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Biodegradation of phenol by *Pseudomonas putida* immobilized in polyvinyl alcohol (PVA) gel

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ABSTRACT

Batch experiments were carried out to evaluate the biodegradation of phenol by *Pseudomonas putida* immobilized in polyvinyl alcohol (PVA) gel pellets in a bubble column bioreactor at different conditions. The bacteria were activated and gradually acclimatized to high concentrations of phenol of up to 300 mg/l. The experimental results indicated that the biodegradation capabilities of *P. putida* are highly affected by temperature, pH, initial phenol concentration and the abundance of the biomass. The biodegradation rate is optimized at 30 °C, a pH of 7 and phenol concentration of 75 mg/l. Higher phenol concentrations inhibited the biomass and reduced the biodegradation rate. At high phenol concentration, the PVA particle size was found to have negligible effect on the biodegradation rate. However, for low concentrations, the biodegradation rate increased slightly with decreasing particle size. Other contaminants such heavy metals and sulfates showed no effect on the biodegradation process. Modeling of the biodegradation of phenol indicated that the Haldane inhibitory model gave better fit of the experimental data than the Monod model, which ignores the inhibitory effects of phenol.

1. Introduction

Petroleum refineries generate huge amounts of wastewater that usually go through a combination of treatment steps to reduce the concentrations of the different contaminants to acceptable discharge levels. The main contaminants of refinery wastewater include phenols, polycyclic aromatic hydrocarbons (PAHs) as well as heavy metals. Of these toxic pollutants, phenols are considered to be among the most hazardous, and they are certainly the most difficult to remove. Phenol may be fatal by ingestion, inhalation, or skin absorption, since it quickly penetrates the skin and may cause severe irritation to the eyes and the respiratory tract. It is listed among the priority organic pollutants by the US Environmental Protection Agency [1]. It is considered to be potentially carcinogenic to humans and may be lethal to fish at concentrations of 5–25 mg/l [2]. The UAE has one of the most stringent environmental regulations, especially those related to discharge levels. Abu Dhabi National Oil Company (ADNOC) has set a desirable limit of phenol discharge concentration of 0.01 mg/l compared to that set by the EPA (USA) of 0.168 mg/l.

It is essential, therefore, that phenol concentrations in refinery effluents be reduced to environmentally acceptable and harmless levels through utilizing effective and practical treatment methods. Many treatment techniques have been employed in the past few years to reduce the concentrations of phenols, including biodegradation, adsorption, ion exchange and the use of bioactive activated carbon. Biological treatment has proved to be the most promising and economical method for the removal of phenol from wastewater. It is believed to lead to complete mineralization of phenol [3] and can handle a wide range of concentrations. The biodegradation of phenols by different types of microbial cultures has attracted the attention of many researchers during the past two decades. Many types of aerobic bacteria, including *Pseudomonas putida*, are believed to be capable of consuming aromatic compounds as the only source of carbon and energy. *P. putida* is a rod-shaped, Gram-negative bacterium that has been known for its ability to degrade organic solvents, especially its high removal efficiency of phenol [4]. Numerous other types of bacteria and biosorbents were reported to be utilized for the biodegradation or the removal of phenol. These include: *Rhodococcus erythropolis* [5]; *Bacillius* sp. [6]; *Alcaligenes faecalis* [7]; *rhizobium Ralstonia taiwanensis* [8]; *Nocardia hydrocarbonoxydans* [9]; *Candida tropicalis* [10] and activated sludge [11].

In recent years, the strain of *P. putida* has been the most widely used type of bacteria for phenol biodegradation. Under aerobic conditions, phenol may be converted by the bacterial biomass to carbon

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dioxide; other intermediates such as benzoate, catechol, *cis–cis*-muconate, β-ketoadipate, succinate and acetate are known to be formed during the biodegradation process [12]. *P. putida* has been studied by many researchers in free and immobilized forms, using different types of bioreactors [2,13–23].

Immobilization of bacterial biomass for the degradation of phenol is an important and effective technique that is usually employed to serve several purposes, including protection of the bacteria from high phenol concentrations as well as ease of separation and reutilization of the biomass. The overall efficiency of the bacterial biomass in the biodegradation of phenol may be affected by many factors such as phenol concentration [24], temperature [25], the presence of other nutrients [26], the presence of other pollutants [27] and bacterial abundance [28].

