Biodegradation of Naphthalene by Free and Alginate Entrapped *Pseudomonas* sp.

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Naphthalene degradation by freely suspended and immobilized cells of *Pseudomonas* sp. isolated from contaminated effluents has been investigated in batch cultures and continuously in a packed bed reactor. Naphthalene concentration was varied from 25 mM to 75 mM, the temperature (30 °C) and pH (7.0) were kept constant. The results showed good acclimation of the strain to carbon source and degradation rate was highly affected by initial concentration. Alginate-entrapped cells have given good yields although initial rates were not as high as those encountered with free cells. A first order exponential decay kinetic model was proposed with values of parameters for each initial concentration. A laboratory scale packed-bed bioreactor was designed using parameters calculated above and continuous experiments were realized at different flow rates (100 to 200 ml/h), with different feed concentrations and operating during 30 days. The conversion at low feed concentrations and low flow rates was complete whereas at high flow rates and high concentrations it was less efficient because of diffusional limitations and short residence time.

Key words: Naphthalene, Biodegradation, Bioreactor

Introduction

Polycyclic aromatic hydrocarbons in nature are considered as environmental pollutants, owing to their inferred recalcitrance to microbial degradation and potential toxicity to higher microorganisms (Atlas and Cerniglia, 1995). Naphthalene is considered to be a primary irritant and the US environmental protection agency (EPA) has classified it as a priority toxic pollutant (EPA 1986, 1994; U.S. DHHS, 1993; Vandermuulen, 1981). Despite its low solubility in water it is frequently encountered in effluents in complex mixtures like petroleum fractions, creosote, and pharmaceutical wastes (Mueller *et al*., 1989). Systematic exposure to naphthalene and its derivatives has been shown to cause several diseases and disturbances to human metabolism (ATSDR, 1990; Amoore and Hautala, 1983). Biological method of treatment has turned out to be a favorable alternative for naphthalene degradation and several reports are available on the removal of naphthalene by different microorganisms (Grund *et al.*, 1992; Kuhm *et al*., 1981). An effective approach to finding a solution to continuous contamination is to concentrate these biodegraders by their immobilization on natural or synthetic supports with high porosity and thus allowing their use in different kinds of bioreactors (fluidized bed, packed bed). Immobilization present many advantages such as the presence of high population density in a limited reactor volume, high conversion rates, limiting substrate inhibition and toxicity to microorganisms by diffusional limitations (Callegari and Francotte, 1986; Trevor, 1992, Willaert *et al.*, 1996). Various methods have been described for the immobilization of bacteria (Klein and Wagner, 1983), and entrapment within polymeric gel matrices has often been successful. The most frequently used matrices are κ-Carrageenan, alginate, and polyacrylamide. Alginate represents however several advantages such as high porosity and chemical stability, with a mild, fast, simple and low cost immobilization method (Fukui and Tanaka, 1982).

In the present study, biodegradation of naphthalene using free suspended and calcium alginate immobilized *Pseudomonas* sp. has been investigated in batch and continuous cultures at different initial concentrations. The apparent kinetic parameters are evaluated and compared for both methods. The extent of diffusion limitations was demonstrated by calculating the experimental effec-

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tiveness factor, Thiele modulus and effective diffusion coefficient.

A laboratory scale packed bed bioreactor was designed by using calculated parameters and operated for different flow rates and different loading concentrations.

Materials and Methods

Microorganism

The investigated bacterium was isolated from a petroleum refinery waste water and identified to be *Pseudomonas* sp. It was able to grow on naphthalene as sole source of carbon and energy in the concentration range 20 to 75 mM. The optimum temperature and pH of growth were 30 °C and 7 respectively.

Mineral medium

For fermentation studies, the mineral salts medium contained (in g per liter): K_2HPO_4 (0.38), $MgSO_4$ $7H_2O$ (0.2), NH_4Cl (1.0), $FeCl_3$ (0.05), $ZnSO_4$ (0.025), NaCl (0.5), yeast extract (0.01). This medium was used for free cell studies. For alginate-entrapped cells, the fermentation medium contained (in g per liter): K_2HPO_4 (0.15), $MgSO_4$ $7H_2O$ (0.2), NH_4Cl (1.0), $FeCl_3$ (0.05), $ZnSO_4$ (0.025), NaCl (0.5), yeast extract (0.01), $CaCl_2$ (0.2). The pH was adjusted to 7.0 and tween80 was added. Naphthalene at various concentrations (20–80 mM) was dissolved in a minimum amount of N,N-dimethyl formamide and added to the medium and with a vigorous agitation a homogenous mixture was obtained.

