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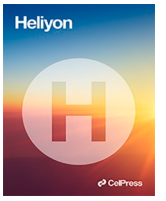

Research article

Investigating the effect of electrosprayed alginate/PVA beads size on the microbial growth kinetics: Phenol biodegradation through immobilized activated sludge

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ABSTRACT

The presence of cyclic organic compounds, including phenol, in the wastewater of many industries has made phenol removal an important issue. Meanwhile, the biological methods of removing phenol have attracted the attention of researchers in recent years. Recently, the use of immobilized microbial cells is proposed as a new approach in industrial wastewater treatment. In this research, the aim is to study the effect of immobilized beads size on the phenol biodegradation efficiency and specific microbial growth rate. For this purpose, electrospray technique was used to immobilize activated sludge in hybrid matrix of alginate and polyvinyl alcohol (PVA). The fabricated alginate/PVA beads were characterized using Fourier transform infrared spectroscopy (FTIR). Evaluation of the results related to the free and immobilized cell systems in the shake flask experiments showed that at low phenol concentrations the immobilized cell system had the same performance as the free cell system, while the immobilized cell system at higher concentrations had a better performance in removing phenol so that at a concentration of 2000 mg/L, removal percentage has increased from 15% to 25–34%. On the other hand, in this survey, the kinetic behavior of activated sludge was in good agreement with Haldane's equation. Moreover, the maximum specific growth rate was measured 0.033 and 0.041 (h^{-1}) beside 544 and 636 mg/L substrate inhibition constant, for free and immobilized cell systems, respectively. This result shows that the phenol biodegradation has been improved by using the cell immobilization technique especially with applying the smaller beads, which is due to improved mass transfer and microbial cell protection from harsh environments.

1. Introduction

There are different types of pollutants in industrial wastewater effluents. Phenol, a known human carcinogen, is used in various industries. Phenol contaminated water poses an important environmental risk due to its high toxicity and low biodegradability that actually causes detrimental effects on human and various ecosystems [1,2]. Furthermore, World Health Organization (WHO) suggests 0.001 mg/L as the permissible limit for phenol concentration in potable water [3,4]. Therefore, phenolic compounds have been listed as priority pollutants by U.S. environmental protection agency (EPA). Diverse technologies including adsorption, coagulation, photo-catalysis, chemical–biological oxidation, etc. have been proposed and applied to remove phenol from wastewater streams [5–7].

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Relatively high cost and low efficiency of phenol removal in its trace levels from wastewaters using these methods caused biological treatment approaches at great attention as the promising techniques in wastewater treatments [8]. Moreover, immobilized microbial cell systems due to stability improvement and advancing the tolerance against harsh environments is a promising technique, which has been recently applied in several environmental studies [9–11]. Previous studies reported that physiological properties of microbial cells could change by immobilization of cells. Hence, the performance of immobilized cells has considerably improved compared to free cells. For instance, Cassidy and coworkers observed high cell density and plasmid stability in immobilized cells systems [12]. In another survey, Zhu reported that the changes in the membrane properties such as increased cell wall thickness and higher DNA content in immobilized cell systems would help the cells to prevail the toxic effects such as acidic or alkaline pH and extreme temperatures in the harsh environments [13]. Willaert observed that the metabolic activities increment of the immobilized cells due to facilitated cell-to-cell communication [14]. Besides, activated sludge due to the presence of a large diversity of bacteria and fungi, offers possibilities for complete mineralization of organic pollutants [15–17]. Moreover, development of low cost carriers with high efficiency for the microbial cell immobilization is sought by many researchers to remediate environmental hazards [18–22]. Therefore, in this field the type and size of the matrices are very substantial, while bead size of 1–5 mm were common in previous researches on the phenol biodegradation [15,23–27]. Besides, it has been stated that reducing the bead size can lead to improved mass transfer of the substrate and increase its biodegradation efficiency [28,29]. Cassidy and coworkers reported that the thin film of liquid which surrounds the microbeads may play an important role in restricting diffusion of oxygen into the beads, and the thickness of this layer decreases as a result of beads size decrement, hence diffusion will be improved [12]. Recently, electrospraying as a controllable technique has gained attention regarding the fabrication of effective microbeads with the diameter of interest. In order to obtain spherical beads with the desired size, various factors like polymer concentration, injection feed rate, voltage, needle size, distance from nozzle tip to collector and gelation time are effective [30,31]. Electrospraying is also used for the fabrication of polymeric microbeads with high surface area and porosity as well as low diameter size [32,33]. Although the effect of bead size on substrate mass transfer rate has been studied so far [28,34–36]. To the best of our knowledge, there has been no report on the effect of bead size on microbial growth kinetics. In addition, until now, different matrices for the removal of contaminants from aqueous solutions have been successfully applied which prepared by both natural (agar, alginate, and chitosan) and synthetic (polyvinyl alcohol, polyurethane, and polyacrylamide) polymers [37–42]. The relative fragility and mass transfer limitation of alginate beads in high polymer concentration limit their usage in pollutants biodegradation, especially by the mixed culture system [12,43,44]. Moreover, PVA beads are fabricated in the presence of boric acid solution which can damage the microbial cells and also agglomerated beads in this technique limit their application [45,46]. Meanwhile, as we have previously reported, long-term stability and reusability of fabricated matrices are crucial issues for environmental application, so the combination of PVA, a synthetic polymer, and sodium alginate, a natural polymer, has drawn attentions [9]. However, no report exists in applying alginate/PVA beads for activated sludge entrapment, apart from two studies in which PVA/alginate cryogel beads were used as carriers [15,37]. It should be noted that although temperature-induced gelation leads to produce strong PVA cryogels with high microporous structure, freezing process of this method could affect cell viability and biodegradation efficiency [9,47].

