 540 nm [20].

3. Results and discussion

3.1. Morphological observation of PVA carrier and PVA-immobilized biomass beads

Fig. 1 shows the optical pictures and SEM microphotographs of the PVA carrier and PVA-immobilized biomass beads. Fig. 1(a) shows the successful entrapped biomass in the PVA carrier. Fig. 1(b) shows

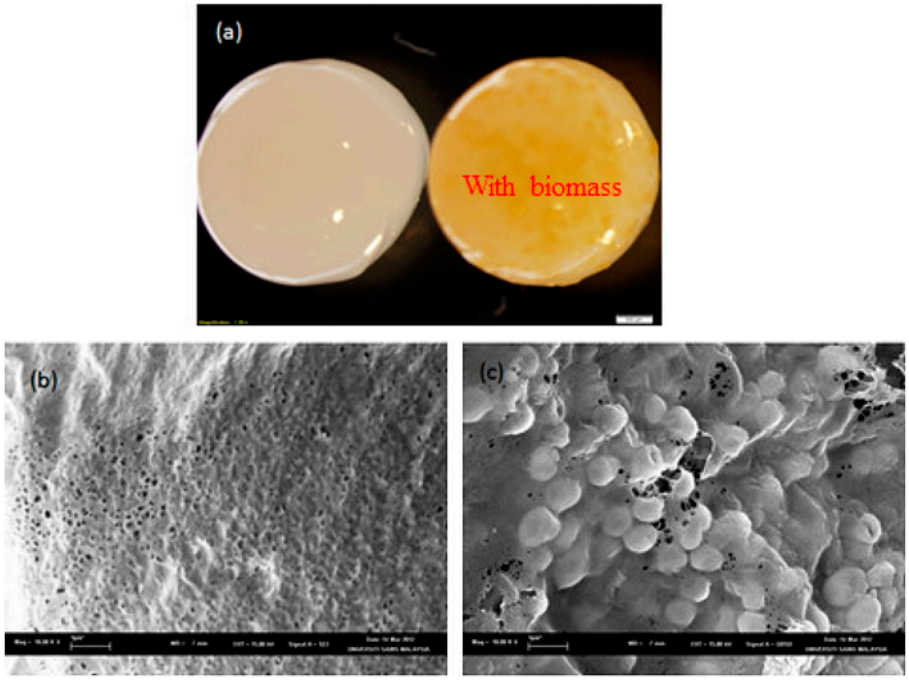

Fig. 1. Optical picture of the PVA–alginate bead with and without biomass (a), SEM images (10 k magnification) of the bead without biomass (b), and with biomass (c).

pores in the PVA carrier indicating that the core of the bead had a porous network structure. This structure provided the pathway for the diffusion of oxygen and toxicant between the external environment and inside the beads. Fig. 1(c) shows the morphological features of biomass in the beads being spherical in shape and successfully entrapped by PVA.

3.2. Effects of various operational factors on the biodegradation rate of o-cresol

3.2.1. Bead size

The effect of beads sizes from 3 to 6 mm on the biodegradation rate of *o*-cresol was evaluated at three different initial *o*-cresol concentrations of 100, 500, and 800 mg/L. The biodegradation rates were determined directly from the slopes of the time courses of *o*-cresol biodegradation by immobilized biomass beads and the results are presented in Fig. 2. It was observed that at the initial *o*-cresol concentration of 100 mg/L, faster rate of *o*-cresol biodegradation was achieved by using progressively smaller beads with 3-mm beads yielding the fastest rate. This finding was consistent with the results of other studies which reported that an increase in particle size caused an increase in diffusion limitations resulting in lower substrate removal rate [21,22]. However, at higher initial *o*-cresol concentrations of 500 and 800 mg/L, the differences in the *o*-cresol biodegradation rates among the four sizes of beads were not so discernible. This indicates that external diffusion as a controlling mechanism is only dominant at low bulk concentration.

The effective factor, η, which represents the extent of diffusion limitation in the immobilized biomass is introduced as the ratio of the biodegradation rate of immobilized biomass (v) with diffusion limitation, and the biodegradation rate of suspended biomass (v_s) without diffusion limitation as shown in Eq. (1).

$$\eta = \frac{v}{v_s} \tag{1}$$

The Thiele modulus, Ø, is related to η as shown in Eq. (2) [21–23]. For small values of Ø, η approaches 1 and the diffusion limitation has no effect on the rate. For Ø > 5 diffusion limitation has a larger effect on the biodegradation rate.

$$\eta = \frac{3}{\varphi}\left[\frac{1}{\tanh\varphi} - \frac{1}{\varphi}\right] \tag{2}$$

