Continuous Degradation of Phenol at Low Concentration using Immobilized *Pseudomonas putida*

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Received 2 December 1998; received in revised form 18 May 1999; accepted 1 June 1999

Abstract

Continuous degradation of phenol from 100 mg L^{-1} to concentrations as low as 2.5 mg L^{-1} using immobilized Pseudomonas putida was established. Increase in dilution rate increased degradation rate but only to 0.6 h^{-1} beyond which effluent phenol concentration began to rise. However, effluent concentration could be held to <10 mg mL^{-1} by proportionally increasing bead quantity when the dilution rate was increased. Under such operation, phenol was removed at rates which increased in proportion to the increase in dilution rate, with 108 mg L^{-1} h^{-1} being the highest degradation rate achieved. At dilution rates above 0.3 h^{-1}, the immobilized cell system studied was better at degrading phenol than a free cell system. Superiority of the immobilized cell system was more pronounced the higher the dilution rate used. Other findings were that pH 5.5 to 6.0, temperatures between 25 - 30°C, and a bead diameter between 1 and 2 mm were found to be optimal for phenol degradation at low levels.

Keywords: Pseudomonas putida; Phenol; Degradation; Immobilization

1. Introduction

Studies on microbial means of treating phenolic effluents date back at least two decades. Although phenol is toxic to fish at concentrations as low as 5 mg L^{-1} [1], most studies have been on the degradation of much higher concentrations. Few authors have presented work on phenol degradation at starting concentrations lower than 250 mg L^{-1} [2-4].

Phenol degradation can be achieved in either batch or continuous mode. In both cases, the major determinant of the rate of degradation is cell number. In continuous culture, the influent phenol concentration and dilution rate controls cell number. When the influent concentration is low, cell number will be corresponding low which in turn means that degradation rates will be low. In order to maintain a high cell number, continuous cultures need to operate at high dilution rates when the influent concentration is low. However, in a free cell system the maximum dilution rate that can be achieved is limited by the need to prevent cell washout. The use of immobilized cells increases the maximum dilution rate that can be achieved before cell washout occurs. Thus, continuous culture with immobilized cells should be capable of degrading phenol at low concentrations because cell numbers can potentially be maintained through the use of higher dilution rates.

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An assessment of previous biodegradation studies with high initial phenol concentrations (>400 mg mL^{-1}) in continuous culture reveals two features. Firstly, what might be considered steady state effluent concentrations of phenol were rarely less than 5 mg mL^{-1} (toxic to fish at this concentration). Secondly, for the most complete removal of phenol, the higher the influent concentration, the smaller the dilution rate needs to be. For example at an influent concentration of 1000 mg mL^{-1}, an effluent concentration of 170 mg mL^{-1} can be achieved if the dilution rate is kept to 0.144 h^{-1}[4]. Another study reported influent and effluent concentrations to be 1000 and 15 mg mL^{-1} respectively at a dilution rate of 0.0072 h^{-1} [5]. Thus, under such conditions, the treatment of high concentration phenol wastewater will be typified by small hydraulic throughputs. A cascade continuous bioreactor system is probably more suitable for treating such wastes where the concentration is reduced step-wise in sequential vessels. Accordingly, studies on the degradation of phenol at concentrations nearer the desired final effluent concentration are worthwhile.

This paper reports on studies of the biodegradation of phenol at an influent concentration of 100 mg L^{-1}, a level lower than has been commonly investigated. *Pseudomonas putida* was immobilized in calcium alginate beads and its capacity for phenol degradation under various dilution rates, bead diameter, pH, and temperatures was studied. A comparison was also made between immobilized cells and free cells in phenol degradation.

The influence of various reaction parameters on phenol degradation at low concentrations has not been widely studied, and it is not known whether the responses seen at high phenol concentrations, will also occur at low concentrations. Thus, effect of dilution rate, reaction temperature and pH were

investigated, and it was thought possible to maintain a steady effluent phenol concentration despite increases in dilution rate, if the quantity of beads was increased in proportion to the increase in dilution rate.

