APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1986, p. 345–351
0099-2240/86/080345-07$02.00/0

Vol. 52, No. 2

Kinetics of Phenol Biodegradation by an Immobilized Methanogenic Consortium†

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Received 19 December 1985/Accepted 22 May 1986

A phenol-degrading methanogenic enrichment was successfully immobilzed in agar as shown by the stoichiometric conversion of phenol to CH_4 and CO_2. The enrichment contained members of three physiological groups necessary for the syntrophic mineralization of phenol: a phenol-oxidizing bacterium, a *Methanothrix*-like bacterium, and an H_2-utilizing methanogen. The immobilization technique resulted in the cells being embedded in a long, thin agar strand (1 mm in diameter by 2 to 50 cm in length) that resembled spaghetti. Immobilization had three effects as shown by a comparative kinetic analysis of phenol degradation by free versus immobilized cells. (i) The maximum rate of degradation was reduced from 14.8 to 10.0 μg of phenol per h; (ii) the apparent K_m for the overall reaction was reduced from 90 to 46 μg of phenol per ml, probably because of the retention of acetate, H_2 and CO_2 in the proximity of immobilized methanogens; and (iii) the cells were protected from substrate inhibition caused by high concentrations of phenol, which increased the apparent K_i value from 900 to 1,725 μg of phenol per ml. Estimates for the kinetic parameters K_m, K_i, and V_{max} were used in a modified substrate inhibition model that simulated rates of phenol degradation for given phenol concentrations. The simulated rates were in close agreement with experimentally derived rates for both stimulatory and inhibitory concentrations of phenol.

Immobilized cells offer several potential advantages for the treatment of processing and waste streams (23). These include the retention of catalytic activity, the protection of cells from the effects of inhibitory substrates, and more efficient substrate mineralization through retention of intermediary products. This last advantage may offer both thermodynamic and kinetic advantages when mineralization involves syntrophic bacteria of methanogenic consortia.

We chose to study the effect of immobilization on the activity of a phenol-degrading methanogenic consortium. Phenol and phenolic compounds are toxic pollutants (9) and inhibitors of biodegradation (6, 7, 15) and have been used as models to test the effect of inhibitory substrates on cellular metabolism and growth kinetics (16, 17). The phenol-degrading methanogenic consortium is postulated to contain three interacting physiological groups of bacteria: a phenol-metabolizer, an H_2-utilizing methanogen, and an acetotrophic methanogen, all of which are required to complete the mineralization of phenol to CH_4 and CO_2 (2, 4). Therefore, with this consortium it should be possible to determine the effect of immobilization on both the degradation of an inhibitory substrate and the kinetic parameters of a catabolic pathway involving more than one species.

Our main goals were to (i) develop a mild immobilization technique convenient to use under anaerobic conditions which would maintain the activity of a variety of syntrophs, (ii) maintain the long-term survivability of cells under toxic concentrations of phenol, and (iii) formulate a kinetic model which describes the rate of biodegradation of toxic substrates for immobilized cells.

Kinetic models are of value in investigating both the capacity and stability of biological processes which utilize inhibitory substrates. We have modified a model based on the Haldane equation, which describes the kinetics of inhibitory substrate utilization (14), to incorporate a substrate limitation parameter for immobilized cells. Experimental data were used to test the success of the model in fitting measured values of kinetic parameters to both stimulatory and inhibitory substrate concentrations.

MATERIALS AND METHODS

Enrichment. The phenol-degrading consortium was enriched from anaerobic digestor sludge obtained from a municipal plant in Jackson, Mich. The enrichment was maintained for 2 years with bimonthly transfers of 25% inocula to fresh revised anaerobic mineral medium (19). The enrichment was grown at 37°C under stationary conditions. Approximately 2 mM phenol was added to the enrichments every 2 days. Phenol was stoichiometrically converted to methane and CO_2.

