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Aerobic batch degradation of phenol using immobilized *Pseudomonas putida*

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The degradation rate of phenol by alginate-immobilized *Pseudomonas putida,* was little affected by alginate concentration between 2 and 4%. Ten degree shifts from 25°C in reaction temperature resulted in approximately 30% slower degradation. Maximal degradation rates appeared to be favored at pH 5.5 - 6.0. The response of degradation rate to increased air flow in the bubble column used was almost linear and an optimal higher than 16 vol.$^{-1}$ vol.$^{-1}$ was indicated although free cells appeared in the reaction medium above 12 vol.$^{-1}$ vol.$^{-1}$. When the initial phenol concentration was raised, degradation rate was not significantly affected until levels higher than 1200 mg mL^{-1} where performance was markedly reduced. Increasing the ratio of bead volume to medium gave progressively smaller increases in degradation rate. At a medium volume to bead volume ratio of 5:1, the maximum degradation rate observed was 250 mg L^{-1} h^{-1}.

Keywords: biodegradation; phenol; *Pseudomonas putida*; immobilized

Introduction

Phenol is a toxic compound even in low concentrations. It is frequently found in the wastes from many modern industrial processes. This can be in the form of atmospheric pollution or in liquid waste. The most common sources of phenol are in the effluents of oil refineries, paper processing plants, resin production, and coal liquefaction [21]. Phenol is typically found in concentrations up to 1.5 g L^{-1} but this can rise to 4.5 g L^{-1} in very polluted waters [5]. It is recommended that exposure should not be more than 20 mg of phenol in an average working day [15]. Phenol is very toxic to fish and has been lethal at concentrations of between 5 and 25 ppm while concentrations as low as 0.1 ppm in surrounding waters can taint the taste of fish [15]. Therefore, the treatment of phenol effluent is important. Numerous microorganisms have been used in the aerobic degradation of phenol. These include *Cryptococcus elinovii* [19, 22], *Fusarium flocciferum* [1, 2], and *Pseudomonas putida* [8, 12, 18]. Of these microorganisms, *P. putida* has been reported to be capable of high rates of phenol degradation [11].

Free cells of *P. putida* were found to tolerate phenol to a concentration of 1.5 g L^{-1} above which more than 50% of the cells were killed [4]. In the same study, cells immobilized in calcium alginate were claimed to degrade 3 g L^{-1} without any obvious inhibition. Fifty percent of free cells of *Escherichia coli* were killed at phenol concentrations below 0.5 g L^{-1} [13]. However, when the *E. coli* was immobilized in calcium alginate, the cells were only inhibited at phenol concentrations above 1.0 g L^{-1}. Therefore, immobilization confers a distinct advantage in the degradation of phenol. This paper reports shake flask and bubble column investigations on the effect of gel polymer concentration, bead/medium ratio, aeration/mixing, temperature, pH and phenol concentration on the batch degradation of phenol by *P. putida* immobilized in alginate beads.

The concentration of sodium alginate commonly used in the preparation of

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immobilized cells has been shown to affect the properties of the gel [9, 14]. However, little research has been performed on the effect of sodium alginate concentration on the degradation of phenol. In a free cell system, cell density will be relatively homogeneous in the medium and cell number in that homogeneity will be determined by substrate concentration. In immobilized cell systems, cell number will also be determined by substrate concentration but the distribution of the cells is determined by the number of immobilized aggregations such as beads. Thus, the ratio of medium volume to bead number (measured as volume in this study) is an important consideration in determining reactor performance. The optimization of aerobic processes with immobilized cells may be complicated due to the problem of providing sufficient oxygen to the cells [20]. Cells entrapped in gels appear to grow only in a narrow outer shell of the bead due to these oxygen limitations [4, 20]. In this study, the rate of air flow was varied. This air flow served both as a source of oxygen and for agitation or mixing of the medium. The agitation of immobilized cells has been reported to cause problems with bead abrasion [4]. Therefore, the optimization of the airflow may be a compromise between maximizing oxygen supply while minimizing the abrasion of the beads. In developing effluent treatment systems, high tolerance to the substrate to be degraded and high degradation rates are obviously desirable. It has been shown that an increase in phenol concentration disproportionately increases the time required for biodegradation [4, 22].