Besides, according to our previous studies, the effect of electrical field in range of 0–15 KV on cell viability was studied after electrospray process [28,48].

Hence, the main objective of this survey is to examine the activated sludge growth kinetic in biodegradation of phenol by free and immobilized activated sludge in aqueous phase shake flask experiments. Moreover, to the best of our knowledge, the effect of bead size on microbial growth kinetic is studied for the first time in this research. For this reason, electrospraying technique has been applied to fabricate alginate/PVA microbeads, potent hybrid matrix for immobilizing the microbial cells, with diameter of 500 μm which cannot be produced by common procedures in microbial cell immobilization.

2. Materials and methods

2.1. Chemicals

For microbial cell immobilization, sodium alginate with a molecular weight of 100–200 kDa and polyvinyl alcohol with molecular weight about 200000 g/mol were purchased from Fluka and Merck, respectively. For culture media, mineral salts in the analytical grade were made of Merck Company. For biodegradation experiments and phenol concentration measurement, phenol, ammonium hydroxide, dipotassium phosphate, potassium dihydrogen phosphate, 4-aminoantipyrine, ferricyanide potassium were obtained from Merck Company. Calcium chloride and sodium citrate for polymer crosslinking and de-entrapment, respectively were obtained from Merck.

2.2. Culture media and inoculum preparation

The activated sludge taken from cellulose industry wastewater (Iran), after preliminary acclimatization to incremental phenol concentrations, was used as the microbial consortium in all free and immobilized cell systems throughout this survey. For this purpose, 100 mg phenol was added to the 1000 mL of mixed liquor containing raw activated sludge, then after 48 h of aeration and 1 h of sedimentation, the supernatant was drained and 1000 mL of fresh culture medium containing phenol (gradually 100–500 mg/L) was added. The culture medium for activated sludge cultivation consisted of (in g/L): KH_2PO_4, 4; Na_2HPO_4, 5; NH_4Cl, 1.5; $MgSO_4{\cdot}7H_2O$, 0.2; $(NH_4)_2(FeSO_4)_2{\cdot}6H_2O$, 0.05; $CaCl_2$, 0.005, $MnSO_4{\cdot}7H_2O$, 0.02.

2.3. Activated sludge immobilization in alginate/PVA beads

In this study, the combination of alginate and PVA has been used for microbial cell immobilization. Sodium alginate (2%) and PVA (12.5%) were dissolved in distilled water by stirring at room temperature and 80 °C, respectively. Then, both solutions were mixed with a volume ratio of 70:30 (alginate/PVA). Besides, 50 mg of activated sludge was collected by centrifuging the 25 mL of mixed liquor (MLSS = 2000 mg/L) at 2000 rpm for 5 min (Hermle Z366, Germany) and was added to 50 mL hybrid alginate/PVA solution to produce microbeads with cell density of 1 mg/mL of polymer suspension. Then the beads produced from 1 mL of polymer solution were added to 20 mL of culture medium containing desired concentration of phenol. In this way, the initial inoculum concentration of immobilized cells in each experiment was 50 mg/L equal to initial concentrations in the free cell system. In this survey, electrospray technique was applied to produce microbeads with various sizes. For this purpose, the syringe pump is fed by a 22 G needle size syringe which is filled by homogeneous mixture. The distance from needle tip to surface of calcium chloride solution (0.2 M) and pump flowrate are set on 10 cm and 5 mL/h, respectively. Meanwhile, a high voltage generator used to induce 0 and 15 kV. Finally, to harden the beads, they were remained in calcium chloride solution as a gelling agent.

2.4. Biodegradation experiments

In this study, to examine the phenol biodegradation efficiency as well as growth rate of activated sludge, 20 mL of culture medium and phenol at different concentrations (100–2000 ppm) as the sole carbon and energy sources were added to100 ml Erlenmeyer flasks. The pH of culture medium was adjusted at 7. Then, flasks were placed in a shaker incubator (Jaltajhiz, Iran) at a temperature of 32 °C and 150 rpm for 72 h. Overall, three sets of experiments were carried out with free and immobilized microbial cells. In the free cell system, each flask was inoculated with 50 mg/L of activated sludge as the initial biomass concentration. Moreover, the performance of immobilized cells was examined at two different bead sizes (4 mm and 500 μm). It is also noteworthy that control or blank experiments were performed without microbial cell inoculation to measure the physicochemical removal of phenol in free and immobilized cell systems. All experiments in this survey, were performed in duplicate and phenol concentration was measured by colorimetric method using spectrophotometer (Jenway 6300, England) at wavelength of 500 nm.