Immobilization of bacterial cells. All media and transfers were made with anaerobic gases and gassing probes. Cells from 1,200 ml of phenol enrichment were collected as pellets by centrifugation at 15,000 × *g* for 15 min. Centrifuge tubes were preflushed with an oxygen-free 80% N_2-20% CO_2 gas mixture. The pelleted cells were suspended and washed in an equal volume of growth medium, recentrifuged, and then suspended in 20 ml of medium. This cell suspension contained approximately 10 mg of protein per ml. A 3-ml portion was then added by syringe to each of three serum bottles (160 ml) containing 50 ml of anaerobic medium. These were used as native cell cultures; the remaining suspension was used for immobilization.

The immobilization matrix was 2% agar. Three 2-m sections of Tygon tubing (inside diameter of 1 mm) were connected to gas lines and were flushed with 80% N_2-20% CO_2 to make the anaerobic matrix. The bulk of the tubing was placed in a double-valved anaerobic jar, which was also flushed with the oxygen-free gas. The distal ends of tubing

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† Journal article 11873 of the Michigan Agricultural Experiment Station.

were inserted into three 25-mm test tubes in a 50°C water bath on a hot plate-magnetic stirrer. The tubes were filled with 3 ml of anaerobic growth medium with 4% agar at 50°C. Three milliliters of cell suspension was then slowly added to the medium while being mixed with a magnetic stir bar. The gas line was disconnected from each section of tubing, and the cell-agar mixture was drawn rapidly into the oxygen-free tubing by suction with a 50-ml syringe. After solidifying, the matrix was slowly ejected by syringe into a preflushed serum bottle (160 ml) containing 50 ml of growth medium. This resulted in a 1-mm diameter agar gel matrix which resembled a long strand of spaghetti. The matrix was washed twice with 50 ml of medium to remove loose cells and fine colloidal particles before the addition of 50 ml of incubation medium. Bottles were then sealed with butyl rubber stoppers. Both native and immobilized cultures contained the same amount of cells.

Experimental procedure. Triplicate serum bottles containing either native or immobilized cells were incubated at 37°C while shaking at 120 rpm. The rate of phenol degradation was assayed by the monitoring of methane production. The range of initial concentrations of phenol was from 10 to 2,000 μg/ml. Methane was quantified by injecting 0.2 ml of culture headspace gas into a Carle model 8515 gas chromatograph equipped with a Porapak Q packed column and a microthermistor detector. All data reported on methane production are means of triplicate bottles. Protein concentrations were determined by a modification (12) of the method of Lowry et al. (10). Microscopy was by phase contrast, and fluorescence was with a Leitz Ortholux microscope. Fluorescence was used to identify methanogenic bacteria. Samples of spaghetti agar for scanning electron microscopy were dehydrated in ethanol and freeze-fractured. Dried specimens were mounted on stubs and coated with gold before being viewed with a JEOL JSM-35-C scanning electron microscope. Samples for thin-section transmission electron microscopy were fixed in 2% glutaraldehyde–0.1 M phosphate buffer (pH 7.2), dehydrated in ethanol followed by propylene oxide, and embedded in epoxy resin. Electron micrographs were taken on a Philips 300 electron microscope.

Kinetic analysis. Andrews (1) and Edwards (3) proposed mathematical growth models based on Monod biokinetics for both batch and continuous cultures of native cells. These kinetic models were based on the Haldane equation and made use of an inhibition constant, K_i, to relate substrate concentrations to specific growth rates. The model was successfully used to predict biomass production during the treatment of phenolic wastes by activated sludge (16, 17). A modification of the basic model was proposed by Neufeld et al. (14):

$$v = V_{max}/[1 + K_m/S + (S/K_i)^n] \quad (1)$$

for which S is the available substrate concentration, v is the specific substrate utilization rate, V_{max} is the maximum substrate utilization rate, K_m is the substrate concentration at 0.5 V_{max}, K_i is the inhibition constant equal to 0.5 V_{max} for inhibitory substrate concentrations, and n is an empirical value describing the order of inhibition.