Immobilization method

Pseudomonas sp. were grown in the mineral medium containing naphthalene (0.1% w/vol) as sole source carbon and energy on orbital shaker at 30 °C. Bacteria were harvested routinely by centrifugation from cultures in early stationary phase (72 h). Alginate (3% w/vol) was dissolved in boiling water and autoclaved at 121 °C for 15 min. The microorganisms from precultures (3 g dry weight for 100 ml-alginate solution) were added and suspended by stirring. This mixture was extruded through a needle into a (0.05 M) $CaCl_2$ solution thus forming beads with a diameter of about 2 mm. After hardening for 1 hour in this solution the beads were washed several times with water.

Batch experiments

For both freely suspended and immobilized cells experiments were conducted in 250 ml shaken flasks as batch reactors at constant temperature 30 °C on a rotary shaker at 150 rpm.

For immobilized *Pseudomonas* sp., 25 g wet beads were placed into 100 ml mineral medium containing the desired amount of naphthalene (25, 50, 75 mM). Samples of the culture broth were taken at the indicated times for analysis of naphthalene. To recover bacteria for determination of growth, 0.1 g of beads were withdrawn then immersed in 1 ml phosphate buffer (1 M, pH = 7) and dissolved by vigorous mixing. For free cells experiments, 0.5 ml innoculum of suspension were inoculated into 100 ml mineral medium (this provided the same ratio as for immobilized cells), and the same culture conditions as for immobilized cells were chosen.

Control experiments were also carried out in order to evaluate abiotic degradation in sterile medium, and showed no significant loss due to evaporation or adsorption by sterile alginate beads.

Continuous experiments

A laboratory packed bed bioreactor containing immobilized cells for continuous removal of naphthalene was designed on the basis of batch calculations for a given diameter and maximum flow rate. It consisted of a cylindrical glass column packed with 350 g beads to a height 40 cm with a 5 cm internal diameter. Mineral medium containing various concentrations of naphthalene at different concentrations (25, 50 and 75 mM) was fed from the top at different flow rates (100, 125, 150, 175 and 200 ml/h), and oxygen was supplied from the bottom of the column at constant pressure. When steady state conditions were attained, samples were withdrawn for analysis.

Analysis

For both batch and continuous experiments, residual naphthalene was determined by HPLC analysis using a UV detector at 276 nm with a Shimadzu spectrophotometer.

Results and Discussion

The bacterium *Pseudomonas* sp. was isolated from a petroleum refinery waste water effluent and naphthalene proved to be a good source of carbon and energy for its growth. Entrapment in alginate was chosen because it is an easy method, where the control of particle size and shape is possible, with lower cell leakage and preserved viability (Manohar and Karegoudar, 1996). Results on batch biodegradation of naphthalene at different initial concentrations with both freely suspended and immobilized *Pseudomonas* sp. are presented as the dimensionless ratio of residual to initial concentration C/Co versus time in Fig. 1A and B.

The data showed that degradation occurred without lag phase, which means good acclimation of bacteria to hydrocarbon source and used medium. By free cells and with an initial concentration of 25 mM there was complete degradation after only four days of incubation, while a maximum amount of 60% was degraded for higher concentrations. This could be explained by substrate inhibition.

The results with immobilized cells in batch cultures are given in Fig. 1(B). The time for complete degradation of naphthalene was 117 and 167 hours with initial concentrations 25 mM and 50 mM respectively, while Co = 75 mM only 80% were degraded even after 15 days of incubation.

The rate of degradation by immobilized cells was less higher than by free cells because of diffusional limitations in alginate beads and intraparticle concentration gradients. This phenomenon was beneficial for limiting the effect of substrate inhibition (Callegari and Francotte, 1986).

A kinetic model for biodegradation of naphthalene by free and immobilized *Pseudomonas* sp. at 30 °C can be used to predict the rate and the yield of biodegradation. The equation has the following form:

$$C/C_0 = Y_0 + A_1 \exp - \frac{t}{t_1} \qquad (1)$$

where C and C_0 are naphthalene concentrations (in mM) at time t and $t = 0$ hours respectively.

With the constants Y_0, A_1, t_1 (hours) determined by least squares method and given for different concentrations for free and immobilized cells in Table I. Accuracy of the model was tested by chi-square test at a confidence level 0.05. Correlation coefficient was greater than 0.985. Fitting curves are given in Fig. 1A and B).