2.5. Analyses

The content of each flask was separately centrifuged (4000 rpm, 3 min) and supernatant was collected for phenol measurement. Then the remaining cells in free cell system and alginate/PVA beads in immobilized cell system were transferred to certain containers for biomass concentration measurements.

2.5.1. Phenol analysis

In this research, phenol concentration was measured by colorimetric method and by 4-aminoantipyrine reagent. Phenolic compounds react with 4-aminoantipyrine at pH level of 7.9 ± 0.1, in the presence of free cyanides. The highest absorption for this aqueous solution occurred at wavelength of 500 nm. In this analytical method the maximum and minimum detection limits were 0.01 and 5 mg/L, respectively. In order to measure phenol content, 2.5 mL of 0.5 N ammonium hydroxide solution was added into 10 mL of sample containing phenol and pH value was rapidly adjusted at 7.9 ± 0.1 by phosphate buffer (pH = 6.8). Then, 1 mL of 4-aminoantipyrine was added to the mixture and well mixed, subsequently 1 mL of ferricyanide potassium solution was added and completely blended and 15 min' time given to complete the reaction. The solution color shade was yellow to red depending on the phenol concentration. Then the solution was centrifuged till having a clear phase [49]. Finally, the optical density of sample was measured at 500 nm and equivalent phenol content was calculated by standard calibration curve. To obtain the standard calibration curve, 5 solutions (phenol concentration of 1–5 mg/L) with a volume of 10 mL were prepared. Then, the absorbance of each standard sample was measured by the above described method at wavelength of 500 nm and the results were plotted versus phenol concentrations. The obtained straight-line relationship between absorbance and concentration was used for phenol measurement. In this study, a control sample containing 10 mL of distilled water was used as a blank and the absorbance of the control sample adjusted on zero at a wavelength of 500 nm.

2.5.2. Measurement of cell dry weight and cell growth yield

In free cell system, after the centrifuge of tubes, the remaining cells were separated and placed in oven at 100 °C to dry. Therefore, after stabilizing the weight, the final weight was measured and cell concentration was derived based on milligrams per 20 mL of culture. For determination of biomass concentration in the immobilized cell system, the separated beads were firstly dissolved by vigorous shaking in 0.3 M sodium citrate solution at pH 5 and microbial cells were separated from culture and measured.

The cells growth yield ($Y_{X/S}$) versus produced cell per consumed phenol (mg/mg) was measured via Eq. (1).

$$Y_{X/S} = \frac{\Delta X}{\Delta S} = \frac{X_t - X_0}{S_0 - S_t} \quad (1)$$

where X_t and X_0 are microbial cell concentrations at times t and 0, respectively and S_0 and S_t are the phenol concentrations at times 0 and t.

2.5.3. Examination of activated sludge growth kinetic

The specific growth rate of activated sludge (1/h) was calculated in the exponential growth phase of the batch experiments based on Eq. (2).

$$\mu = \frac{1}{X}\frac{dX}{dt} = \frac{\ln\left(\frac{X_2}{X_1}\right)}{t_2 - t_1} \tag{2}$$

where X_1 and X_2 are the activated sludge concentrations (mg/L) at times t_1 and t_2, respectively [50]. Then, the Andrews-Haldane kinetic model was applied to determine the correlation between μ and initial concentration of phenol based on Eq. (3).

$$\mu = \frac{\mu_{max} S}{K_s + S + \frac{S^2}{K_I}} \tag{3}$$

where μ_{max} is the maximum specific growth rate, K_S is the half saturation constant, and K_I is the inhibition constant [51,52]. In order to determine the bio-kinetics parameters, nonlinear regression analysis of MATLAB software (version R2021b) was used to solve Eq. (3).

2.5.4. FTIR and scanning electron microscopy (SEM)

FTIR spectra of the hybrid alginate/PVA matrix was obtained by FTIR spectrometer (JASCO FT/IR-4700, Japan) and compared with alginate and PVA polymers. In addition, to investigate the structure of microbeads, SEM test of their cross section was performed (SEM, Hitachi, SU3500).

2.5.5. Statistical analysis

For statistical analyses GraphPad Prism software (version 9.0, California, USA) was applied. The significance status of variations on the studied responses was evaluated by Fisher's F-test, and a p-value of less than 0.05 was considered to indicate a significant effect.