The main objective of this study is to assess the biodegradation of phenol by *P. putida* immobilized in polyvinyl alcohol (PVA) gel matrix at different conditions in a bubble column bioreactor. The article examines many important factors that have not been addressed in the literature including the immobilization of *P. putida* in a PVA gel matrix, the effect of other contaminants on the biodegradation rate of phenol as well as the effect of particle size and hence mass transfer limitations on the biodegradation rate.

2. Materials and methods

2.1. Preparation of microbial culture

A special strain of the bacterium *P. putida* was obtained in an Amnite (P300) cereal form from Cleveland Biotech Ltd., UK. A 100 g of the cereal was mixed in a 1000 ml of 0.22% sodium hexametaphosphate buffered with Na_2CO_3 to a pH of 8.5. The mixture was homogenized in a blender for about 1 h, decanted and kept in the refrigerator at 4 °C for 24 h. Bacteria slurry was prepared by four consecutive steps of low speed centrifugation at 6000 rpm for 15 min. The supernatants were collected and centrifuged again at 10,000 rpm for 20 min. The precipitated amount from the three centrifugations (which contains the harvested bacteria cells) were collected, suspended as slurry in distilled water and kept in the refrigerator for subsequent immobilization. A similar extraction procedure was found to be effective for the extraction of microorganisms from soil [29].

2.2. Immobilization

Polyvinyl alcohol gel was used for immobilizing the bacteria cells. As a synthetic polymer, PVA has better mechanical properties, and it is more durable than Ca-alginate which is biodegradable and can be subject to abrasion [30]. A homogenous 10 wt% PVA viscous solution was prepared by mixing 100 g of PVA powder with 900 ml of distilled water at about 70–80 °C. The 10% mixture is known to result in a good quality polymer matrix with high porosity [31]. The formed mixture was allowed to cool to room temperature before adding 10 ml of the previously prepared bacterial suspension. It was then well stirred for 10–15 min to insure the homogeneity of the whole solution. The mixture was then poured into special molds and kept in a freezer at −20 °C for 24 h before it was transferred to the refrigerator and allowed to thaw at about 4 °C. This gives the gel lower thawing rate and enhances the crystalline area formation, which increases the mechanical strength of the formed polymer. The freezing–thawing process was repeated 3–4 times for 5 h for each cycle. This improves the cross-linking in the polymerized PVA gel structure. The frozen molds were then cut into the specified sizes, washed with distilled water to remove any uncross-linked chains, and then sent for acclimatization.

Table 1
Composition of nutrient mineral salt medium

Component	Concentration (mg/l)
$MgSO_4 \cdot 7H_2O$	300
K_2HPO_4	250
$CaCl_2 \cdot 2H_2O$	150
$(NH_4)_2CO_3$	120
$FeSO_4 \cdot 7H_2O$	3.5
$ZnSO_4 \cdot 7H_2O$	1.3
$MnCl_2 \cdot 4H_2O$	0.13
$CuSO_4 \cdot 5H_2O$	0.018
$CoCl_2 \cdot 6H_2O$	0.015
$Na_2MoO_4 \cdot 2H_2O$	0.013

2.3. Acclimatization of bacteria

A portion of the cut PVA gel (containing immobilized bacteria) was placed in a 1 l solution containing 100 mg/l of glucose (as an easily biodegradable source of organic compounds) in addition to other essential mineral nutrients (with concentrations shown in Table 1) with continuous aeration. The bacteria cells were activated by gradually increasing the concentration of glucose to 1000 mg/l over a period of 5 days. The activation of the bacteria was confirmed through microscopic analysis as well as observed reduction in the glucose concentration. Once activated, the bacteria cells were then slowly acclimatized to phenol concentrations of up to 300 mg/l. This process was achieved by gradual increase in the phenol concentration (10 mg/(l day)) in combination with a gradual reduction in the glucose concentration. The increase of phenol (accompanied with the decrease of glucose) is made after confirming that the bacteria has consumed all the organics in the previous batch (both glucose and phenol) and washing the PVA gel pieces with distilled water before being placed in a solution containing the new concentrations of phenol and glucose. This process allows the biomass to gradually adapt to the biodegradation of phenol as a substitute organic compound.