As the diameter of gel beads immobilizing living cells is increased, the problem of mass and gas transfer in the gel matrix becomes increasingly significant. Further, micro-environments can develop within beads and these may alter intra-particle growth, metabolism and product formation [6,7]. A bead diameter of 1 mm is suggested if uniform distribution of cells within the beads is desired [8]. Other studies indicate that cell growth is restricted to the outer 0.3 - 0.5 mm of a bead [7]. At low substrate concentrations, the concentration gradient within beads may be so poor that sufficient substrate may not be available for cellular processes. The obvious answer to the problem is to decrease bead diameter. Therefore, examination of bead diameter may allow improvement in reaction rates.

2. Materials and Methods

2.1 Microorganism

The culture used was *Pseudomonas putida* ATCC 11172 from the American Type Culture Collection (Maryland, USA). The culture was maintained on Nutrient Agar slants at 25°C.

2.2 Inoculum Production Medium (IPM)

The medium used for inoculum production was the mineral salts medium of Hill and Robinson [3]. It comprised (mg L-1): phenol, 500; KH2PO4, 840; K2HPO4, 750; (NH4)2SO4, 488; NaCl 60; CaCl2, 60; MgSO4, 60; FeCl3, 60. The phenol was added after autoclaving. For the preparation of IPM agar plates , 11.0 g L-1 of agar was added.

2.3 Reaction Mediu.

The components of the Reaction Medium were (mg L-1): phenol, 100; KH2PO4, 105.5; K2HPO4, 95; (NH4)2SO4, 488; NaCl, 60; CaCl2, 2775; MgSO4, 60 and $FeCl_3$, 60.

2.4 Phenol determination

Phenol concentrations were determined by a modified colorimetric technique, based on a standard method for phenol [9]. Twenty-fold dilutions were made to the reagents ammonium hydroxide, potassium ferricyanide and 4-aminoantipyrine, to enable the use of 5 mL phenol samples instead of 100 mL. The modified method was confirmed to have a comparable linear response to the original method between 0 and 10 mg mL^{-1} of phenol.

2.5 Sterilization

All media and apparatus were autoclaved at 121°C for 15 minutes.

2.6 Production of inoculum for use in the preparation of immobilized cells

Three 3-day old colonies of *P. putida* incubated at 25°C on IPM agar were transferred into 5 mL of IPM and incubated statically for 48 h at 25°C. This culture was subsequently transferred into a 250 mL screw-capped Erlenmeyer flask containing 45 mL of IPM. The loosely capped flasks were incubated for 24 h in an orbital shaker at 25°C and 150 rpm.

2.7 Production of immobilized cells.

Inoculum culture from the previous step was mixed into 3% w/v alginate (Manugel GMB kindly supplied by Kelco AIL, Sydney). The ratio of inoculum culture to alginate solution was 1:9. Beads were produced by pumping the inoculum/alginate suspension through a hypodermic needle from a height of 5 cm into a slowly stirred solution of 0.1 M $CaCl_2$. The type of needle used routinely was Terumo Neolus 25G which produced beads approximately 2 mm in diameter. Where bead size was varied for one study, 18G was used for 3 mm beads and 30G for 1 mm beads. Beads were allowed to harden in the $CaCl_2$ for approximately 30 min, after which they were washed twice in distilled water, drained and placed directly into the bioreactor with Reaction Medium. The amount of immobilized cell material available for any reaction was quantified by the volume of the inoculum/alginate mix used to make the beads.

2.8 Bubble column bioreactor.

The glass bioreactor was 3.0 cm in internal diameter and 410 mL in working volume. Continuous culture was effected by the addition of fresh medium through a port at the top of the bioreactor using a pump and spent medium left via an overflow weir. The bioreactor was fluidized and aerated via a downward pointing sparger (single-orificed, 0.5 mm in diameter) 0.5 cm from the bottom of the bioreactor. Air was filtered online with a sterile 0.2 μm cellulose acetate filter (Millipore Corp., Massachusetts, USA) to exclude microorganisms. Air was humidified by passage through a vessel containing sterile distilled water. Loss of liquid in the column was minimized by manipulating the temperature of the humidifier.