3. Results and discussion

3.1. Morphology and size of alginate/PVA beads

As shown in Fig. 1(a) and (b), the results of investigating bead size showed that with increasing the voltage from 0 to 15 KV, the bead size has significantly decreased from 4 mm to 500 μm, that represents the electric field considerably affect the size of beads. This finding is in line with our previous observation for alginate beads [30]. Besides, the brownish color of alginate/PVA beads in Fig. 1(a)

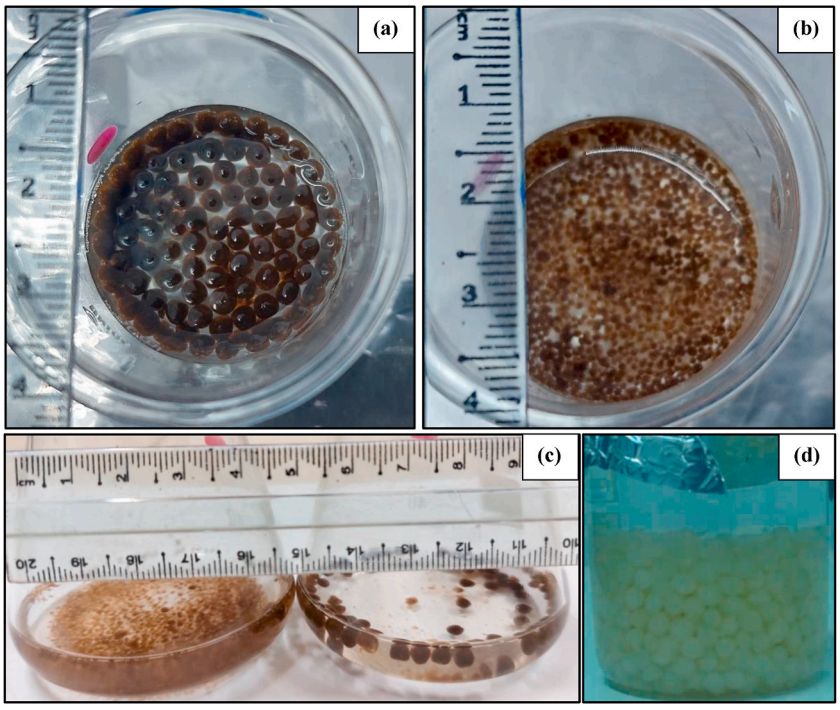

Fig. 1. Images of electrosprayed micro-beads at various voltages (a) 0 KV (b) 15 KV (c) 0 and 15 KV in phenol biodegradation experiments at time zero (d) 0 KV without activated sludge.

and (b) indicates that the activated sludge has been effectively entrapped in the hybrid matrix. As shown in Fig. 1(c), at the beginning of the biodegradation process, slight microbial cell release was observed. Therefore, the concentration of released cells from micro-beads was gravimetrically measured and its amount was almost 2 mg/L which is equivalent to 4% of the initial immobilized cells in alginate/PVA beads. In addition, in the literature it has been mentioned that a portion of the microbial cells can be released from the carriers as a result of cell growth, and this release after adaptation to the surrounding environment can assist biodegradation process [53–55]. Fig. 1(d) shows image of alginate/PVA beads without activated sludge. Besides, SEM images in Fig. 2(a) and (b) show that the microbial cells were successfully entrapped in alginate/PVA hybrid matrix. Therefore, it can be concluded that the microbial cell entrapment in the hybrid matrix has been effectively occurred.

Besides, according to the appearance of the immobilized beads in the culture medium, they were stable during the process without any breakage. Furthermore, in this survey, beads stability was evaluated by shaking in tap water up to 1 month, no breakage was observed. However, alginate-PVA microbeads have been sequentially recycled for the biodegradation of hydrocarbons in the aqueous phase [56,57], it should be noted that some researchers have mentioned poor mechanical stability and dissolution of alginate/PVA hybrid beads in a short period of time [58,59]. Although, in this study, the formed beads have maintained their initial structure and their quantity have remained constant at the end of first stage, but further research is needed to investigate the amount of cell release and stability of the beads during repeated batch cultivation.

3.2. FTIR

In this study, FTIR spectroscopy provides data about the chemical structure of PVA and alginate polymers as well as occurred reactions after mixing and crosslinking. As shown in Fig. 3, in case of PVA polymer, the main peaks were appeared at wavenumbers of 3337, 1632 and 1082 cm^{-1} which can be related to OH stretching vibrations, C═O vibrations and *C*–O stretching, respectively.

Moreover, for alginate/PVA microbeads, OH stretching vibrations with highly reduced intensity were observed at wavenumber of 3289 cm^{-1} that demonstrates the OH groups concentration was decreased as the possible hydrogen bonding interactions between OH groups of alginate and PVA polymers. Yang et al. (2022) observed a similar trend in OH stretching vibrations for polyvinyl alcohol (PVA)-polydopamine (PDA) microspheres compared to FTIR spectra of pristine PVA [60]. Therefore, this phenomenon can possibly improve the microbeads structure as well as cells entrapment in hybrid matrix and reduce microbial cells leakage from the beads.