Materials and Methods

Microorganism. *Pseudomonas putida* ATCC 11172 was used for this work. Cultures were maintained on Nutrient Agar (NA) slopes for short term storage and L-dried [16] for the long term.

Media. A Mineral Salts Medium (MSM) [11] was used as the standard growth medium. MSM comprised (mg L^{-1}): KH_2PO_4, 840; K_2HPO_4, 750; $(NH_4)SO_4$, 488; NaCl, 60; $CaCl_2$, 60; $MgSO_4$, 60; and $FeCl_3$, 60. Filter (0.22 μ Millipore GS; Millipore Corp., Bedford, MA, U.S.A.) sterilized phenol was added after the medium was autoclaved (121°C; 15 minutes). Media concentrations were adjusted to compensate for the volume of water in beads in all immobilized cell experiments.

Inoculum. All inocula were incubated at 25°C. A 48 h old, isolated colony on NA was inoculated into 5 ml of MSM (500 mg L^{-1} phenol) in a 20 mL McCartney bottle. After 48 h, the culture was transferred into 45 ml MSM (500 mg L^{-1} phenol) in a 250 ml Erlenmyer flask (cotton sheik closure). Flasks were incubated in a New Brunswick Innova 4330 gyratory shaker (orbit diameter of 3 cm) at 150 r.p.m. for 24 h after which the culture was ready for use for the preparation of immobilized cells.

Immobilization. All equipment and materials used in immobilization were sterilized at 121°C for 15 minutes. Sodium alginate (Manugel GMB, kindly supplied by Kelco AIL, Sydney, Australia) at 2% (w/v) was used except in the study on the effect of alginate concentration where 2, 3 and 4% were tested. After sterilization of the alginate, inoculum was added at a rate of 10% (v/v) at room temperature. The alginate/cell mixture was aseptically extruded through 25G (Neolus, Terumo) needle into a stirred solution of sterile 0.1 M calcium chloride. The height of the needle and rate of stirring of the calcium chloride solution were adjusted so that spheroidal beads were obtained. Beads were left to harden in the calcium chloride solution for 15 minutes. The amount of beads containing immobilized *P. putida* used in each batch run was quantified as the volume of the alginate/bacterial mix prior to gelation (referred to as the total bead volume).

Phenol degradation. The effect of alginate concentration used in immobilization was studied using triplicated shake flask cultures where 50 mL of MSM (500 mg L^{-1} phenol) were added to 20 mL of beads in 250 mL Erlenmeyer flasks. Flasks were incubated at 25°C and shaken at 150 r.p.m. in a New Brunswick Innova 4330 gyratory incubator. All other studies were conducted using a bubble column (internal diameter = 3.0 cm; single sparger orifice = 0.5 mm; height of sparger orifice from reactor bottom = 0.5 cm). Bubble column batch runs typically used 50 mL of beads to 125 mL of MSM except in the study of the effect of total bead volume to medium volume ratio when medium volume was varied. MSM contained 500 mg L^{-1} of phenol except in the study on the effect of phenol concentration. Air was supplied at 8 vol. vol.$^{-1}$ min^{-1} except in the study on the

effect of the rate of air flow and that on the effect of medium volume to total bead volume ratio (in calculating the rates of air flow, volume of the medium without beads was used). In the latter case, the standard air flow was 8 vol. vol.$^{-1}$ min^{-1} at a medium volume to total bead volume ratio of 5 and flow was adjusted proportionally when the medium volume was changed. Reaction temperature was held at 25°C except in the study on the effect of reaction temperature. The pH of the reaction was uncontrolled except in the study on the effect of medium pH where a pH controller adding 0.1 M H_2SO4 or 0.1 M NaOH maintained the set pH. In both shake flask and bubble column studies immobilized *P. putida* was allowed to be acclimated to the reaction conditions by incubation in at least one batch culture before measurements were taken in subsequent batch runs. Between batches, under aseptic conditions, spent medium was drained, the beads were washed with MSM (equivalent to twice the total bead volume) and then new medium was added, after which the following batch run was commenced. Batch runs were repeated a minimum of three times (not counting the initial batch for acclimation).

Assay. Phenol concentrations were estimated using a standard colorimetric assay [17] where phenolic compounds react with 4-aminoantipyrine in the presence of potassium ferricyanide to form a colored antipyrine dye. Sample intervals were 0.5 to 1.5 h apart depending on the rapidity at which phenol was degraded (this in turn depended on the variable being tested). Between 4 and 9 sample points were made in each batch and sampling was ended when the phenol concentration was reduced to around 25 mg L^{-1}.

