Journal of Industrial Microbiology & Biotechnology (1999) 22, 121–126

http://www.stockton-press.co.uk/jim

Aerobic batch degradation of phenol using immobilized *Pseudomonas putida*

AM Hannaford[1] and C Kuek[2]

Department of Biological Sciences, The University of Western Sydney Macarthur, PO Box 555, Campbelltown, NSW 2560, Australia

Alginate concentrations between 2 and 4% had little effect on the degradation rate of phenol by alginate-immobilized *Pseudomonas putida*. Ten-degree shifts from 25°C resulted in approximately 30% slower degradation. Maximal degradation rates were 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 vol^{-1} was indicated, although free cells appeared in the reaction medium above 12 vol 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 total bead volume to medium volume gave progressively smaller increases in degradation rate. At a medium volume to total bead volume ratio of 5:1, the maximum degradation rate was 250 mg L^{-1} h^{-1}.

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

Introduction

Phenol is a toxic compound even at low concentrations. It is frequently found in the wastes from many 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 [20]. 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 human exposure should not be more than 20 mg of phenol in an average working day [21]. Phenol is toxic to fish and has been lethal at concentrations of 5–25 ppm while concentrations as low as 0.1 ppm in surrounding waters can taint the taste of fish [21]. Therefore, the treatment of phenol effluent is important. Numerous microorganisms have been used in the aerobic degradation of phenol. These include *Cryptococcus elinovii* [18,22], *Fusarium flocciferum* [1,2], and *Pseudomonas putida* [8,12,17]. Of these microorganisms, *P. putida* has been reported to be capable of high rates of phenol degradation [11].

Free cells of *P. putida* tolerated 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 *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 immobilized cells affects 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 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 [19]. Cells entrapped in gels appear to grow only in a narrow outer shell of the bead due to these oxygen limitations [4,19]. In this study, the rate of air flow was varied. This air flow served both as a source of oxygen and for mixing the medium. The agitation of immobilized cells can cause problems due to bead abrasion [4]. Therefore, optimization of the airflow may be a compromise between maximizing oxygen supply while minimizing abrasion. In developing effluent treatment systems, high tolerance to the substrate to be degraded and high degradation rates are desirable. An increase in phenol concentration disproportionately increases the time required for biodegradation [4,22].

Materials and methods

Microorganism

Pseudomonas putida ATCC 11172 was used. Cultures were maintained on nurtient agar (NA) slopes for short-term storage and L-dried [15] for the long term.

[1]Present address: Fort Dodge Australia Pty Ltd, PO Box 1122, Penrith, NSW 2750, Australia
[2]Correspondence: Dr C Kuek, Dept of Biological Sciences, The University of Western Sydney Macarthur, PO Box 555, Campbelltown, NSW 2560, Australia
Received 24 November 1998; accepted 27 January 1999

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_4SO_4, 488; NaCl, 60; $CaCl_2$, 60; $MgSO_4$, 60; and $FeCl_3$, 60. Filter sterilized (0.22-μm pore size Millipore GS; Millipore Corp, Bedford, MA, USA) phenol was added after the medium was autoclaved (121°C; 15 min). Media concentrations were adjusted to compensate for the volume of water in beads in experiments involving immobilized cells.

Inoculum

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 Erlenmeyer flask (cotton sheik closure). Flasks were incubated in a New Brunswick Innova 4330 gyratory shaker (orbit diameter 3 cm) at 150 rpm 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 min. 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 25-gauge needle (Neolus, Terumo, Melbourne, VIC, Australia) 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 min. The amount of beads containing immobilized *P. putida* used in each batch 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 concentrations used in immobilization was studied using triplicate 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 rpm 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_2SO_4 or 0.1 M NaOH maintained the pH. In studies involving shake flasks or bubble columns, immobilized *P. putida* was 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 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 [16] where phenolic compounds react with 4-aminoantipyrine in the presence of potassium ferricyanide to form a colored antipyrine dye. Sample intervals were 0.5–1.5 h apart depending on the rapidity at which phenol was degraded (this in turn depended on the variable being tested). Between four and nine 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 through the course of 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 (mg L^{-1}); 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 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 data from the experiment on the effect of medium volume to total bead volume ratio.

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* (Figure 1).