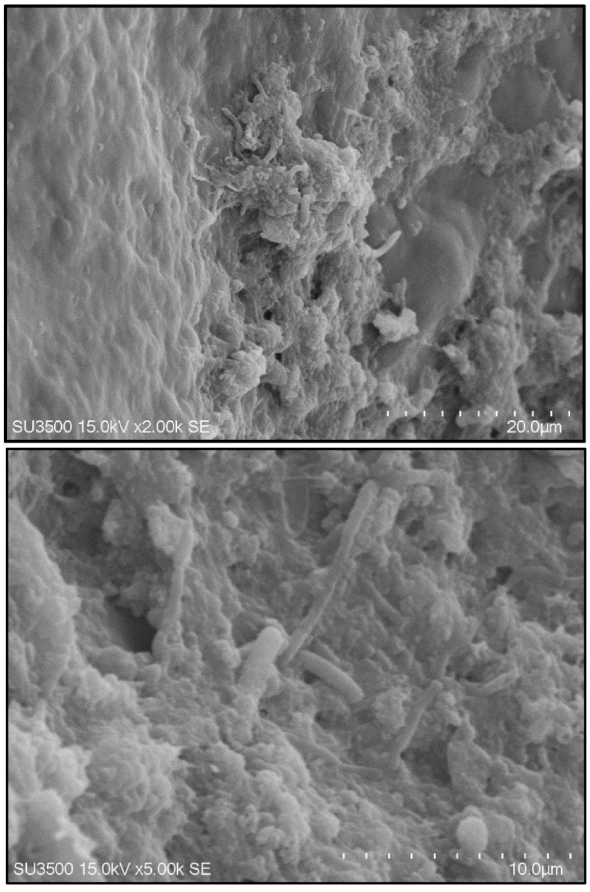

Fig. 2. SEM images of the interior structures of alginate/PVA hybrid beads at two different magnifications (a) 2K (b) 5K.

Moslemy and co-workers reported that matrices with minimized cell release are suitable for bioreactor application as a result of cell washout prevention from the bioreactor [61].

3.3. Examination of phenol biodegradation

In this study, enhanced biodegradation by immobilized cells compared to free cells was observed at high concentrations of phenol (Fig. 4). According to the most recent studies, free and immobilized cell systems degraded similar amounts of the pollutants at the low concentrations [62,63]. For instance, Zinjarde and Pant observed that although both systems degraded similar amounts of crude oil at low concentration of 0.5% w/v, the immobilized cells in agar-alginate beads degraded more crude oil than the free cells at high concentrations ranges between 1 and 2.5 %w/v [63]. Moslemy and coworkers observed similar biodegradation rate of gasoline for two free and immobilized cell systems at gasoline concentrations lower than 100 ppm, while with increasing gasoline concentration from 200 ppm to 600 ppm, the biodegradation rate was significantly increased in the immobilized cell system compare to free cell [62]. Also, Fig. 4(a–c) illustrates that pollutant removal percent decreases by increase in initial phenol concentrations as a result of phenol inhibitory effects at higher concentrations. Similarly, researchers have declared that lag-phase duration increases by rise in initial concentrations [64] in which total consumed phenol is not dedicated for new cell generation. In the other word, more substrate consumption is applied in production of required enzymes to prevail phenol inhibitory effect and as well as energy production to produce hydroxylase enzyme. Therefore, at the initial stages of biodegradation, biomass production rate is limited but speed up by increase in phenol consumption and inhibitory effect decline [65]. Finally, it can be concluded that at initial stages of phenol biodegradation at high concentrations, specific growth rate and biomass production yield is very low which increase by inhibitory effect weakening due to phenol consumption. Additionally, control experiments were carried out in the absence of microbial cell and presence of beads without microbial culture at initial phenol concentration of 2000 mg/L, in free and immobilized cell systems, respectively to examine the contribution of phenol removal by physicochemical phenomena. According to the results, negligible phenol loss of 1.9% and 2.4% were observed in free and immobilized cell system, respectively.

3.4. Determination of cell dry weight and growth yield

Fig. 5(a–c) shows the variations of the cell dry weight at different phenol concentrations versus times. According to Fig. 5(a–c), the highest amount of biomass was observed in low concentrations of phenol while, in concentrations higher than 500 mg/L, the changes in dry weight of cell are insignificant.

For more investigations, the amount of activated sludge growth yield was calculated at various initial phenol concentrations and shows in Table 1. Similarly, the results of Table 1 show that the growth yield significantly decreases with increasing phenol concentration from almost 2 to 0.05 (F-value = 378.6, p-value = 0.0008 < 0.05). This result is in accordance with previous study reporting a sharp decrease in yield coefficient ($Y_{X/S}$) with phenol concentration increment [52]. It is also noteworthy that the growth yield has not significantly changed in all three systems (F-value = 1.488, p-value = 0.2718 > 0.05). In the other words, according to p-value>0.05, we can conclude that free and immobilized cells do not show significant differences on growth yield.