The influence of diffusionnal limitations on biodegradation rate could be quantified by calculating from experimental data using Eqn. (1) the experimental effectiveness factor (η) and Thiele modulus Φ (Mavituna, 1979; Bucholz, 1982). The mean values of η = 0.546 and Φ = 1.37 (details of calculations not shown but available on request) showed the magnitude of mass transfer effects and diffusional limitations on overall kinetic. Mass transfer resistance reduces the reaction rate by about 55%. These data were used for the estimation of the resistance to diffusion of naphthalene

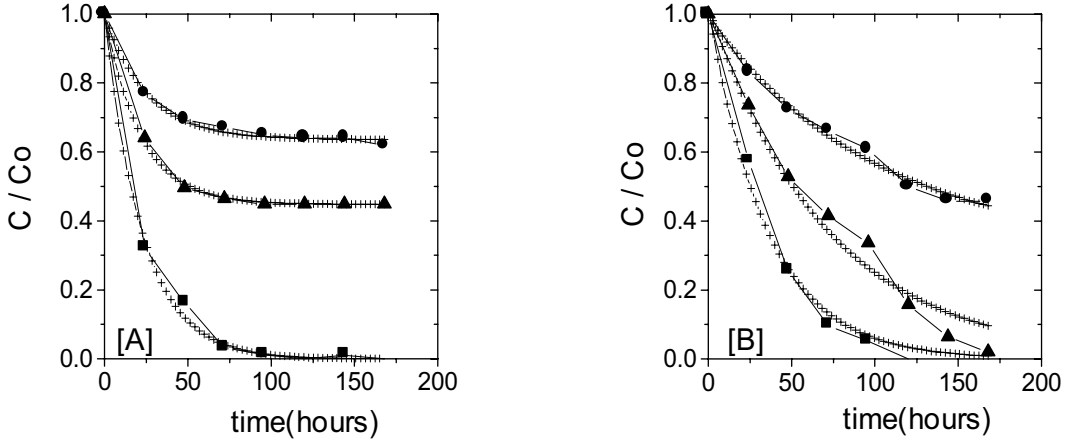

Fig. 1. Biodegradation of naphthalene by free (A) and alginate entrapped (B) cells of *Pseudomonas* sp. at different initial concentrations. (—■—) 25 mM (—▲—) 50 mM (—●—) 75 mM (+++) model. T = 30 °C; pH = 7.0.

Table I: Experimental values of half-reaction times ($t_{1/2}$) and model parameters for the reaction of biodegradation of naphthalene by free and immobilized *Pseudomonas* sp.

				T°= 30 °C, pH = 7				
Initial concentration [mM]	Free cells				Immobilized cells			
	$t_{1/2}$ [hours]	Y_0	A_1	t_1 [hours]	$t_{1/2}$ [hours]	Y_0	A_1	t_1 [hours]
25	18.1	0.000	0.996	22.7	29.5	0.000	1.021	35.3
50	48.1	0.444	0.558	22.06	53.6	0.000	1.021	71.1
75	–	0.636	0.362	25.44	122.0	0.317	0.673	100.74

in alginate beads by calculating effective diffusion coefficient (D_{eff}) assuming a first order kinetic (Zumriye and Gultaç, 1999). The obtained value $D_{eff} = 5\times10^{-7}$cm^2/sec was compared to naphthalene diffusivity in pure water calculated from Wilke and Chang equation at 30 °C (Perry, 1984), $(D_e)_{water} = 1.1\times10^{-6}$ cm^2/sec.

Reactor design

The conversion in a packed bed reactor with plug flow in steady state conditions can be described by the following set of equations (Webb *et al.*, 1986; Winterbottom and King, 1999):

$$-\varepsilon \frac{dC}{dt} = J(C)A_s \tag{2}$$

$$\text{with} \quad J(C) = -D_{eff}\left(\frac{dC}{dr}\right)_{rp} \tag{3}$$

$$A_s = \frac{3\,(1-\varepsilon)}{r_p} \tag{4}$$

Where

- t: residence time of the liquid in the bed (seconds),
- C: naphthalene concentration (mol/cm^3),
- $J(C)$: flux to the particles of the component to be converted (naphthalene) (mol/cm^2 sec),
- r: radial distance (cm),
- r_p: particle radius (cm),
- ε: bed voidage (for packed bed ε = 0.4).