Once the maximum concentration (300 mg/l) of phenol is reached, the concentration of glucose would be dropped to zero. At this stage, the bacteria are considered fully acclimatized to phenol with concentrations up to 300 mg/l as the only source of organic compounds. The PVA pellets with the phenol acclimated bacteria were then used for the experimental study to evaluate the effect of different parameters on the biodegradation rate. A 300 ml of these pellets were placed in a 1 liter glass bubble column with 700 ml of synthetic phenol solution. The concentration of the mineral nutrients, which were added at the beginning of each run, was always kept constant at 82.6 mg/l.

2.4. Reagents

Analytical grade phenol was purchased from BDH Chemicals, UK. Synthetic phenol solutions were prepared for the desired concentration in distilled water before each experimental run. The solutions were always kept in brown flasks inside a dark cabinet to avoid light oxidation of the phenol. All other chemicals and PVA powder were of analytical grade and were also obtained from BDH, UK.

2.5. Analytical methods

Phenol concentration in the biomass free samples was determined quantitatively using Chrompack Gas Chromatograph, Model CP9001. The accuracy of the analyzer was checked to be within ±0.5 mg/l and confirmed for low concentrations (less than 50 mg/l) using a Shimadzu UV Spectrophotometer, Model UV-2450. Mea-

surements for each phenol sample were carried out in duplicates and a standard solution was used to recheck the accuracy of the GC after every 4 h of continuous operation. During the acclimatization period, the activity of bacteria was monitored by following the consumption of glucose using HPLC (Alliance, Model 2695).

3. Results and discussion

All experimental results reported in the next sections were based on averaging results of repeated experimental runs (duplicates), with the standard deviation ranging from 2 to 7% of the reported average.

3.1. Effect of temperature and pH

Experiments were carried out to assess the effect of temperature and solution pH on the biodegradation of phenol. All other parameters were kept constant, while varying the temperature from 20 to 40 °C and pH from 4 to 9. The bubble column bioreactor was kept in a water bath, where the temperature can be controlled within ±1 °C. The initial solution pH was adjusted using few drops of either HCl or NaOH depending on the desired value. The total operating volume of the bubble column reactor was fixed at 1 liter; the PVA pellets represented 30% of the reactor volume, with the balance being phenol solution with an initial concentration of 150 mg/l. The initial pH at this concentration was about 7.1.

The reduction in phenol concentration was found to be linear with time, indicating a constant biodegradation rate that depended heavily on temperature. A plot of the degradation rate versus temperature is shown in Fig. 1. Clearly, the activity of *P. putida*, and consequently its ability to degrade phenol, is optimized at about 30 °C. Higher temperatures seemed to negatively affect the activity of the bacteria and hence hindered its biodegradation capabilities. It is believed that sudden exposure to temperatures higher than 35 °C may have detrimental effect on the bacterial enzymes that are usually responsible for the benzene ring cleavage, which is the main step in the biological degradation process. On the other hand, exposure to lower temperatures is expected to slow down the bacterial activity. In addition, the inhibitory effect of phenol on the bacteria is known to be enhanced at low temperatures [25]. This is especially true for high phenol concentrations. The observed sensitivity of the degradation rate to any deviation outside the optimum temperature range was reported in the literature [32] and it is believed to be due the higher production rate of metabolites at 30 °C [15].

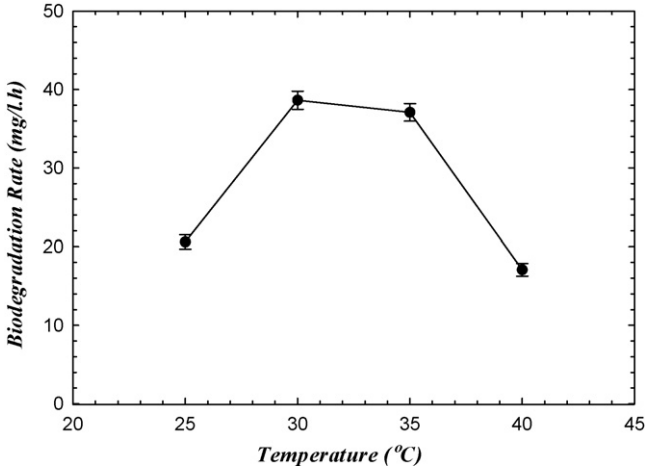

Fig. 1. Variation of biodegradation rate with temperature. Initial pH 7.1; PVA volume = 300 ml; initial phenol concentration = 150 mg/l.