For immobilized cells, the available substrate concentration, S', may be decreased from S because of diffusion-limited mass transport. The extent of the limitation depends upon the type of immobilizing matrix used, the presence of other constituents in the matrix (e.g., cells, organic matter, etc.), and the physical properties of the substrate (22). We have altered equation 1 to describe the utilization of an inhibitory substrate by either native or immobilized microorganisms:

$$v = V_{max}/[1 + K_m/(S \cdot X) + (S/K_i)^n] \quad (2)$$

for which X is an empirical parameter ranging from 0 to 1 that changes S to S' to reflect the limited substrate concentration available to immobilized cells (S'). For native cells, $X = 1$. For immobilized cells, X equals an empirical value such that $S' = S \cdot X$.

Initial estimates of V_{max}, K_m, K_i, and X were obtained from initial velocity experiments with substrate concentrations of 10, 20, and 30 μg of phenol per ml. Values for V_{max} and K_m were estimated directly from Lineweaver-Burk plots of the resultant data. The data are mean values from repeated experiments with two separately grown consortia. An estimation of K_i was then made with equation 2 with a value of 1 for n, the estimated values for V_{max} and K_m, and the observed initial velocity, v, at an inhibitory substrate concentration of 1,000 μg of phenol per ml for native cells and 2,000 μg of phenol per ml for immobilized cells. Thus, by use of the Lineweaver-Burk plot and equation 2, we obtained estimates for V_{max}, K_m, and K_i. These values were used to set the range of the model program for estimating V_{max}, K_m, and K_i.

Because rates are dependent on substrate concentration, the difference in the rates of methane production between native and immobilized cells was used to obtain a value for X to convert S to S'. Since the ratio between the degradation rates of immobilized and native cells (v'/v) is a constant, S'/S must also be a constant equal to both v'/v and V'_{max}/V_{max}. Given that $X = S'/S$ (equation 2), the ratio of V'_{max}/V_{max} was used to calculate a value for X. Values for n were estimated by fitting the model to experimental data.

The parameter X was used to alter S in K_m/S and not in $(S/K_i)^n$ (equation 2) because X could be estimated only for low substrate concentrations which were not inhibitory. Also, at low substrate concentrations, $K_m/S > (S/K_i)^n$, and thus a change in S by a factor of X alters determinations of rates only at low concentrations. At higher substrate concentrations $(S/K_i)^n > K_m/S$, and a different value of X would be required to alter S. This value could not be determined by our methodology and was not used in the model.

A range of values was established around the estimated kinetic parameters. These were used in a program implemented in BASIC that simulated datum points for a plot of v versus S as per equation 2 by using all permutations of the parameters within the ranges. The simulated data were compared with experimental data by a least-squares analysis. Kinetic parameters were then selected for which the mean square deviation between simulated and experimental data was minimal. Because equation 2 contains four constants, an almost infinite number of numerical combinations would suffice to fit the data. By first obtaining independent estimates for three of the constants, we were able to set a range for each value such that the kinetic parameters selected by the program were the best estimations obtainable for V_{max}, K_m, K_i, and X with all the available data.

RESULTS

Phenol-degrading consortium. The spaghetti agar containing the cells broke into 2- to 50-cm lengths when initially placed on the rotary shaker but then maintained both integrity and phenol-degrading activity for over 1 month. Observation of the agar matrix by scanning electron microscopy