As the external diffusion is dominant at low initial substrate concentration, the effect of external diffusion on *o*-cresol biodegradation was kinetically important at the initial *o*-cresol concentration of 100 mg/L wherein there was no significant inhibition. The calculation of the effectiveness factor, η, and the Thiele modulus, Ø, for different bead sizes was done by using Eqs. (1) and (2), respectively and the values of η and Ø are presented in Table 1. It was observed that all the experimental effectiveness factors obtained for the immobilized cells were lower than 1.0 indicating that the biodegradation rate was diffusion dependent. The values of the Thiele modulus generated were much greater than 5 signifying that the overall biodegradation rate was influenced by intraparticle mass transfer resistance [21]. This could be envisaged from the pore structure prepared by the entrapment method which was a series of tortuous, interconnecting pathway of polymeric entanglement. The impenetrable parts of the polymer significantly increase the path length for the movement of *o*-cresol to the biomass for biodegradation resulting in significant mass transfer resistance. Thus, the external diffusion is one of the limiting processes for the biodegradation by

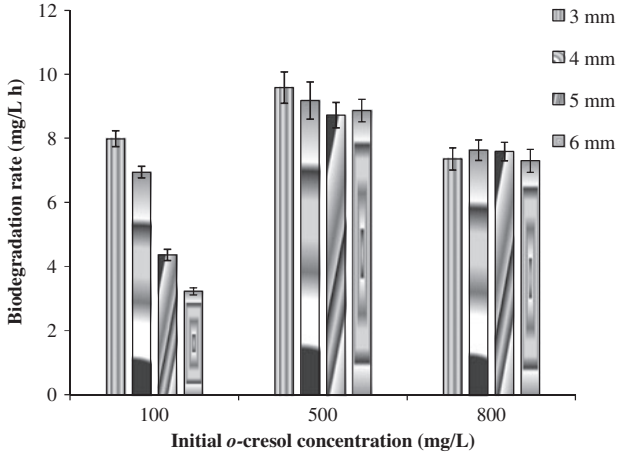

Fig. 2. Effect of beads size (3–6 mm) on the biodegradation rate of *o*-cresol at three different initial *o*-cresol concentrations of 100, 500, and 800 mg/L.

Table 1
The effect of immobilized bead size (d) on the biodegradation rate (v), experimental effectiveness factor (η), and the Thiele modulus (Ø)

d (mm)	v (mg/L h)	Effectiveness factor (η)	Thiele modulus (Ø)
Suspended	57.23 ± 2.33	–	–
3	7.99 ± 0.25	0.140 ± 0.002	20 ± 0
4	6.95 ± 0.18	0.121 ± 0.001	24 ± 1
5	4.37 ± 0.18	0.079 ± 0.001	39 ± 1
6	3.23 ± 0.11	0.061 ± 0.001	49 ± 2

PVA–alginate immobilized biomass at low bulk concentration.

3.2.2. Initial *o*-cresol concentration

The 4-mm immobilized biomass beads were chosen for the study due to the relative ease in preparation and less diffusion limitations. The time courses of the biodegradation of *o*-cresol by using the suspended biomass and the immobilized biomass beads at various initial *o*-cresol concentrations are shown in Fig. 3 (a) and (b), respectively. Fig. 4 shows the effect of initial *o*-cresol concentration on the biodegradation rate of *o*-cresol for the suspended and immobilized biomass systems. It was observed that the biodegradation rate in the suspended biomass system increased when the initial *o*-cresol concentration was increased until a maximum rate of around 70 mg/L h was reached at the initial *o*-cresol concentration of 250 mg/L. Beyond that, the biodegradation rate decreased gradually with the increase in the *o*-cresol concentration indicating an inhibitory effect exerted by the phenolic compound. Cresol degradation by suspended biomass has commonly been known to be inhibited by high concentration of cresol resulting in a longer lag time for the biodegradation process [5–7].