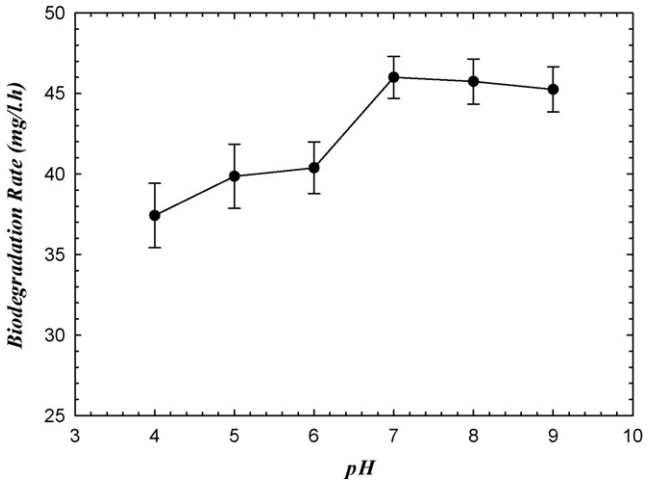

Fig. 2. Effect of the initial solution pH on the biodegradation rate of phenol; PVA volume = 300 ml; initial phenol concentration = 150 mg/l; T = 30 °C.

It is worth noting here that the biodegradation of phenol produces CO_2 as a gaseous product, which contributes to reducing the solution pH due to the formation of carbonic acid. This was observed experimentally for all runs, reducing the pH in most cases by about 2. The experimental results for the effect of the initial solution pH on the concentration and the biodegradation rate of phenol are shown in Fig. 2. These results reveal that the biodegradation rate increases with solution pH reaching a maximum at a pH of 7. The rate declines slightly for pH values higher than 7 and then stabilizes as shown in the figure. These behaviors are consistent with those reported in the literature for the biodegradation of phenol [23].

3.2. Effect of phenol concentration

Initial phenol concentration plays an important role in the biodegradation process, since some hydrocarbon contaminants, including phenol are known to have inhibitory effect on the activity of the biomass. Experiments were carried out at different initial phenol concentrations ranging from 5 to 150 mg/l. The temperature was fixed at 30 °C and the volume of PVA pellets in the reactor was kept at 300 ml or 30 vol% of the total working volume.

Fig. 3 presents the experimental results of the effect of phenol concentration on the biodegradation process. It is noticeable again

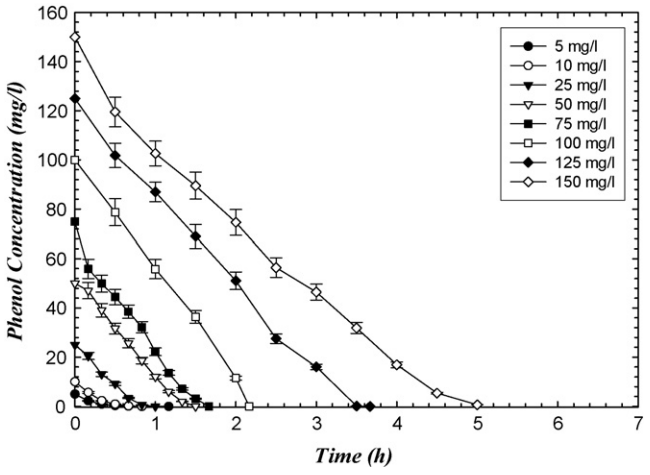

Fig. 3. Variation of phenol concentration with time; PVA volume = 300 ml; T = 30 °C.

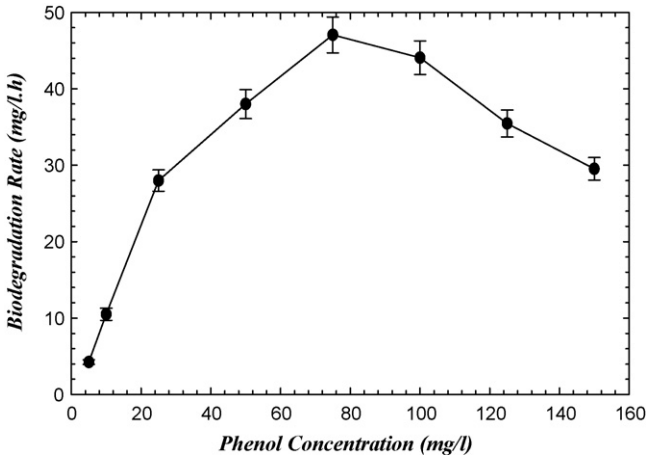

Fig. 4. Biodegradation rate at different phenol concentrations; PVA volume = 300 ml; $T = 30\,^{\circ}C$.