Results

Phenol concentrations determined at intervals in a batch run were used to derive *R* which is defined as:

$$R_b = \frac{[P]_c - [P]_a}{T_c - T_a}$$

Where:
R = the rate of phenol degradation (mg L^{-1} h^{-1})
[P] = phenol concentration
T_a = the time at which the sample before that at time b was taken (h)
b = the time at which R is calculated (h)
T_c = the time at which the sample after that at time b was taken (h)
R_{max} is the highest R value calculated for any one batch run and this value was used as the comparative measure of phenol degradation capability under a given bioreactor condition. The relationship between the time course of phenol concentration and R is shown as an example in Table 1 which presents some data from the experiment on the effect of medium volume to total bead volume ratio.

Table 1 Effect of medium volume to total bead volume ratio on aerobic degradation of phenol by immobilized *P. putida* ATCC 11172: Phenol concentration measured through time and the derived degradation rate (*R*). The highest *R* value in a batch is designated R_{max} (marked with an asterisk).

Medium Volume: Total Bead Volume = 1.25:1			Medium Volume: Total Bead Volume = 2.5:1			Medium Volume: Total Bead Volume = 5:1		
Time (h)	Phenol (mg L^{-1})	*R* (mg L^{-1} h^{-1})	Time (h)	Phenol (mg L^{-1})	*R* (mg L^{-1} h^{-1})	Time (h)	Phenol (mg L^{-1})	*R* (mg L^{-1} h^{-1})
0	628		0	535		0	668	
0.5	505	282	1.0	517	177	2.0	148	250*
1.0	346	285	2.0	182	291*	2.5	43	173
1.5	220	297*	2.5	80	165	2.75	18	
2.0	49	191	3.0	17				
2.5	29							

Effect of alginate concentration. Between 2 and 4%, the concentration of alginate used in the preparation of gel beads appeared to make little difference in the rate of phenol degradation by immobilized *P. putida* (Fig. 1).

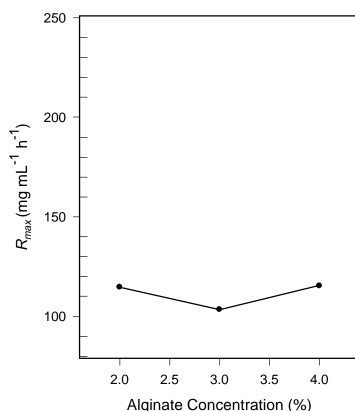

Fig. 1 The effect of alginate concentration used in entrapment on the maximum degradation rate (R_{max}) *of phenol by immobilized* Pseudomonas putida ATCC 11172.

Effect of temperature. Phenol degradation proved to be significantly sensitive to incubation temperature. Ten degree shifts up or down from 25°C resulted in approximately 30% loss of degradation rate (Fig. 2).

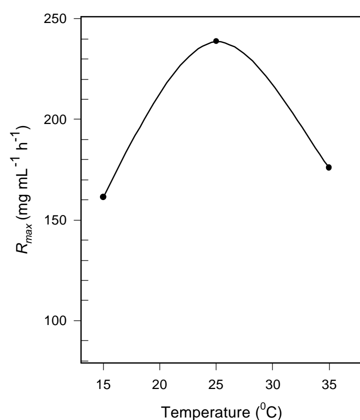

Fig. 2 The effect of reaction temperature on the maximum degradation rate (*Rmax)* of phenol by immobilized *Pseudomonas* putida ATCC 11172.

Effect of pH. Slightly acidic conditions appear to be better for higher rates of phenol degradation (Fig. 3). Degradation at pH 7.5 although conducted, is not reported because the presence of a viscous, gelatinous material in the culture liquor made sample withdrawal difficult and could have resulted in mixing and aeration conditions which were significantly different from the batches controlled at the other pH values.

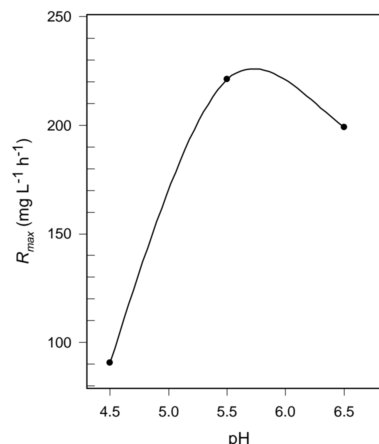

Fig. 3 The effect of medium pH on the maximum degradation rate (R_{max}) of phenol by immobilized *Pseudomonas putida* ATCC 11172.