3.5. Determination of kinetic parameters

Kinetic parameters were obtained by fitting the experimental data through the least squares technique. This method has various advantages like easy application, simple computation, and minimal difference between the residuals sums of squares to the line of best fit [66]. According to the laboratory data (Fig. 6), the maximum specific growth rate was observed at 100 mg/L as the minimum phenol

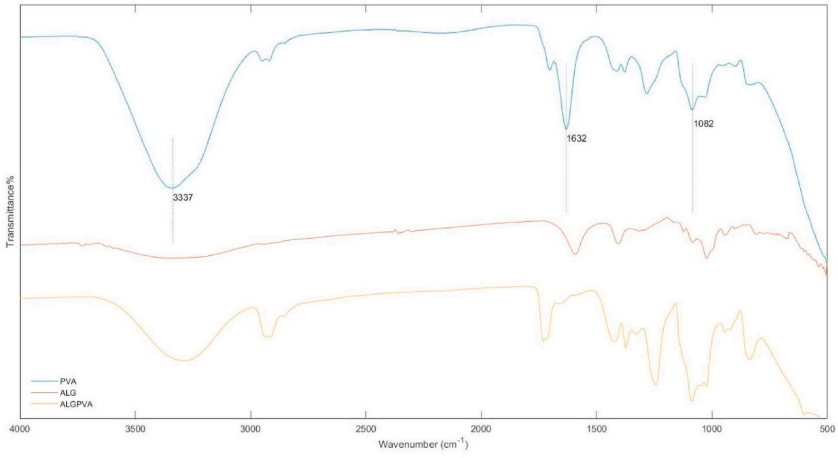

Fig. 3. FTIR spectra of pristine materials (Alginate and PVA) and electrosprayed microbeads after crosslinking.

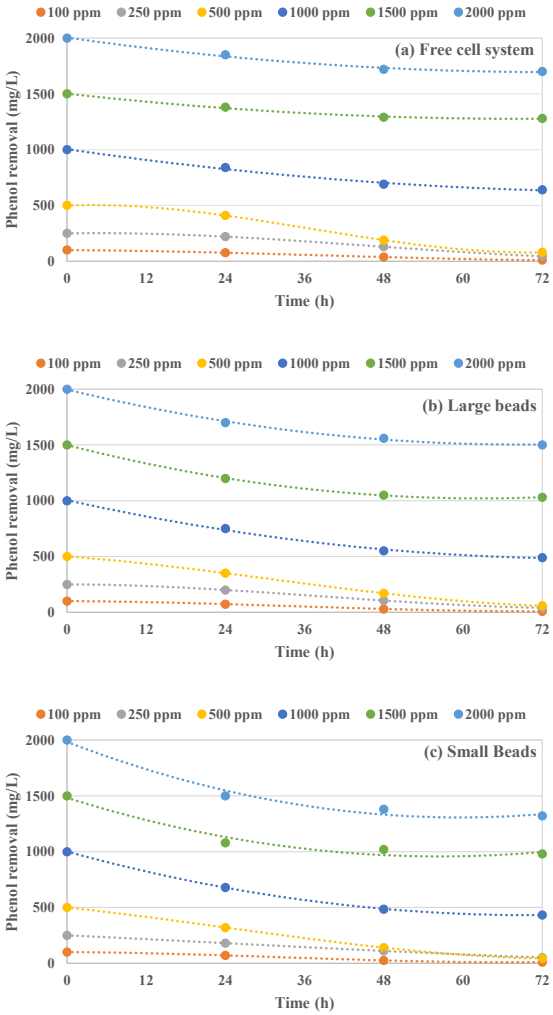

Fig. 4. Phenol removal at various concentrations for (a) free cell (b) immobilized cells with small size of beads (c) immobilized cells with small size of beads.

concentration for three different cases of free microbial cells and immobilized cells in two different sizes of alginate/PVA beads. Moreover, lower specific growth rate can be justified as a consequence of high inhibitory effect of phenol in microbial growth. In this study, in shake flask experiments, phenol is considered as the sole microbial growth inhibitor and aeration in shake flask is assumed constant and sufficient for constant oxygen level controlling. In case that substrate is the microbial growth inhibitor and initial concentration of inhibitory substrate stands at higher levels, Haldane equation as a general model is used to develop the appropriate correlation for dynamic growth rate behavior. As shown in Fig. 6(a–c), in all three cases, specific growth rate data at various initial substrate concentrations fitted well into Haldane equation. The results show that there is no significant difference between various specific growth rates at lower phenol concentrations (<250 ppm) for all three systems (F-value = 2.914, p-value = 0.1675 > 0.05), while for phenol concentrations higher than 500 ppm, the growth rate in the immobilized cell system had a significant difference with the free cells (F-value = 20.35, p-value = 0.0021 < 0.05), and also with the decrease in the bead size, the specific growth rate significantly increased. Similarly, in several previous studies have been reported that immobilized cells have shown superior efficiencies compared to free cells at harsh environments such as high pollutant concentrations, inappropriate temperature, and pH [7,22,38,41,67,68]. For instance, Liu and co-workers reported that cell growth in free cell system had been inhibited at phenol concentrations higher than 500 mg/L while immobilized cell system tolerated the toxicity of phenol in high concentrations [69]. Namane and coworkers reported that slow substrate diffusion and weakening of the alginate beads were observed by increase in bead sizes from 3 to 5 mm [70].