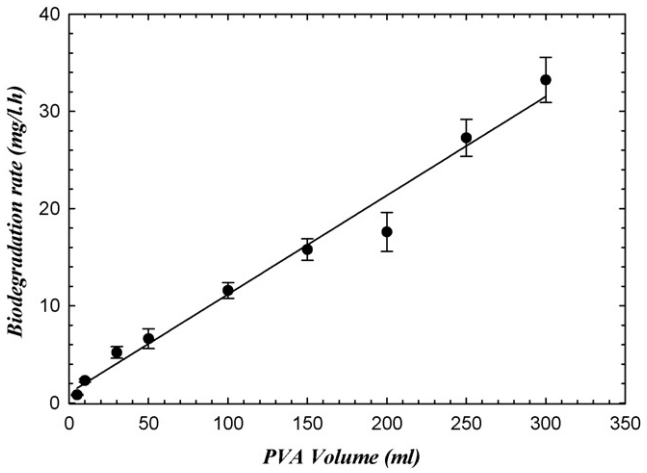

Fig. 5. Variation of biodegradation rate with PVA volume; $T = 30\,^{\circ}C$; initial phenol concentration = 150 mg/l.

that the reduction in phenol concentration is practically linear with time, and therefore, the degradation rate is constant for all initial concentrations of phenol. Although the biodegradation rate does not change with time, it increases with increasing the initial concentration of phenol, reaching a maximum at 75 mg/l as shown in Fig. 4. Any increase in the initial phenol concentration beyond this value reduces the removal rate of phenol. This can be attributed to the inhibitory effect of phenol. On the other hand, the lower biodegradation rate at low phenol concentrations is believed to be due to mass transfer control, where less phenol is accessible for the biomass.

Mass transfer was reported to play an important role as a controlling mechanism for the biosorption of heavy metals at low concentrations [33]. Therefore, it is expected that the biodegradation of phenol may have a similar controlling mechanism.

3.3. Effect of PVA volume

The amount of PVA pellets (particles), which contain the active biomass, in the bioreactor plays an important role in the biodegradation process. Experiments were carried out to evaluate the effect of PVA volume on the biodegradation rate; the initial phenol concentration and temperature were kept constant at 150 mg/l and $30\,^{\circ}C$, respectively. Also, the total working volume of the reactor was kept constant at 1000 ml.

Since the amount of PVA in the reactor can be related directly to the amount of the biomass, it is expected that as the PVA volume increases, the biodegradation rate will increase. This was confirmed experimentally as shown in Fig. 5 for PVA volumes ranging from 5 to 300 ml. Although larger PVA volumes were not tested, it is expected that more PVA particles in the bioreactor will hinder particle movement and mixing and, consequently, reduce the biodegradation rate.

The effect of PVA gel volume can also be expressed in terms of the specific biodegradation rate or the uptake of phenol (mg phenol/(g PVA h)) for different masses of PVA. The optimum phenol uptake was found to be 2.2 mg/(g h) at about 1 g of dry PVA, which is equivalent to a volume of 10 ml of pellets (1% of the total reactor volume). This value was estimated at a fixed rate of aeration. It is expected, however, that this optimum value would change slightly with the rate of aeration. This optimum value represents a very important parameter for economical consideration in designing an industrial process for wastewater treatment.

3.4. Effect of PVA particle size

In this part of the study, attempts were made to evaluate the effect of PVA pellet size on the biodegradation rate of phenol. A 300 ml of PVA cubes were divided into three equal parts. Pellets in the first part (100 ml) were left at their original size (about 10 mm); those in the second part were divided into two equal halves; while those in the third group were divided into four equal parts (quarters). Batch experiments were then carried out for two initial phenol concentrations (5 and 150 mg/l) at $30\,^{\circ}C$.