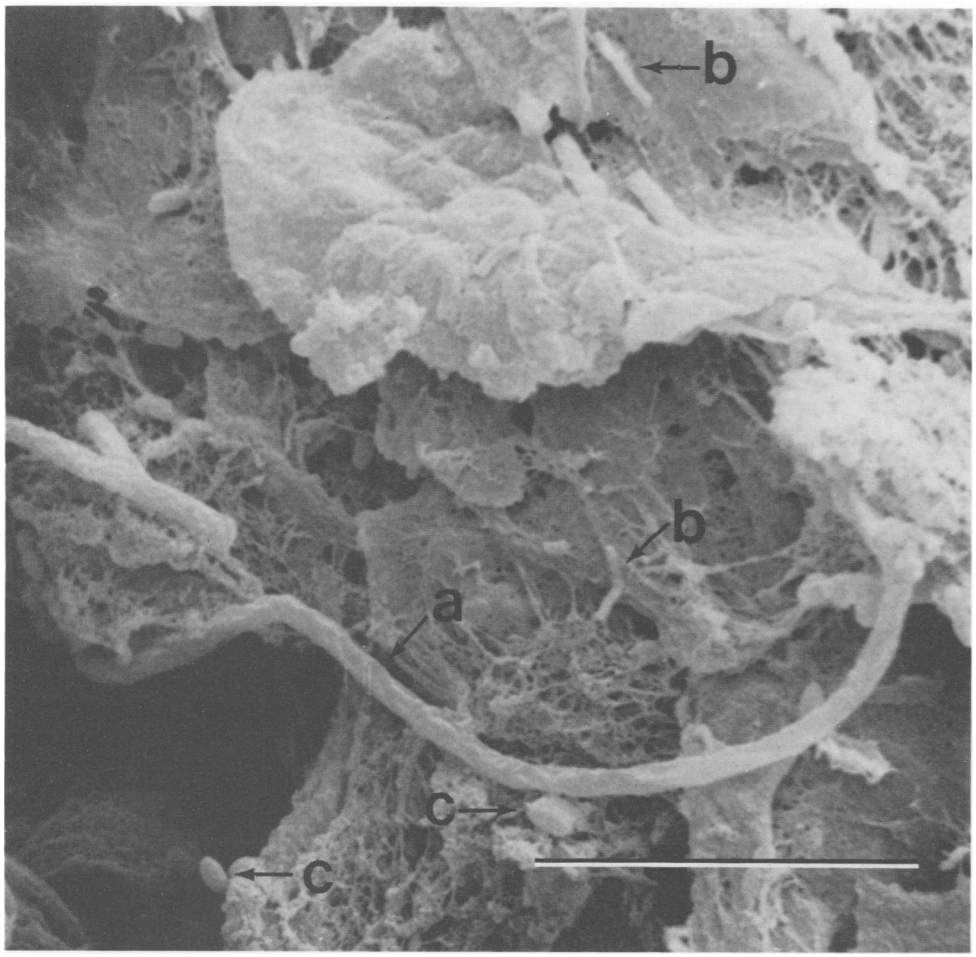

FIG. 1. Scanning electron micrograph of a freeze-fractured surface of the agar matrix containing cells of the phenol-degrading consortium. Cells present are the proposed *Methanothrix*-like organism (a), H_2-utilizing methanogen (b), and the proposed phenol-oxidizing bacterium (c). Bar = 10 μm.

showed the cells to be in an undisrupted state (e.g., Fig. 1). Stoichiometric conversion of phenol to CH_4 and CO_2 was evidence that the consortium remained active after immobilization.

Three distinct bacterial morphotypes which dominated the liquid enrichments were also evident within the agar matrix (Fig. 1). These appeared to be the following three physiological types: an acetoclastic methanogen, recognized by its *Methanothrix*-like morphology; an H_2-utilizing methanogen which also fluoresced; and phenol-oxidizing bacterium. The proposed phenol oxidizer was oval (0.8 by 1.2 to 3.3 μm) (Fig. 2a) and stained gram negative. These cells had an undulating membrane (Fig. 2b) similar to that of two strains of anaerobic benzoate-oxidizing bacteria (13, 20). Approximately 80% of the bacteria in the enrichment had the morphology of the proposed phenol-oxidizing bacterium as shown by random counts under phase microscopy. Both benzoate and *p*-cresol were rapidly metabolized to CH_4 and CO_2 in the enrichment (data not shown), indicating that the phenol-oxidizing bacterium might use these substrates as well.

The rate of phenol degradation was affected by immobilization of the cells; the type of effect depended on the concentration of phenol initially available. At concentrations of 200 μg of phenol per ml (Fig. 3) or less (data not shown), a lag in methane production occurred for native cells but not for the immobilized consortium. At 500 μg of phenol per ml or more, neither system exhibited an apparent lag (Fig. 4). The rate of phenol degradation for native cells decreased at concentrations above 500 μg/ml and for immobilized cells at concentrations above 1,000 μg/ml. At 2,000 μg/ml of phenol, native cells were completely inhibited, whereas the immobilized cells maintained some activity.