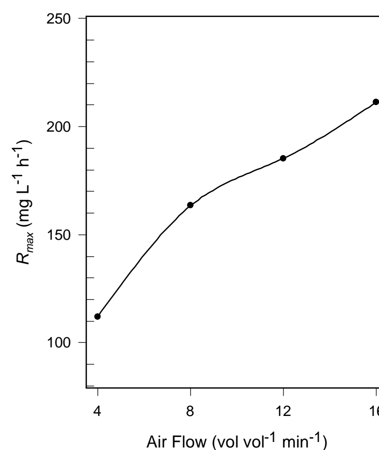

Fig. 4 The effect of rate of air flow on the maximum degradation rate (*Rmax)* of phenol by immobilized *Pseudomonas* putida ATCC 11172.

Effect of air flow. Degradation rate was found to be responsive to increased aeration/mixing and the best rate appears to be higher than the maximum air flow tested (Fig. 4). However, growth of free cells in the culture was observed at 12 and 16 vol. vol.$^{-1}$ min.$^{-1}$ Thus, rates beyond 16 vol. vol.$^{-1}$ min.$^{-1}$ were not investigated. As a check, control medium was sparged at the highest rate tested and it was verified that passage of air alone does not remove phenol.

Effect of phenol concentration. The response of degradation rate to initial phenol concentration appears to have two stages. At initial phenol concentrations between 500 to 1200 mg L^{-1}, increased phenol meant only slight deterioration in degradation rate (Fig. 5), indicating insensitivity to phenol in that range. Beyond 1200 mg mL^{-1}, the negative effect of phenol was more marked.

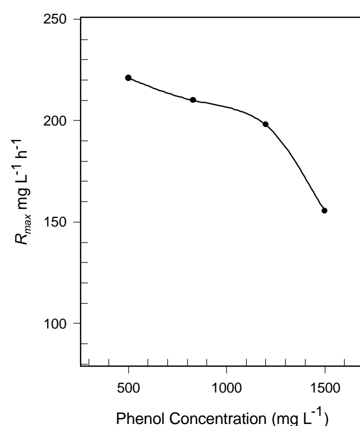

Fig. 5 The effect of initial phenol concentration on the maximum degradation rate (R_{max}) of phenol by immobilized *Pseudomonas putida* ATCC 11172.

Effect of medium volume to total bead volume. The R_{max} associated with a medium volume to total bead volume ratio of 5 was arbitrarily chosen as the base value. Maximum degradation rates associated with other ratios were expressed as percentages of the base value. The relationship between the rate of phenol degradation and the ratio between medium volume and bead volume was found to be near linear when medium volume was between 2× and 10× the total bead volume (Fig. 6). Decreasing the volume of medium in relation to the amount of bead increased the rate of phenol degradation but reduction of the ratio gave progressively smaller increases in degradation rate. When medium volume was reduced below twice the total bead volume, linear improvement in degradation rate ceased.

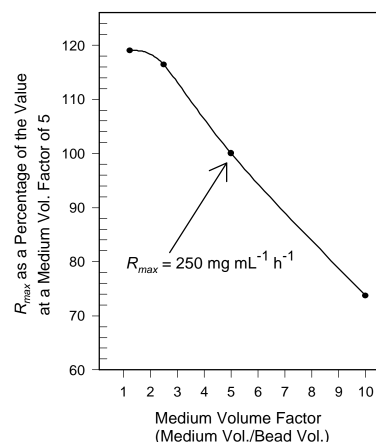

Fig. 6 The effect of the ratio of medium volume to total bead volume on the maximum degradation rate (R_{max}) of phenol by immobilized putida ATCC 11172.

Discussion

The lack of effect of alginate concentration between 2 and 4% indicates that at the concentrations tested, mass and gas transfer through the gel matrix were similar *i.e.* substrate and inhibition kinetics were not affected. These findings are at variance with those of an earlier work where phenol degradation was higher at 5 than 3% alginate [4]. On the basis of the results reported here there appears to be little advantage in using concentrations higher than 2%. However, if beads are required to be used in long reactions, it is likely that higher concentrations of alginate would be desirable as the resulting beads would be more resistant to wear.