The reduction in phenol concentration for the three pellet sizes is shown in Fig. 6. It is clear that pellet size has no effect on the biodegradation of phenol for high initial phenol concentration. This is expected since PVA particles are known to have high porosity and hence the resistance to mass transfer is negligible especially for high bulk concentrations as is the case here. However, for low initial phenol concentration, the biodegradation rate was slightly improved by reducing the pellet size as shown in the figure. The particle movement and hence mixing inside the bioreactor were improved for smaller particles. This highlights the importance of external diffusion as a controlling mechanism, which usually dominates at low bulk concentrations.

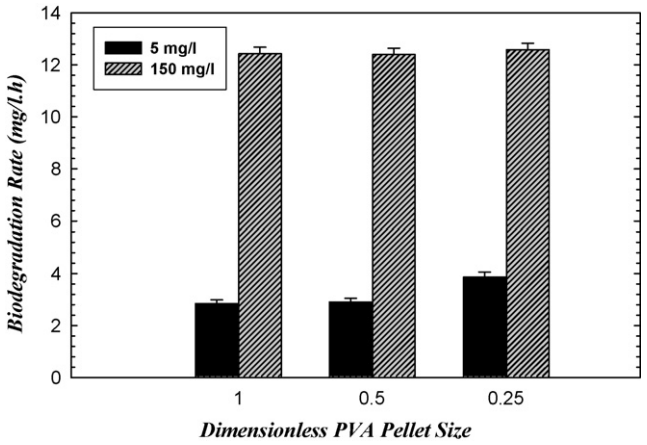

Fig. 6. Biodegradation of phenol for different PVA pellet sizes and different initial phenol concentrations; PVA volume = 100 ml; $T = 30\,^{\circ}C$.

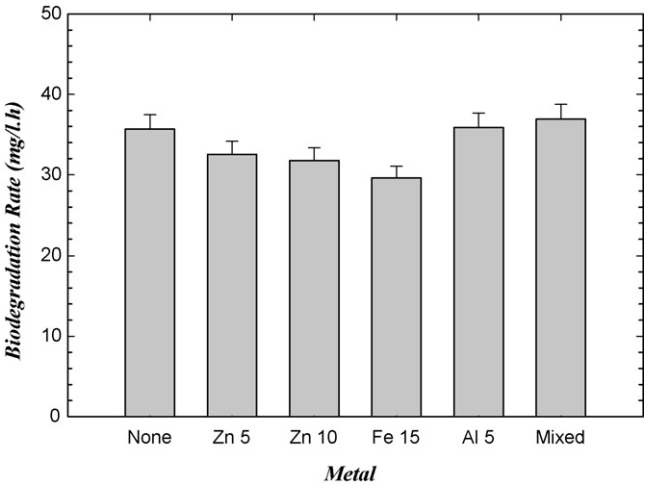

Fig. 7. Effect of heavy metals on the biodegradation rate; initial phenol concentration = 150 mg/l; PVA volume = 300 ml; $T = 30\,^{\circ}C$.

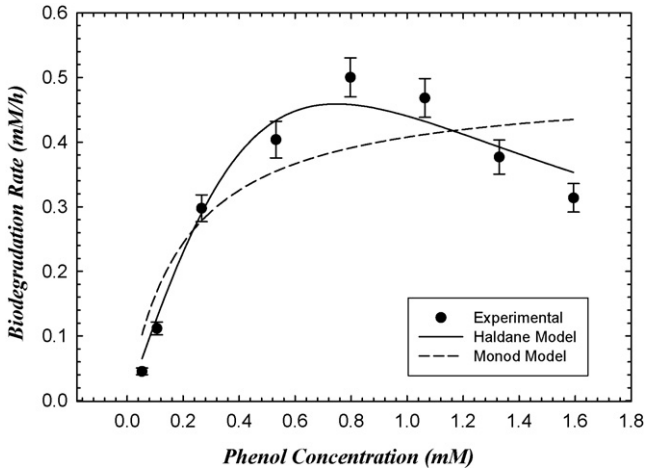

Fig. 8. Comparison of the experimental data with fitted models; PVA volume = 300 ml; $T = 30\,^{\circ}C$.

3.5. Effect of other contaminants

The presence of other contaminants in the wastewater can have detrimental effects on the activity of the bacteria and therefore may hinder its ability to biodegrade phenol. Chemical analysis of a typical refinery wastewater indicated that it contained other major contaminants, including sulfates and heavy metal ions such as iron, aluminum and zinc. The effect of these contaminants on the biodegradation of phenol was assessed for different concentrations. The experimental results clearly indicated that these contaminants had negligible effect on the biodegradation rate of phenol as shown in Fig. 7.