2.9 Reaction conditions.

Reaction pH was maintained by the automatic addition of 0.1 M HCl or 0.1 M NaOH regulated by a pH controller. The electrode was sterilized by immersion in 70% (v/v) ethanol for 15 min before use. Electrode drift was corrected daily by comparisons of pH controller readings against an

external pH meter. Air was supplied to the column at 1.0 L min.$^{-1}$. Reaction temperature was maintained by water flow from a recirculating water bath through the water jacket of the reactor. Temperature was maintained at 25°C, pH at 6.5, the ratio of bead volume to volume of medium was 1:11, and the dilution rate was 0.6 h^{-1} unless otherwise indicated. In order that performance of the beads is as uniform as possible (with minimal effect from cell history), newly prepared batches of beads were always used for each level of a reaction parameter being tested. An effluent phenol concentration that is maintained for at least six residence times was deemed to be the steady state value that phenol concentration was attenuated down to. All the effluent phenol concentrations reported and phenol degradations rates derived therefrom, were at steady state. Five millitre samples were collected from the efflux stream and cells were removed using 0.22 μm Millipore GS membrane filters. After determination of its pH, the sample was assayed for phenol.

3. Results

Phenol degradation rate, *R*, was used as a measure of the rate of phenol removal.

$$R = (S_i - S_e) \cdot D$$

Where: S_i = influent phenol concentration

S_e = effluent phenol concentration at steady state

D = the dilution rate

In common with most other studies on phenol biodegradation, the unit used for *R* in this paper is mg L^{-1} h^{-1}. This unit can be converted to kg m^{-3} day^{-1} (amount degraded per unit reactor volume per unit time) using a multification factor of 0.024.

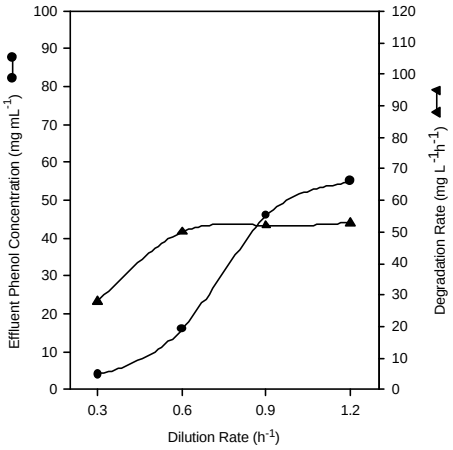

Fig. 1. The effect of dilution rate on the effluent concentration and rate of degradation of phenol by immobilized *Pseudomonas putida* ATCC 11172.

Degradation rate of phenol responded to dilution rate in two steps. Degradation rate increased to a maximum at a dilution rate of 0.6 h^{-1} and did not respond to higher rates up to 1.2 h^{-1} (Fig. 1). The lack of response of degradation rate to increased dilution rate was accompanied by a corresponding increase in phenol concentration in the effluent. thus allowing effluent phenol concentration

In the system tested, dilution rate can be increased from 0.15 to 1.2 h^{-1} without increasing effluent phenol concentrations (maintained relatively steady around 10 mg mL^{-1} or below) as long as the quantity of beads was also increased in proportion (Fig. 2). The increases in bead quantity increased degradation rate linearly in direct proportion to increment in dilution rate, to be held steady.

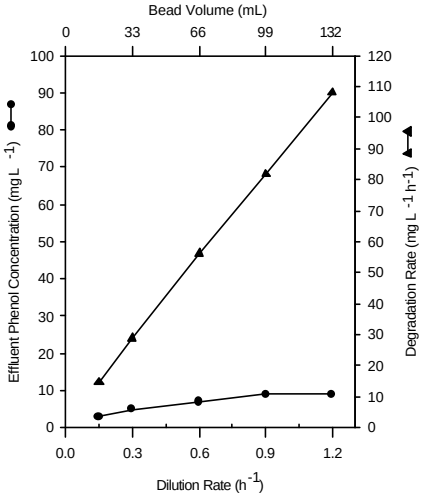

Fig. 2. The effect of increasing bead volume in direct proportion to dilution rate on the effluent concentration and rate of degradation of phenol by immobilized *Pseudomonas putida* ATCC 11172.

At dilution rates between 0.6 and 1.2 h^{-1}, immobilized cells were better than free cells in degrading phenol *i.e.* degradation rates were higher and effluent concentrations of phenol were lower (Fig. 3 *cf.* Fig. 1). With higher dilution rates, free cells began to form large flocs. This caused some fluctuation in the total volume of reaction because the overflow outlet was intermittently impeded. At the highest dilution rate, growth appeared rapidly on the bioreactor walls and so the results at this rate are not considered to be indicative of truly free cell bioreaction.