From mass balance and assuming no external mass transfer resistance and boundary conditions $C = C_0$ (at $r = r_p$); and $C = 0$ (at $r = 0$) we obtain:

$$\left(\frac{dC}{dr}\right)_{r_p} = \frac{3\Phi C}{r_P}\left(\frac{1}{\tanh 3\Phi} - \frac{1}{3\Phi}\right) \tag{5}$$

and therefore the residence time will be obtained by integrating Eqn. (2):

$$t = -\frac{\varepsilon}{9\,(1-\varepsilon)}\frac{r_p^2}{D_{eff}\,\Phi^2\eta}\ln\,(1-f) \tag{6}$$

where f is the conversion defined as: f = concentration (exit of the bioreactor)/concentration (inlet of the bioreactor); for $f = 0.0001$; $t = 3.8$ h.

Thus the reactor volume will be: V_R = (flow rate F (cm^3/hour)); (residence time t (hours)).

The maximum chosen flow rate is: $F = 200$ cm^3/h, so we find $V_R = 758$ cm^3.

For an internal diameter of the reactor: D_R = 5 cm, the height of the bed will be: $H_R = 38.6$ cm. The actual height will be taken as 40 cm.

Continuous Experiments with Immobilized *Pseudomonas* sp.

Data on continuous removal of naphthalene in a packed bed bioreactor with different flow rates (100, 125, 150, 175, 200 ml h^{-1}) and different initial concentrations are presented in Fig. 2. At low concentrations, naphthalene is completely depleted and the biodegradation yield does not depend on flow rates. An increase in flow rate for high concentration range (Co > 50 mM) led to partial degradation between 70 and 90% due to poor mass transfer, diffusional limitations and short residence time. External mass transfer limitations of naphthalene and oxygen should be taken into consideration during design calculations. The yield could be increased by recycling, increasing the bed height , decreasing particle size, realizing a high degree of turbulence and increasing mass transfer by the use of fluidized bed bioreactor .The bioreactor was efficiently operated for 30 days.

Conclusion

Naphthalene biodegradation by using free and immobilized *Pseudomonas* sp. entrapped in Ca-alginate beads was investigated in a batch system and continuously in a packed bed reactor at con-

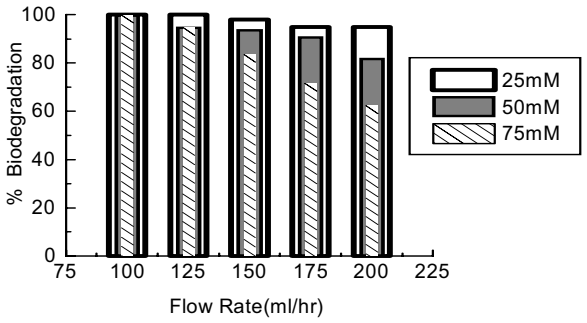

Fig. 2. Continuous biodegradation of naphthalene by immobilized *Pseudomonas* sp. in a packed bed reactor at different flow rates and different concentrations.

stant temperature 30 °C and pH 7.0 for the concentration range 25 mM to 75 mM.

Batch results showed in both cases that biodegradation was highly affected by initial concentration and entrapped microorganisms obtained higher yields. Diffusional limitations in alginate beads protected bacteria against substrate inhibition. A statistical first order exponential decay model was proposed in order to evaluate the rate of biodegradation. The estimated constants of the model were tested for significance and it was found that equation fits experimental data adequately. The effect of diffusion resistance on biodegradation rate is very significant and should not be ignored in any engineering analysis. The obtained data were used for the design of a continuous packed bed bioreactor under the assumptions of a plug flow and steady state conditions. The reactor height was obtained for the maximum flow rate of 200 ml/h and a column diameter $D_R = 5$ cm.

Calculating the dimensionless numbers (D_{eff}/u $H_R \ll 0.01$ where u is the flow velocity in cm/sec) and ($H_R/d_p \gg 50$ where d_p is particle diameter) showed that the plug flow assumption was correct (Buchholz, 1982, Webb *et al.*, 1986). Continuous experiments were carried out for different flow rates at different feed concentrations. Results showed that maximum degradation of naphthalene was obtained at low feed concentration 25 mM while for higher concentrations the influence of flow rate was not negligible because intraparticle convection effect becomes significant. Limited conversion at high flow rates was caused by other factors such as external mass transfer limitations of naphthalene and oxygen supply. Studying the effect of these factors and others such as bead size and flow conditions could refine the research work. The effect of external mass transfer and radial diffusion should also be considered in order to obtain better design parameters.

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