Kinetics of phenol degradation. The kinetic parameters V_{max}, K_m, K_i, and X were compared between native and immobilized consortia. Rates of methane production were obtained during the initial 45 min of linear productivity with 10, 20, and 30 μg of phenol per ml. These rates were used to construct Lineweaver-Burk plots (Fig. 5). Low phenol concentrations were used to avoid substrate inhibition. The substrate range was constrained by two factors. Phenol concentrations greater than 40 μg/ml apparently inhibited metabolism by native cells, while below 10 μg/ml our instrumentation did not allow accurate estimations of CH_4 production rates. The fit of linear plots in double-reciprocal coordinates was taken as support for the Michaelis-Menten model of substrate dependence and as evidence that these phenol concentrations were not inhibitory. Inhibition would

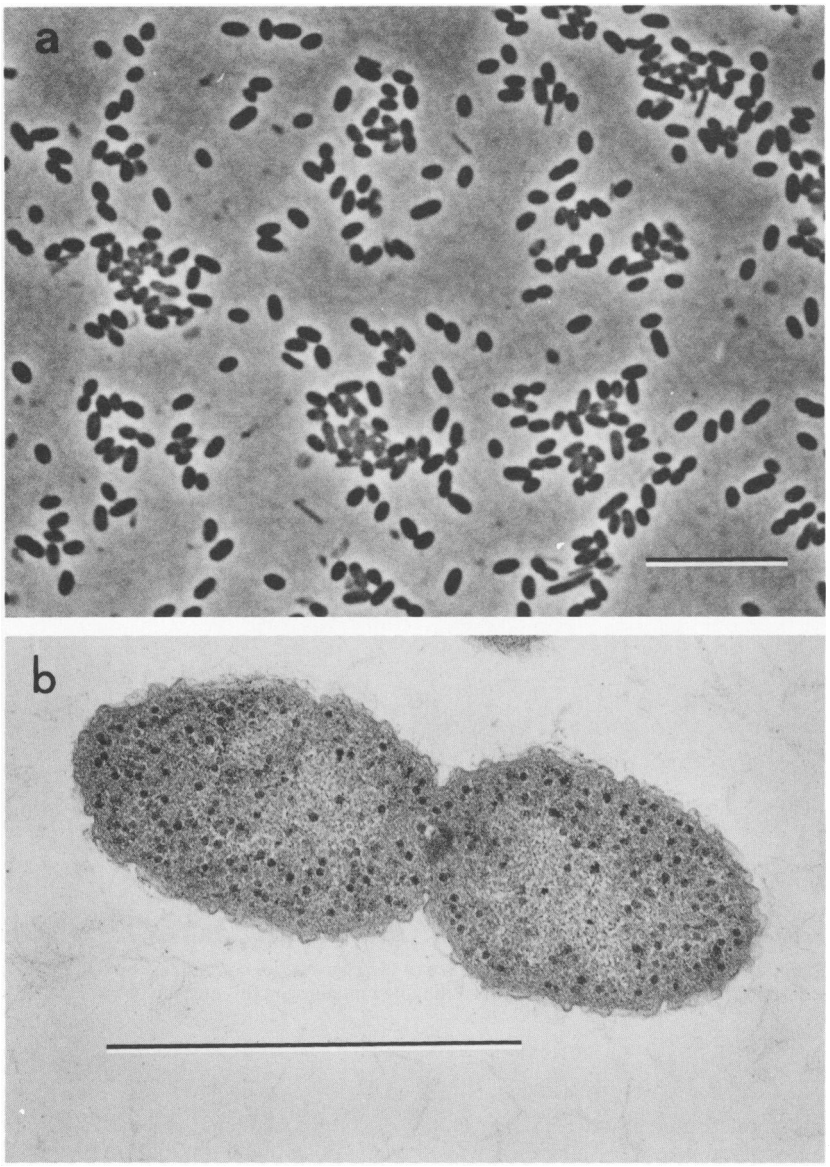

FIG. 2. (a) Phase-contrast photomicrograph of the consortium, showing the shape and numerical dominance of the organism proposed to be the phenol-oxidizing bacterium. Both single cells and cells in division are shown. Bar = 10 μm. (b) Transmission electron micrograph of a thin section of the proposed phenol-oxidizing bacterium.

have been expressed as higher reciprocal rates due to a decrease in the degradation rate with higher phenol concentration.