Degradation of phenol was best when pH was held between 5.5 and 6.5 (Fig. 4) with effluent phenol concentration at a minimum of <10 mg mL^{-1} on the more acidic side of the band. Tolerance to acidic conditions fell sharply between pH 5.0 to 5.5. Half a pH unit separated optimal degradation and almost non-existent degradation (effluent phenol

concentration approached the influent concentration at pH 5.0). Degradation also diminished above pH 6.5 although not as sharply as below pH 5.5.

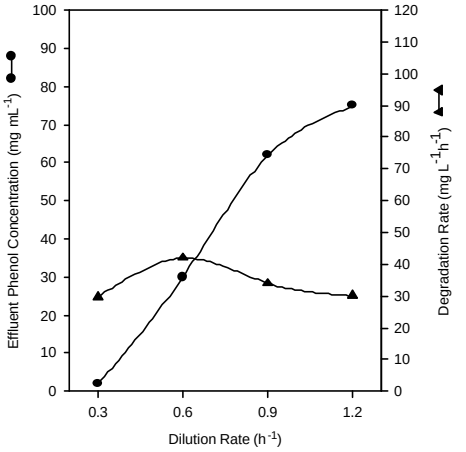

Fig. 3. The effect of dilution rate on the effluent concentration and rate of degradation of phenol by free cells of *Pseudomonas putida* ATCC 11172.

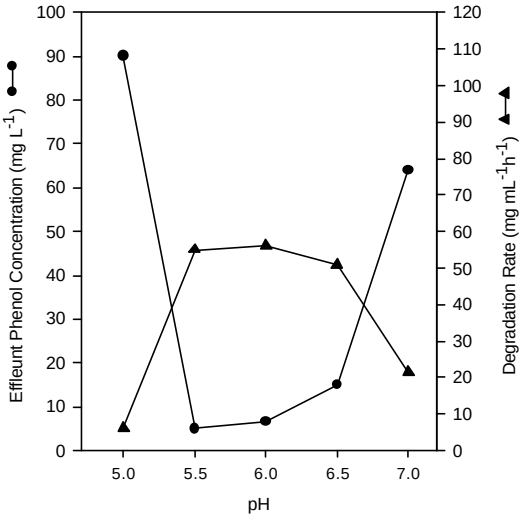

Fig. 4. The effect of pH on the effluent concentration and rate of degradation of phenol by immobilized *Pseudomonas putida* ATCC 11172.

The temperature profile indicates an optimum between 25°C and 30°C for phenol degradation (Fig. 5) where effluent phenol concentrations were again below 10 mg mL^{-1} when at optimum. Higher temperatures appear more detrimental to phenol degradation than do lower temperatures.

Differences in bead diameter in the range tested had apparently marginal effect in absolute terms for measured phenol concentration and degradation rate (Fig. 6).

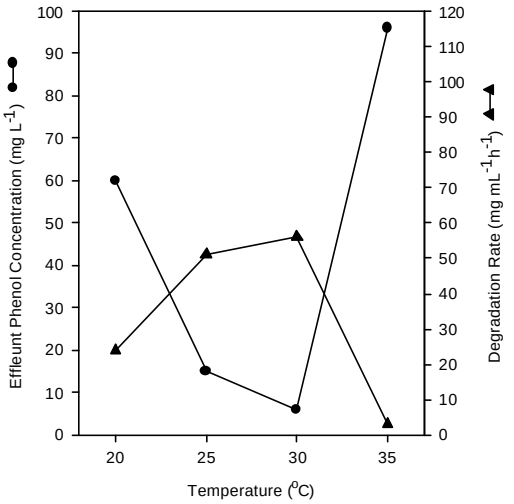

Fig. 5. The effect of temperature on the effluent concentration and rate of degradation of phenol by immobilized *Pseudomonas putida* ATCC 11172.