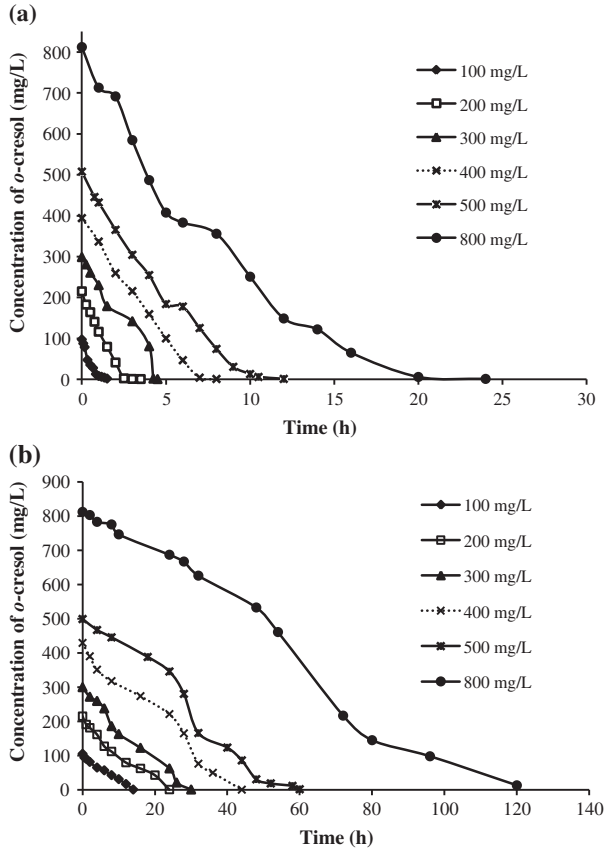

Fig. 3. Time courses of the biodegradation of *o*-cresol by using (a) suspended biomass and (b) immobilized biomass beads at different initial *o*-cresol concentrations.

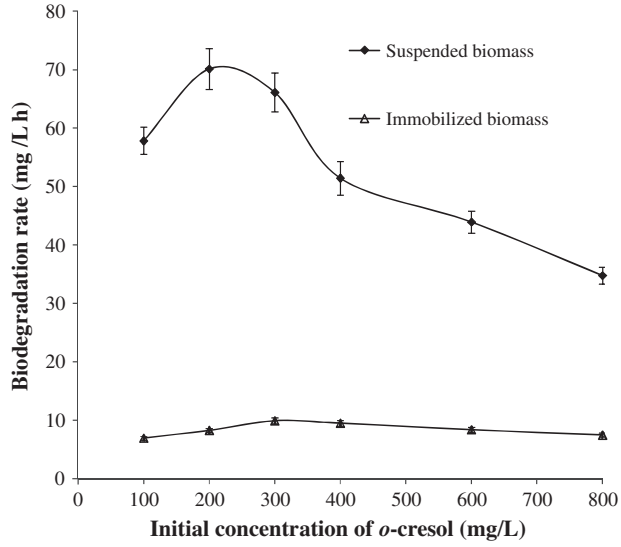

Fig. 4. Effect of initial *o*-cresol concentration on *o*-cresol biodegradation rate for the suspended and immobilized biomass systems.

In contrast, the biodegradation rate in the PVA–alginate immobilized biomass system maintained a fairly constant value under the inhibitory condition when the initial *o*-cresol concentration was above 250 mg/L (Fig. 4). Although the immobilized biomass system exhibited slower substrate biodegradation rate as compared to the suspended biomass, it seems reasonable to conclude that the immobilized biomass are able to withstand higher initial *o*-cresol concentrations without the loss of biomass viability. A plausible explanation for the trend is the substrate dilution provided by the PVA carrier due to diffusion resistance. Thus, the amount of *o*-cresol available to the biomass was significantly limited by the diffusion process resulting in a less toxic environment with respect to the immobilized biomass. The protection enhancement of immobilized living cells has also been reported by other researchers [23,24].

3.2.3. Initial pH

Biodegradation rate is affected by the activity of the enzyme which is strongly influenced by the pH. Fig. 5 shows the biodegradation rates of 100 mg/L of *o*-cresol using suspended and immobilized biomasses at various pH values. It was observed that the biodegradation rate in the immobilized biomass system was fairly constant for the studied range of pH 3–11. In

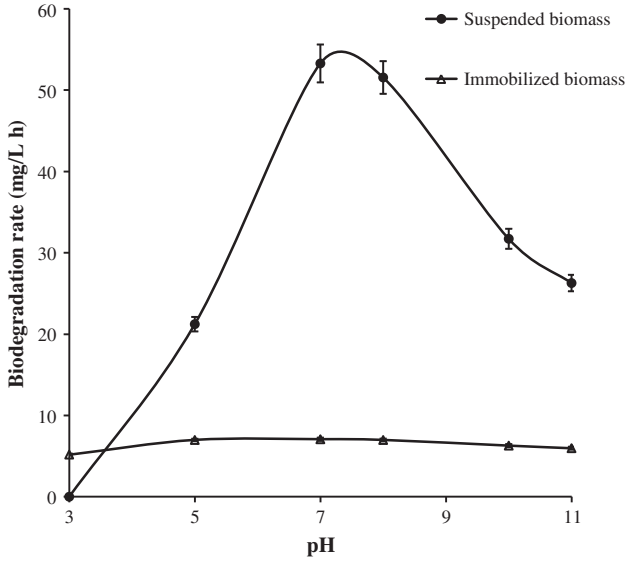

Fig. 5. Effect of initial pH on the *o*-cresol degradation for the suspended and immobilized biomass.