The linearity between the rate of methane production and phenol concentration (Fig. 5) indicated that S (equation 2) was the rate-limiting factor. The rates for immobilized cells, v', were proportionately less than for native cells, v, probably owing to mass transport limitation of substrate which reduced S to S', the apparent initial substrate concentration for immobilized cells. The estimated kinetic parameters from these plots are listed in Table 1.

Model. The modified model of Neufeld (equation 2) was used to obtain kinetic parameters (Table 1) which best fit a series of experimental rate determinations. The values obtained from the model were used to construct simulated curves for the degradation rate versus the concentration of phenol for both native and immobilized cells. The curves shown in Fig. 6 are superimposed on the experimental data. Both plots show an area of substrate stimulation (a), saturation (b), and inhibition (c). Immobilized cells did not attain rates as high as those of native cells but were protected from the inhibitory effect of high phenol concentrations. For example, at 1,000 μg of phenol per ml the rate of phenol degradation by native cells was reduced by approximately 40% from the observed maximum, while the maximum rate for immobilized cells was unaffected (Fig. 6). This protection was also indicated by the K_i values (Table 1) of 900 (native) versus 1,725 μg/ml (immobilized). The accuracy of this model for predicting phenol oxidation rates was further supported, since the peak of activity occurred for both native and immobilized cells at the predicted $[S = (K_m K_i)^{0.5}]$ phenol concentrations of 285 and 282 μg/ml, respectively.

The experimental degradation rate for native cells at 1,500 μg/ml appeared to cause the simulated curve to overestimate

rates for region c (substrate inhibition). If the curve was computed without this value it approached a rate of zero at 2,000 μg/ml.

DISCUSSION

We were successful in devising a form of immobilization mild enough to maintain the syntrophic activity necessary for phenol biodegradation with minimal exposure of cells to oxygen. Many immobilizing agents, e.g., polyacrylamides, either may be toxic (11), use oxidative polymerizing agents (8), or cause cell disruption (25). We therefore used cell entrapment in agar, since both agar and alginate gel immobilization methods are relatively mild and maintain anaerobic cellular activity (8, 18). Observation of many samples by scanning electron microscopy (e.g., Fig. 1) demonstrated the good physical condition of the cells of the phenol-degrading enrichment. Stoichiometric production of methane from phenol demonstrated activity by all three physiological groups.

The spaghetti agar was of a consistent size (1 mm in diameter), which is important for comparative kinetic studies. The immobilized cells also maintained long-term (>1 month) phenol-degrading activity. Thus, our immobilization method is useful both for laboratory screening of anaerobic cellular activity and for determining the kinetic changes associated with immobilization.

The effect of immobilization on the methanogenic consortium was twofold. It protected the cells from inhibitory concentrations of phenol and altered the kinetic characteristics of the food chain. The latter is shown by a decrease in the apparent K_m (Table 1), the lag period (Fig. 3), and the rate of phenol mineralization (Fig. 5). Since activity was measured as methane production, we feel that these effects were due to the retention of methanogenic substrates during the mineralization of phenol in close proximity to interacting cells within the agar matrix. This is in contrast to results from studies involving single enzymatic reactions. A kinetic analysis of β-glucosidase activity with a pure culture of immobilized *Alcaligenes faecalis* showed that the apparent K_m increased above that for the free enzyme (24). This occurred while the apparent V_{max} decreased and reflected the restriction of substrate transport across the immobilizing matrix. In our study this same restriction likely caused the retention of methanogenic substrates and is reflected in the decrease in the apparent K_m.