Table 2 shows the coefficients and constant values of Haldane equation fitted on phenol biodegradation by free and immobilized cells. Regardless of lower specific growth rate, applied activated sludge had considerable capability in phenol biodegradation. Similarly, Arutchelvan and coworkers assessed the phenol biodegradation using *Bacillus brevis* in concentrations of 750–1750 mg/L in

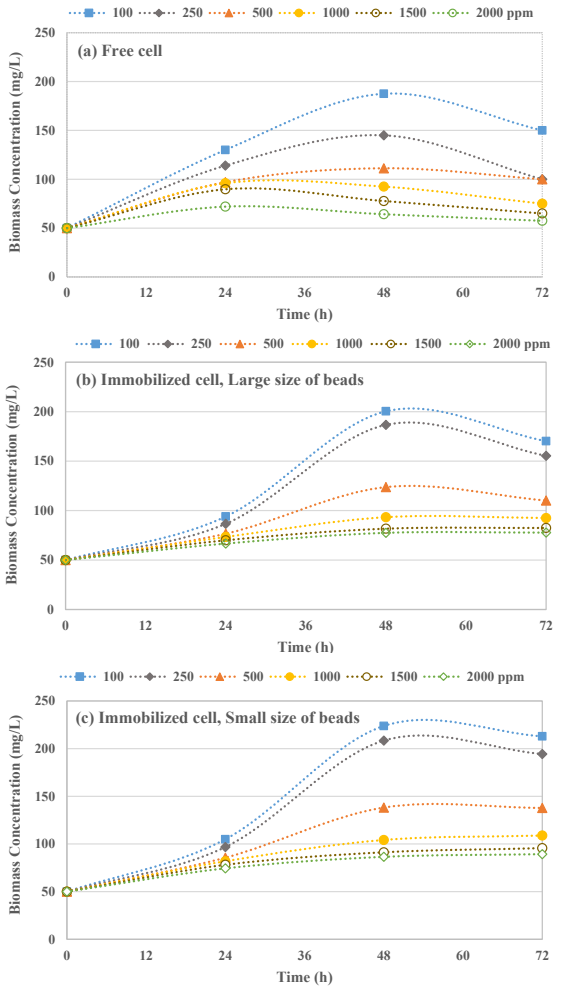

Fig. 5. Variation of biomass concentration in biodegradation at various phenol concentrations for (a) free cell (b) immobilized cells with small size of beads (c) immobilized cells with small size of beads.

Table 1
Growth yield of activated sludge at various initial phenol concentrations in biodegradation of phenol by free and immobilized cell systems.

Phenol conc. (mg/L)	100	250	500	1000	1500	2000	Mean[a]	Standard Deviation
Free cell	2.218	0.792	0.198	0.138	0.133	0.051	0.58833	0.84244
Immobilized cell, large beads	2.149	0.943	0.224	0.097	0.071	0.063	0.59117	0.83417
Immobilized cell, small beads	2.32	1.129	0.245	0.105	0.077	0.059	0.65583	0.91179
Mean[b]	2.2290	0.95467	0.22333	0.11333	0.093667	0.057667	–	–
Standard Deviation	0.086029	0.1688	0.023544	0.021733	0.034195	0.0061101	–	–

[a] There is no significant difference between the means of any pair (between row).
[b] The means of the following pairs are significantly different (between column).

which μ_{max} and K_s were obtained in range of 0.026–0.078 h^{-1} and 2.2–29.31 mg/L, respectively [71]. Stoilova and coworkers, in a similar study, reported 0.0169–0.233 h^{-1} and 916–1013 mg/L for μ_{max} and K_s in phenol biodegradation in range of 300–1000 mg/L by *Aspergillus awamori* [72]. According Table 2, maximum specific growth rate (μ_{max}) in free cell, immobilized cells in 4 mm and 500 μm beads were measured 0.033, 0.039 and 0.041, respectively. This result proved that by beads size decrease, inhibitory effect of phenol decreases and specific growth rate increases. Moreover, substrate inhibition constant (K_I) and highest substrate affinity constant (Ks) were maximum for small beads. This result showed that the smaller size of the beads made the microbes less sensitive to substrate inhibition and on the other hand improved the potential of phenol biodegradation. Previous studies have shown that by reducing the gel particle size, the substrate diffusion limitation has decreased [28,36]. Therefore, it can be concluded that by decreasing beads size, substrate diffusion and microbial growth were increased. Besides, as shown in Fig. 4 the phenol biodegradation time at various initial