Effect of temperature

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

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							

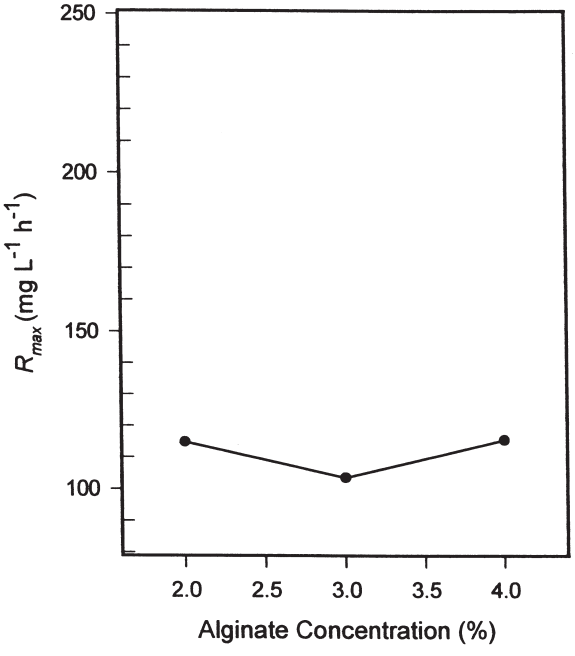

Figure 1 The effect of alginate concentration used in entrapment on the maximum degradation rate (R_{max}) of phenol by immobilized *Pseudomonas putida* ATCC 11172.

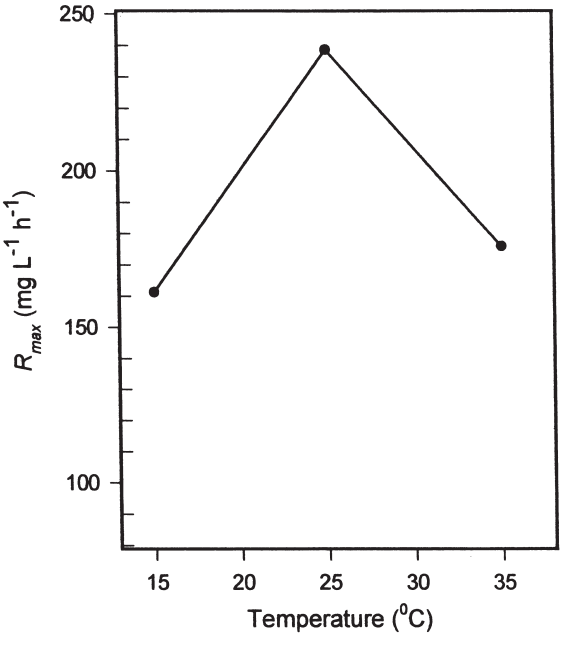

Figure 2 The effect of reaction temperature on the maximum degradation rate (R_{max}) of phenol by immobilized *Pseudomonas putida* ATCC 11172.

Effect of pH

Slightly acidic conditions were better for higher rates of phenol degradation (Figure 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.

Effect of air flow

Degradation rate was responsive to increased aeration/mixing and the best rate appears to be higher than the maximum air flow tested (Figure 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, sterile 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 (Figure 5), indicating insensitivity to phenol in that range. Beyond 1200 mg ml^{-1}, the negative effect of phenol was more marked.

Effect of medium volume to total bead volume

The R_{max} associated with a medium volume to total bead volume ratio of 5 was arbitarily 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 near linear when medium volume was between 2× and 10× the total bead volume (Figure 6). Decreasing the volume of medium in relation to the amount of bead increased the

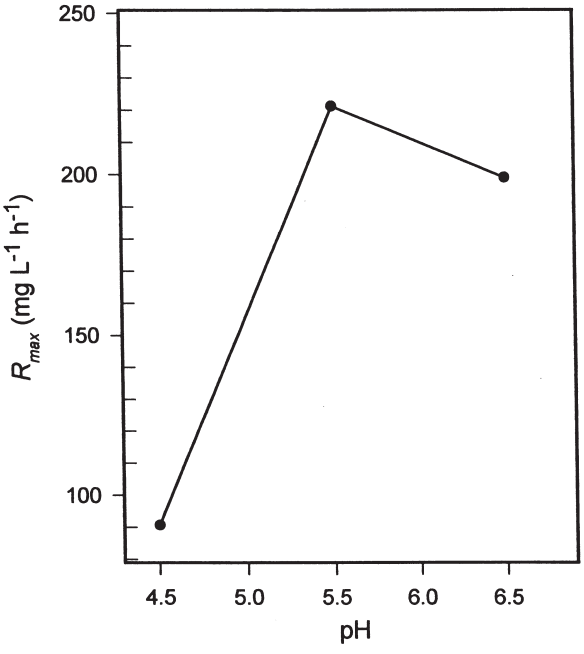

Figure 3 The effect of medium pH on the maximum degradation rate (R_{max}) of phenol by immobilized *Pseudomonas putida* ATCC 11172.