Studies have shown that substrate diffusion through agar and water are apparently similar, but in our experiment an

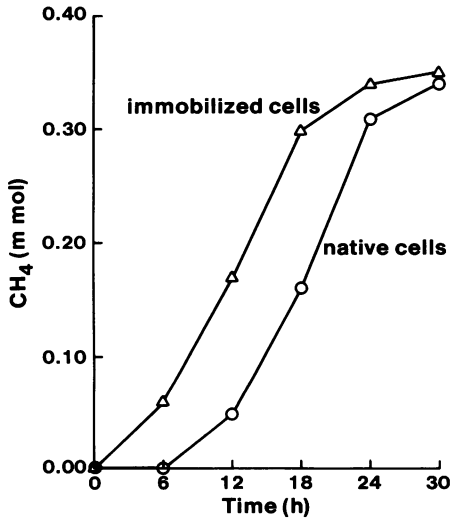

FIG. 3. Temporal production of methane from 200 μg of phenol per ml by native (○) and immobilized (△) cells. Complete conversion of phenol should yield 0.35 mmol of CH_4.

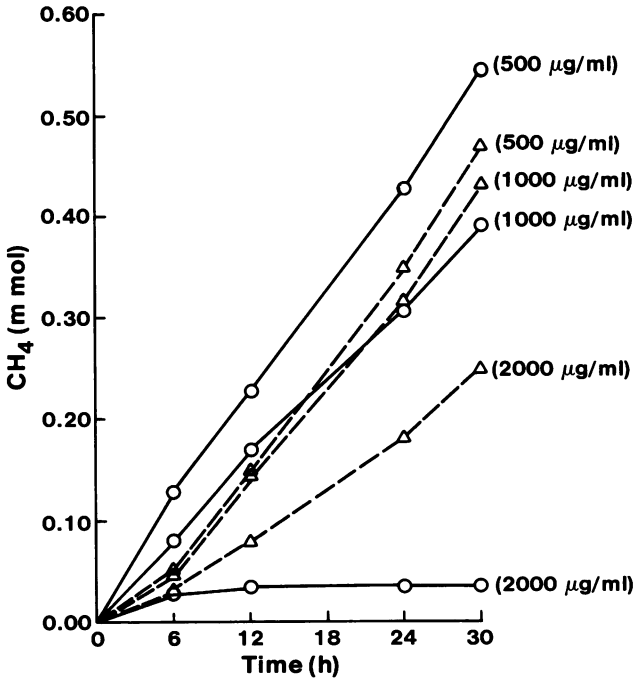

FIG. 4. Temporal production of methane from phenol biodegradation by native (○) and immobilized (△) cells. Initial phenol concentrations are in parentheses.

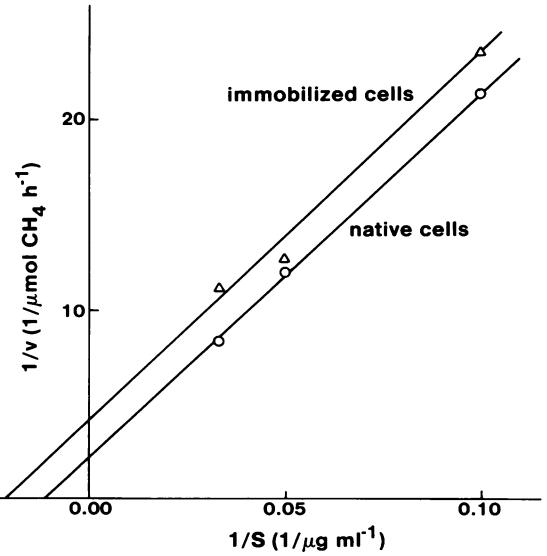

FIG. 5. Lineweaver-Burk plots of initial methane production rates by native (○) and immobilized cells (△). Rates are mean values of duplicate cultures. Initial phenol concentrations were 10, 20, and 30 μg/ml. V_{max} and K_m estimates were obtained from these plots. Regression coefficients: $r^2 = 0.999$ for native cells, and $r^2 = 0.986$ for immobilized cells.

TABLE 1. Kinetic parameters for phenol utilization by native and immobilized cells

Cell culture	Estimation method	V_{max} (μg/ml per h)	K_m (μg/ml)	K_i (μg/ml)	X^a	n^b
Native	Lineweaver-Burk	14.0	78	1,400	—[c]	—
	Model	20.0	90	900	—	2.5
Immobilized	Lineweaver-Burk	7.8	47	1,780	0.6	—
	Model	12.0	46	1,720	0.6	3.6

[a] Empirical parameter used to change the real substrate concentration of the culture, S, to the apparent substrate concentration in the immobilizing matrix, S'.