It is important to note here that the concentrations of these contaminants were chosen to be within or slightly higher than those found in a typical refinery wastewater. Although iron seems to have a slightly negative effect on the biodegradation rate (Fig. 7), this can be neglected since the biodegradation rate seems to be unaffected when the bacteria are exposed to a mixture of these metals ions as shown in the figure.

The effect of sulfates on the biodegradation rate was also assessed using sodium sulfate at concentrations ranging from 100 to 1000 mg/l. The experimental results indicated that the addition of sodium sulfate had no effect on the biodegradation rate even at the high concentration of 1000 mg/l. The concentrations of heavy metals and sulfates were also measured at the end of each experimental run and found to be unchanged, with the exception of a slight reduction (about 10%) in the concentration of iron, which was attributed to adsorption on the PVA. This was confirmed by carrying out similar experiments with blank (bacteria-free) PVA particles that showed the same reduction in the concentration of iron. These blank experiments also confirmed that other factors such as phenol evaporation and adsorption on the PVA pellets did not have any contribution to the reduction of phenol concentration and the overall biodegradation rate.

3.6. Modeling of the biodegradation process

Modeling any biodegradation process involves relating the specific growth rate of the biomass to the consumption rate of the substrate (contaminant). Based on material balance, the specific consumption rate of the substrate can be expressed as follows:

$$Q_S = -\frac{dS}{X\,dt} = \frac{\mu}{Y} \tag{1}$$

where Q_S is the specific consumption rate (mg/(mg h)); S is the substrate concentration (mg/l); X is the biomass concentration (mg/l); Y is the cell mass yield (g/g); μ is the specific growth rate (h^{-1}).

Two of the most widely used models for the biodegradation of phenol are the Monod model and the Haldane model. The first considers phenol as non-inhibitory compound and, therefore, neglects the inhibitory effect; whereas the second takes into consideration the inhibitory effect of phenol. The Monod model can be expressed as

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

The Haldane inhibitory model can be expressed as

$$\mu = \frac{\mu_{max} S}{K_s + S + (S^2/K_i)} \tag{3}$$

where μ_{max} is the maximum specific growth rate (h^{-1}); K_s is the half saturation coefficient (mg/l); K_i is the inhibition constant for cell growth (mg/l).

These two models can be used to predict the variations of the biodegradation rate (Q) with initial phenol concentrations, utilizing the relation in Eq. (1) and assuming that Y is constant over the concentration range. This assumption is valid if the phenol concentration is much higher than K_s (i.e. $S \gg K_s$). The two models were fitted to the experimental data using SigmaPlot non-linear regression [34], which uses the Marquardt–Levenberg algorithm [35] to find the parameters that give the best fit between the data and the model equations. The models are compared with the experimental data in Fig. 8. The Haldane inhibitory model obviously gives a much better fit than the Monod model. This reiterates the main finding that phenol has a considerable inhibitory effect on the biodegradation capabilities of *P. putida*, especially at concentrations higher than 75 mg/l.

4. Conclusions

Batch experiments were carried out in a bubble column bioreactor to evaluate the biodegradation of phenol by *P. putida* immobilized in PVA gel pellets at different conditions. The experimental results indicated that the biodegradation capabilities of *P. putida* are highly affected by temperature, initial phenol concentration and the abundance of the biomass. The biodegradation rate is optimized at a temperature of 30 °C and an initial phenol concentration of 75 mg/l. Higher phenol concentrations inhibit the biomass and reduce the biodegradation rate. Although the biodegradation

rate increased linearly with the amount of PVA, the phenol uptake per mass of PVA reached a maximum at a PVA volume of 10 ml within a total volume of 1 liter. At high phenol concentration, the PVA particle size was found to have negligible effect on the biodegradation rate. However, for low concentrations, particle size seemed to show a small effect, which highlights the importance of mass transfer-controlled mechanisms. An extensive evaluation of mass transfer effects utilizing effectiveness factor will be carried out in future studies. Other contaminants such as heavy metals and sulfates showed no effect on the biodegradation process. Modeling of the biodegradation of phenol indicated that the Haldane inhibitory model gave better fit of the experimental data than the Monod model, which ignores the inhibitory effects of phenol. These experimental results can surely be used to design a practical and economical biodegradation process for the removal of phenol from wastewater.

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