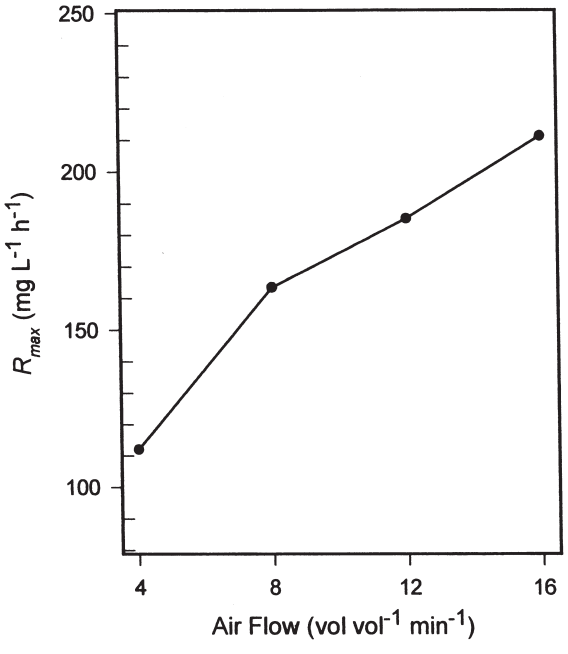

Figure 4 The effect of air flow on the maximum degradation rate (R_{max}) of phenol by immobilized *Pseudomonas putida* ATCC 11172.

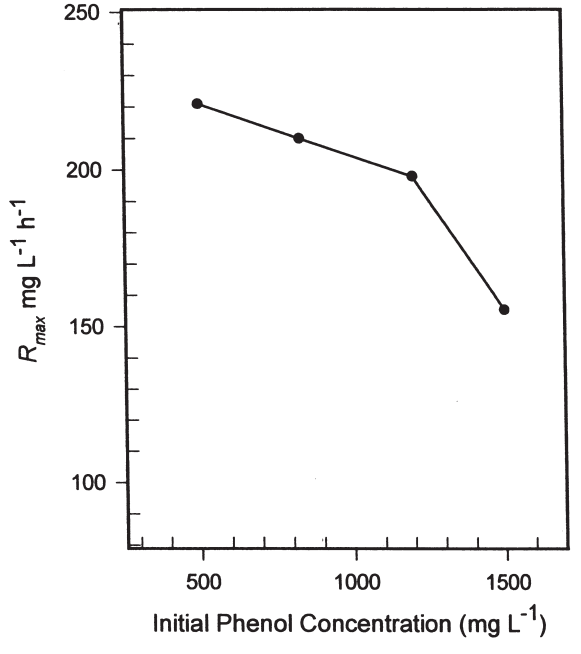

Figure 5 The effect of initial phenol concentration on the maximum degradation rate (R_{max}) of phenol by immobilized *Pseudomonas putida* ATCC 11172.

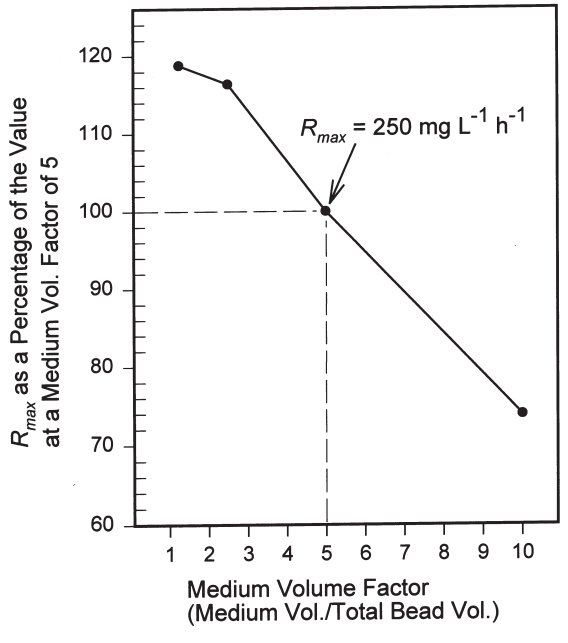

Figure 6 The effect of the ratio of medium volume to total bead volume on the maximum degradation rate (R_{max}) of phenol by immobilized *Pseudomonas putida* ATCC 11172.

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.

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 ie substrate and inhibition kinetics were not affected. These findings are a 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 exam-

ined significantly reduced degradation rate, it is more likely that in a controlled bioreactor, temperature deviations would be smaller than those tested ie 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 eg when the initial phenol concentration is raised 3-fold from 500 mg L^{-1} (Figure 5) or when the ratio of bead to medium is doubled from 1:5 (Figure 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 operationally difficult due to the appearance of gelatinous material in the medium. 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 which 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 whether dilution may be necessary). This study shows that the best degradation rates are obtained at phenol concentrations of up to 1200 mg ml^{-1}. 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 increased by using more beads in proportion to medium volume. However, the diminishing improvement in degradation rate as bead volume is increased means that in a phenol treatment system, 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. 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 (Figure 6) were due to problems with fluidization/mixing medium volumes which were too small to freely suspend the beads. While 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) are also important. 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.