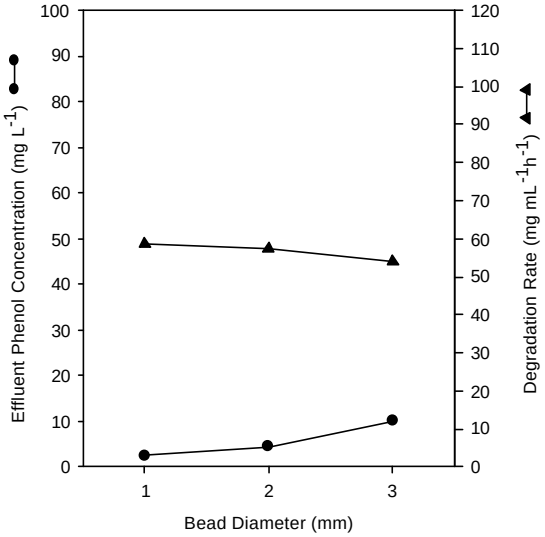

Fig. 6 The effect of bead diameter on the effluent concentration and rate of degradation of phenol by immobilized *Pseudomonas putida* ATCC 11172.

Bead diameter (mm)	Std. error of S_e (mg L^{-1})	Std. error of R_s (mg L^{-1} h^{-1})
1	±0.09	±0.05
2	±0.20	±0.10
3	±0.40	±0.20

However, in relative terms, if 1 mm is considered as the starting size, each millimeter increase in diameter was accompanied by an

approximate doubling of the effluent phenol concentration. The surface area to volume ratio of a bead and the number of beads are critical determinants of the degradation capacity of the bioreactor. Surface area to volume ratio may be expressed as:

$$\frac{A_b}{V_b} = \frac{4\pi r^2 N_b}{V_b} \qquad (1)$$

and the number of beads as:

$$N_b = \frac{V_b}{\frac{4}{3}\pi r^3} \qquad (2)$$

Therefore, substituting for N_b in (1) :

$$\frac{A_b}{V_b} = \frac{4\pi r^2}{V_b} \frac{V_b}{\frac{4}{3}\pi r^3}$$

which simplifies to :

$$\frac{A_b}{V_b} = \frac{3}{r} \qquad (3)$$

where A_b = Total bead surface area

N_b = Number of beads

V_b = Total volume of beads

r = bead radius

Therefore, the surface area to volume ratio of the beads is proportional to the reciprocal of bead radius and thus the bead diameter. When examined in relation to the reciprocal of bead diameter, the change in degradation rate was not linear, unlike the change in the surface area to volume ratio (Fig. 7). Degradation rate improved as diameters were reduced from 3 mm but this improvement was halted as diameters approach 2 and then 1 mm. Standard error for the *Se* and *R* data points in Figures 6 and 7 were calculated by using values from 6 different samples taken at steady state. The resultant error bars proved too small for the figures and were therefore tabulated in the figure legends.

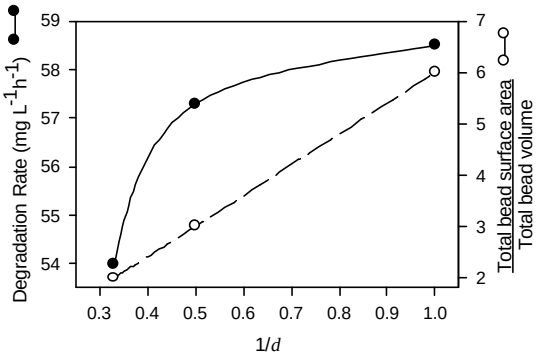

Fig. 7 The relationship between bead diameter and degradation rate of phenol by immobilized *Pseudomonas putida*. (*d* = bead diameter).