contrast, the biodegradation rate in the suspended biomass system increased rapidly when the pH was increased from 3 to 7 and reached the maximum value between the pH values of 7 and 8. The rate decreased rapidly when the pH was above 8. It was observed from Fig. 5 that the activity of suspended biomass was almost completely inhibited at pH 3. In contrast, the biodegradation rate in the immobilized biomass system at pH 3 was only decreased by 26% as compared to that at pH 5. Without the protection from PVA carrier, the suspended biomass was directly exposed to pH fluctuation resulting in partial or complete inhibition of the bioactivity. In contrast, the fluctuation of pH was significantly mitigated by the diffusion process through the PVA carrier resulting in better tolerance of immobilized biomass compared with the suspended biomass towards the fluctuation of initial pH condition.

3.2.4. Initial biomass concentration in the beads

Biomass with concentrations varying from 0.5 to 2.5 g/100 mL gel was immobilized in the beads. The effect of the biomass loading in the beads on the biodegradation rate is shown in Fig. 6. It was evident that the *o*-cresol biodegradation rate was affected by the initial biomass loading. The biodegradation rate increased as the biomass loading was increased until the rate reached a fairly constant value of 2.0–2.5 g wet wt/100 mL gel. Higher biomass loading might lead to saturation within the beads where only limited space was allowed for cell growth [25,26].

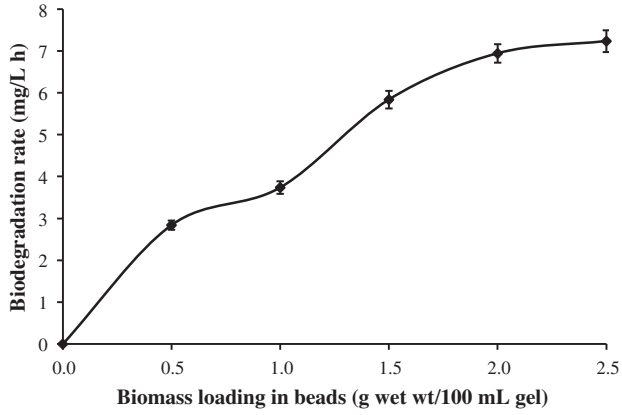

Fig. 6. Effect of the biomass loading concentration beads on the *o*-cresol biodegradation rate.

Biomass saturation could result in severe mass transfer limitation and lead to unexpected lower biodegradation rate. Also, with increasing number of cells in the beads, the oxygen consumption was possibly faster than the oxygen diffusion into the immobilized cells resulting in lower biodegradation rate as well [27]. Hence, the initial biomass concentration in the PVA–alginate gel was fixed at 2.0 g wet wt/100 mL gel throughout the study.

3.3. Reusability of beads

The biodegradation rates of *o*-cresol with the number of cycles of use at various initial *o*-cresol concentrations are shown in Table 2. A progressive decrease in the biodegradation rate with the cycle of use at the initial *o*-cresol concentrations of 100 and 400 mg/L could be explained by the increase of bead size (from 4 to 5 mm) due to the growth of biomass during the recycling process [28]. The growth of biomass could lead to biomass saturation within the bead resulting in severe mass transfer limitations and thus, lower removal rate. In addition, the increase in the initial *o*-cresol concentration would result in more growth of biomass and earlier disintegration of beads leading to reduced number of cycles of use.

Table 2
The biodegradation rates of *o*-cresol with the number of cycles of use at various initial *o*-cresol concentrations

Initial *o*-cresol concentration (mg/L)	Biodegradation rate (mg/L h)		
	Cycle 1	Cycle 2	Cycle 3
100	7.29 ± 0.37	6.61 ± 0.25	6.00 ± 0.21
400	9.66 ± 0.51	7.07 ± 0.40	(*)
800	7.47 ± 0.38	(*)	(*)

(*) indicates disintegration of the immobilized beads.

4. Conclusions

This study clearly shows the merits of using immobilized biomass in good separation of the biomass and the treated solution, and better tolerance towards more extreme operating conditions. In the present study, the effects of bead size, initial *o*-cresol concentration, initial pH, and initial biomass concentration in beads on the biodegradation rate of *o*-cresol using immobilized biomass were evaluated. Comparing the bead sizes from 3 to 6 mm, it was found that beads of the smallest size yielded the highest *o*-cresol biodegradation rate though the difference in the rates became less discernible at higher initial *o*-cresol concentration. The bioactivity of the immobilized biomass was less affected by higher initial *o*-cresol concentrations and extreme pH conditions compared with that of the suspended biomass. In addition, the immobilized biomass beads could be used up to three cycles, but only at relatively lower initial *o*-cresol concentration.

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