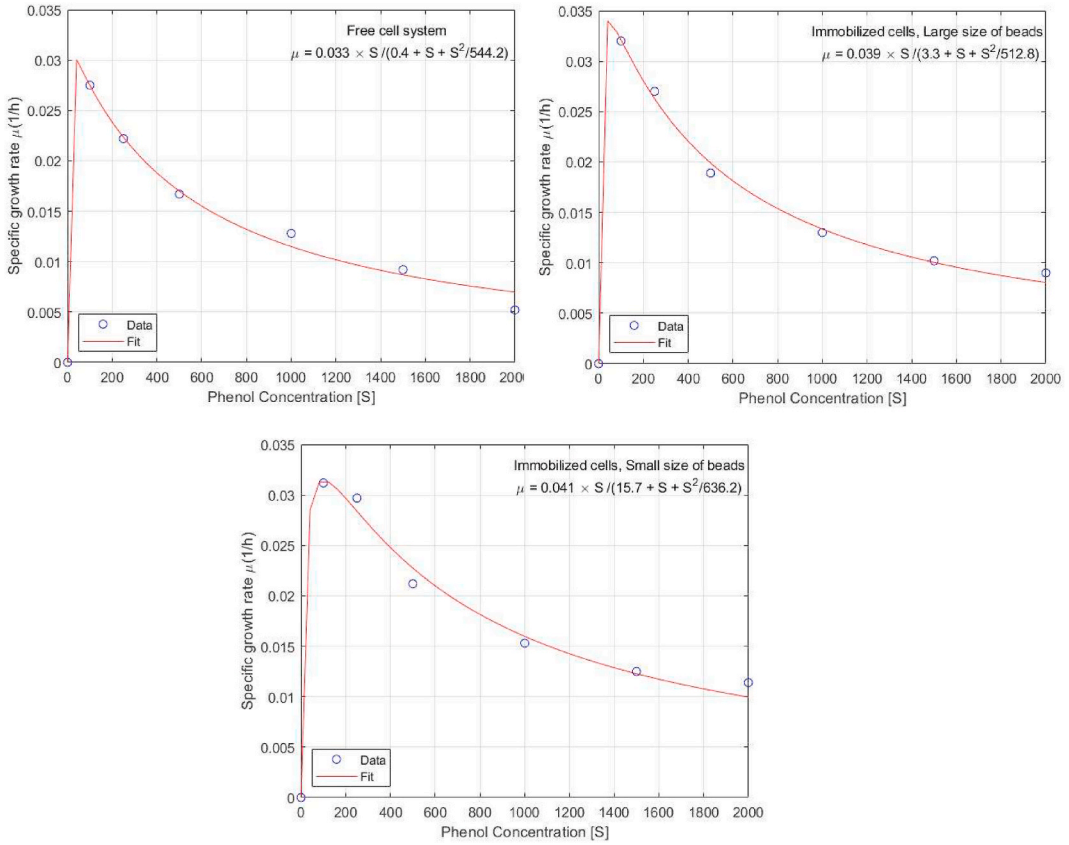

Fig. 6. Variation of specific cells growth rate with different initial phenol concentrations (a) free cell system (b) immobilized cells with small size of beads (c) immobilized cells with small size of beads.

Table 2
Kinetic parameters obtained from Haldane model in biodegradation of phenol by free and immobilized activated sludge.

Constants	μ_{max} (1/h)	K_s (mg/L)	K_I (mg/L)	SSE[a]
Free cell	0.033	0.4	544.2	5.2335e-06
Immobilized cell, large beads	0.039	3.3	512.8	2.5968e-06
Immobilized cell, small beads	0.041	15.7	636.2	6.7470e-06

[a] Sum-Squared-Error.

concentrations was shorter for beads with a size of 500 μm compared to the other two systems. This result is consistent with the calculated K_I constants in this study as well as previous studies which reported longer phenol biodegradation time at lower K_I values.

Besides, according to previous research the critical substrate concentration ($C_{critical}$) can be determined for self-inhibitory compounds as Eq. (4) [51].

$$C_{critical} = \sqrt{K_s * K_I} \tag{4}$$

Therefore, in the present survey, critical concentrations of phenol were 15, 41 and 100 mg/L for free cell, immobilized cells of 4 mm and 500 μm beads, respectively. These results confirmed that, the increase in the phenol biodegradation rate was caused by relative overcome to self-inhibitory effect by using immobilized cells. This may be attributed to protection of cells from fluid shear stress and harsh environments by their immobilization by the entrapment technique [10,73].

4. Conclusions

In this research, the effect of bead size of hybrid alginate/PVA on the specific microbial growth rate of activated sludge in the phenol biodegradation was evaluated for the first time. For this purpose, the electrospray technique as a controllable manner has been used by which microbeads of arbitrary size can be produced. Results showed that the immobilized microbial cell system had a higher efficiency of phenol removal compared to the free cell system, especially for high concentrations of phenol. In this study, kinetic

behavior of activated sludge growth was in a good agreement with Haldane's equation and maximum specific growth rate was observed for small beads size of immobilized cells. Therefore, by producing small size of microbeads using electrospray method, it is possible to profit many advantages including higher biodegradation efficiency and specific growth rate in the presence of growth limiting substrates. These benefits can help to use immobilized cells in the bioreactors and prevent cell washout phenomenon. Overall, in this survey, a new advantage of the cell immobilization technique for the industrial application of immobilized microbial cells has been presented.

Author contribution statement

Ali Partovinia: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Elham Vatankhah: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Data availability statement

Data will be made available on request.

Additional information

No additional information is available for this paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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