Reaction conditions for phenol degradation by immobilized *Pseudomonas* sp have been previously reported. However, the limits of conditions for degradation are often not determined. Reaction pHs between 6.5 and 7.0 have been reported 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 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].

References

1 Anselmo AM, JMS Cabral and JM Novais. 1989. The adsorption of *Fusarium flocciferum* spores on celite particles and their use in the degradation of phenol. Appl Microbiol Biotechnol 31: 200–203.
2 Anselmo AM, M Mateus, JMS Cabral and JM Novais. 1985. Degradation of phenol by immobilized cells of *Fusarium flocciferum*. Biotechnol Lett 7: 889–894.
3 Babu KS, PV Ajithkumar and AAM Kunhi. 1995. Mineralization of phenol and its derivatives by *Pseudomonas* sp strain CP4. World J Microbiol & Biotechnol 11: 661–664.
4 Bettman H and HJ Rehm. 1984. Degradation of phenol by polymer entrapped microorganisms. Appl Microbiol Biotechnol 20: 285–290.
5 Bond RG and CP Straub. 1974. Handbook of Environmental Control, Vol IV, CRC Press, USA.
6 Chitra S, G Sekaran and G Chandrakasan. 1996. Immobilized mutant strain of *Pseudomonas pictorum* for the degradation of phenol in wastewater. J Gen Appl Microbiol 42: 355–361.
7 Chitra S, G Sekaran, S Padmavathi and G Chandrakasan. 1995. Removal of phenolic compounds from wastewater using mutant strain of *Pseudomonas pictorum*. J Gen Appl Microbiol 41: 229–237.
8 Ehrhardt H and HJ Rehm. 1989. Semicontinuous and continuous degradation of phenol by *Pseudomonas putida* P8 adsorbed on activated carbon. Appl Microbiol Biotechnol 30: 312–317.
9 Gilson CD, A Thomas and FR Hawkes. 1990. Gelling mechanisms of algniate beads with and without immobilized yeast. Process Biochem 6: 104–108.
10 Gonzalez BG and TG Herrera. 1995. Biodegradation of phenol by free and immobilized cells of *Pseudomonas putida*. Acta Microbiologica Polonica 44: 285–296.
11 Hill GA and CW Robinson. 1975. Substrate inhibition kinetics: phenol degradation by *Pseudomonas putida*. Biotechnol Bioeng 17: 1599–1615.
12 Hutchinson DH and CW Robinson. 1988. Kinetics of the simultaneous batch degradation of *p*-cresol and phenol by *Pseudomonas putida*. Appl Microbiol Biotechnol 29: 599–604.
13 Keweloh H, HJ Heipieper and HJ Rehm. 1989. Protection of bacteria against the toxicity of phenol by immobilization in calcium alginate. Appl Microbiol Biotechnol 31: 383–389.
14 Kierstan M, G Darcy and J Reily. 1982. Studies on the characteristics of alginate gels in relation to their use in separation and immobilization applications. Biotechnol Bioeng 24: 1507–1517.
15 Lapage SP, JE Shelton, TG Mitchell and AR Mackenzie. 1970. Culture collections and the preservation of bacteria. In: Methods in Microbiology, Vol 3A (Norris JR and DW Ribbons, eds), pp 135–228, Academic Press, London.

16 Martin RW. 1949. Rapid colorimetric estimation of phenol. Nature 21: 1419–1420.
17 Molin G and I Nilsson. 1985. Sand administration as an instument for biofilm control of *Pseudomonas putida* ATCC 11172 in chemostat cultures. Biotechnol Bioeng 27: 117–120.
18 Morsen A and HJ Rehm. 1990. Degradation of phenol by a defined mixed culture immobilized by adsorption on activated carbon and sintered glass. Appl Microbiol Biotechnol 33: 206–212.
19 Ogbonna JC, M Matsumura and H Kataoka. 1991. Effective oxygenation of immobilized cells through the reduction in bead diameters: a review. Process Biochem 26: 109–121.
20 Tibbles BJ and AAW Baecker. 1989. Effects and fate of phenol in simulated landfill sites. Microbial Ecology 17: 201–206.
21 Wallace J. 1991. Phenol. In: Kirk-Othmer Encyclopedia of Chemical Technology (Kroschwitz JI, ed), pp 592–602, John Wiley & Sons, New York.
22 Zache G and HJ Rehm. 1989. Degradation of phenol by a coimmobilized entrapped mixed culture. Appl Microbiol Biotechnol 30: 426–432.