While the extreme ends of the temperature range examined were found to significantly reduce degradation rate, it is more likely that in a controlled bioreactor temperature deviations will be smaller than those tested *i.e.* less than ±10°C. Thus, a less significant loss in performance in controlled reactions is indicated. However, reaction temperature appears to be a relatively important operating variable because the 30% deterioration found at 15 and 35°C is

only matched when the changes are relatively large in other operating variables *e.g.* when the initial phenol concentration is raised 3 fold from 500 mg L^{-1} (Fig. 5) or when the ratio of bead to medium is doubled from 1:5 (Fig. 6).

The sharp drop in phenol degradation at pH 4.5 suggests that pH adjustment of acidic effluent will be required. On the other hand, reaction at pH 7.5 was found to be operationally difficult due to the appearance of gelatinous material in the medium during culture. This material was not medium component(s) precipitating out at pH 7.5 (verified by control media held at pH 7.5). The material is probably bacterial polymer(s) either preferentially synthesized or dropping out of solution at pH 7.5.

The vigorous aeration at ≥ 12 vol. $vol.^{-1}$ min^{-1} probably caused excessive bead abrasion which released cells into the media to proliferate. Higher alginate concentrations are indicated for such high aeration rates. Thus, the degradation rates at ≥ 12 vol. $vol.^{-1}$ min^{-1} were likely the result of reaction by a mixture of immobilized and free cells. Therefore, the degradation rates at 4 and 8 vol. $vol.^{-1}$ min^{-1} are better indicators of the performance of immobilized *P. putida.* Nevertheless, the effect of air flow appears not to produce a typical dissolved oxygen response with saturation at high aeration rates. This is probably because in pneumatically agitated bioreactors aeration is coupled with mixing. Thus, even when dissolved oxygen may be saturated, increased air flow increases mixing and this may independently alter the bioreaction.

The choice of initial phenol concentration in a phenol removal system is likely to be determined by consideration of the desired speed of the reaction (or time available for reaction), and the concentration of phenol to be treated (and thus whether dilution may become a necessary procedure). The results in this study show that the best degradation rates are obtained at a phenol concentration of up to 1200 mg mL^{-1} (below which degradation rates were similar). Beyond this, a significant decrease in performance will have to be accepted if it is necessary to treat phenol solutions of typical (1500 mg L^{-1}) to high (4500 mg L^{-1}) concentrations [5].

Degradation rates may be manipulated to increase by simply using more beads in proportion to medium volume. However, as shown in this study, the diminishing improvement in degradation rate as total bead volume is increased means that in a phenol treatment system, the choice of a ratio of medium to beads will be significantly affected by the trade off between degradation rate and cost in producing and maintaining a certain amount of immobilized cells. In any event, the extent to which increasing the proportion of immobilized cells can improve degradation rate is limited because it is thought that the poorer improvements at ratios between 1.25 and 2.5 (Fig. 6) was due to problems with fluidization/mixing medium volumes which were too small to freely suspend the beads. While bead quantity (total bead volume) affects degradation rate by determining total cell number, mass and gas transfer rates as determined by depth of gel matrix (bead diameter) will also be of importance. Thus, the effect of distributing the same total volume of beads into different bead sizes would be a useful study to further refine the data obtained in the current study.

Numerous reaction conditions for phenol degradation by immobilized *Pseudomonas* sp. have been previously reported. However, whilst degradation may have been found to proceed, the limit of conditions are often not determined. In the case of reaction pH, values between 6.5 and 7.0 have been reported by others for effective phenol degradation [3, 10] whereas lower values are indicated in this study (around pH 5.5). With reaction temperature, degradation has been effected by others at between 30 and 34°C [6, 7] whereas lower values are again indicated in this study (around 25°C). Much higher initial phenol concentrations than those used in this study have been reported to be successfully degraded. However, this study shows that in terms of relative degradation rates, initial concentrations of 1.5 g L^{-1} [3], 2 g L^{-1} [7] and 3 g L^{-1} [4, 6] are in the range where increases in phenol concentration decrease degradation rate more markedly than those kept below 1.2 g L^{-1}. Finally, the current study supports previous observations of a disproportional increase in degradation times with increased phenol concentration [4, 22].

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