1/*d*	Std. error of R_s (mg L^{-1} h^{-1})
0.33	±0.05
0.50	±0.10
1.0	±0.21

4. Discussion

Increase in dilution rate increased degradation of phenol but only when it was kept below 0.6 h^{-1} beyond which effluent concentration of phenol began to rise (Fig. 1). This saturation in the response curve was also encountered by Lakhwala et al [4] who attributed the effect to an increase in oxygen demand by a cell population which had increased in size. However, the experiment where bead volumes were increased in proportion to increased dilution rate (Fig. 2), suggests differently. Degradation rate was found to respond linearly when bead volumes and dilution rate were increased together. Degradation rates are an indication of cell numbers on the basis of the relationship between substrate consumption and cell numbers. Thus, it could be said that total population size in the reactions increased in direct proportion to increased dilution rate and bead volumes. This was despite aeration rates that were kept the same across the different dilution rates and bead volumes examined. Thus, a better explanation for the response found with a fixed number of beads when dilution rate is increased may simply be that the cells were substrate saturated. This saturation appears to be relieved when the total population size is increased such as by using more beads. Within the range of dilution rates tested, there appeared to be no factor limiting phenol degradation rate when bead volumes were kept in proportion. However, it is likely that at some rate higher than 1.2 h^{-1}, aeration rate (oxygen supply and bead fluidization), bioreactor geometry (mixing and gas transfer), and washout of beads will no longer allow a linear response of degradation rate to

increased dilution rate.

The immobilized cell system was better for degrading phenol than the free cell system at dilution rates >0.3 h^{-1} (Fig. 2 *cf.* Fig. 3). Superiority of the immobilized cell system is more pronounced the higher the dilution rate used. This superiority is probably based on higher (on the basis of the amounts of phenol degraded) total cell numbers available for bioreaction (assuming that any beneficial physicochemical effect of immobilization is of lesser significance). The loss of degradation rate with free cells at dilution rates greater than 0.6 h^{-1} was probably related to two factors. The first was the observation of the formation of flocs the size of which became larger the higher the dilution rate used. Flocs would conceivably be less efficient than free cells in phenol degradation on the basis of mass transfer and gas exchange. Secondly, at the higher dilution rates, washout especially of the unflocculated cells would contribute to loss of total degradative capability.

Little difference in the ability of immobilized *P. putida* to degrade phenol was observed between pH 5.5 and 6.5. However, the bacterium is sensitive to small changes outside the limits where a deviation of half a pH unit resulted in a minimum of 50% drop in degradation rate. This indicates that regulation of pH must be a feature of phenol degradation for effluents with pH <5.5 or >6.5.

Phenol degradation proceeded optimally between 25 and 30°C. As with pH, the system was relatively sensitive to deviations outside the optimal range where a 5°C change at either end of the range resulted in decreases in phenol degradation rate of at least 50% at the lower end, and almost 100% at the higher end. As with pH, temperature control is indicated for phenol biodegradation systems to maintain high performance.

That degradation rate should decrease when bead diameter is increased is predictable on the basis of lower mass and gas transfer because of increased distances in the gel matrix and the decrease in the surface area to volume ratio of the bead. This probably explains the claim that cell growth is restricted to the outer 0.3 to 0.5 mm of bead [7], at least for larger beads. This being the case, the poorer degradation rates of larger beads (when the total bead volume is kept the same) would be due to relatively smaller cell populations per bead. The implication is that smaller diameters are better. However, it is also predictable as this study suggests, that an optimal bead diameter exists lesser than which improvement of degradation rates ceases. An explanation for this can be made on the basis that below a certain bead diameter, diffusion across the gel matrix will cease to be an influence. A diameter of 1 mm has been suggested if beads are to be uniformly [8] and maximally populated. Below the critical bead diameter, reaction rates within the beads would be similar, and because the total bead volume was kept constant in this study, changes in bead diameter would simply change how the total cell population is distributed amongst beads but not the population size. This means that reactions would be mediated by the same total number of cells resulting in similar degradation rates. A further speculation is that in beads of less than critical diameter, conditions for the immobilized cells would approach those available to free cells, thus obviating the physicochemical differences that might be beneficial for bioreaction.

Effluents are likely to contain substances other than phenol. The performance of the system reported may be attenuated in the presence of metabolic inhibitors or competing substrates and any such effect may be studied using concocted effluents containing a range of selected materials. Of the few reports on biodegradation at comparable influent phenol concentrations using real effluent, degradation rates estimated from data published were 3.8 mg L^{-1} h^{-1} (oil shale ash leacheate; *Pseudomonas putida;* aerobic process) [10] and 22.5 mg L^{-1} h^{-1} (phenolic wastewater; unspecified acclimated-phenol bacterial population; aerobic process) [11]. Thus, the best degradation rate reported in this paper of 58. 5 mg L^{-1} h^{-1} (Fig. 6) are significantly better and is indicative of the potential of an unattentuated system.

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