[b] Empirical value which describes the order of substrate inhibition and is obtained by fitting the kinetic model to rate data.

[c] —, Not determined.

apparent limitation of mass transport occurred within the immobilizing agar matrix relative to the medium. This conclusion is based on the obvious reduction in both the apparent V_{max} and the apparent K_m for immobilized versus native cells. Furthermore, there is some evidence that porosity and diffusional characteristics of similar gels, e.g., alginate, may be reduced by incorporation of other substituents into the immobilizing matrix (22).

The response of methane production to phenol concentration (Fig. 4 and 6) was similar to that for previous batch culture studies (6). Three distinct regions of methane-producing activity were evident in the range of 10 to 2,000 μg of phenol per ml (Fig. 6). Region a demonstrated first-order kinetics, and the initial methane production rates increased with phenol concentration. Immobilized cells had lower rates, probably due to mass transport limitations of phenol in the agar matrix. In region b both native and immobilized cells reached a maximum rate of activity. For native cells, this equaled the rate predicted by the Lineweaver-Burk estimation of kinetic parameters (14 μg of phenol per h). In contrast, the maximum rate of activity for immobilized cells (10 μg of phenol per h) was greater than predicted because the Lineweaver-Burk analysis was affected by diffusion-limited mass transport at low phenol concentrations. Higher concentrations of phenol might have increased S'. Region b included a wider range of substrate concentrations for immobilized cells than for native cells. This was a result of the protection against substrate inhibition afforded by immobilization. While the phenol concentrations of region c inhibited activity, immobilized cells were protected. At 2,000 μg of phenol per ml, native cells were completely inhibited, whereas immobilized cells maintained about one-half of their phenol-degrading activity. This protection from phenol inhibition also is evident from a comparison of the K_i values (Table 1).

Our model-derived value of K_i for immobilized cells was 1,725 μg of phenol per ml, whereas for native cells it was 900 μg/ml, which is comparable to the K_i values of 500 to 700 ppm (500 to 700 μg/ml) and 966 ppm (966 μg/ml) found by Pearson et al. (15) and Neufeld et al. (14), respectively. Our results indicated that immobilization might be an alternative to the dilution treatment now used by many facilities for lessening the effect of toxic substrates. This is important considering that wastewater from coal conversion techniques may contain phenolic compounds up to 7,600 ppm (7,600 μg/ml) (5). Other microbial processes should be amenable for immobilization, since agar gels allow the diffusion of substrates with molecular weights of less than 2×10^4 g/mol (22). As an example, we have successfully immobilized polyethylene glycol-degrading methanogenic cocultures (unpublished results).

Our diffusion-limited rate data were successfully used in a kinetic model designed to describe substrate-inhibited activity. Kinetic growth models for inhibitory substrates have been tested (14, 21), but an empirical model which incorporates a mass transport limitation has not. Our modification of the model of Neufeld (14), equation 2, was made to compensate for the limiting effect that immobilization should have upon apparent substrate concentrations and substrate-dependent kinetics (24). An analysis of the model shows why this is true. At low substrate concentrations, $K_m/(S \cdot X) > (S/K_i)^n$ and the equation reduces to its simple Michaelis-Menten function. In this form, V'_{max} is overestimated for immobilized cells without X to effectively change S to S'. With X, V_{max} is reduced to V'_{max} by the percentage underestimated by the Lineweaver-Burk analysis. Simulated values for v' then reflect activity at S', which was the case, as the close agreement between our experimental data and simulated data shows (mean square deviation = 1.9).

At high inhibitory substrate concentrations, $(S/K_i)^n > K_m/(S \cdot X)$ and the inhibition term decreases V_{max}. We did not obtain an estimate for the apparent substrate concentration at high values of S, but if S' could be obtained, its incorporation would perhaps be beneficial. Not altering $(S/K_i)^n$ did not disturb the close fit of the experimental data and the simulated curve. Thus, the importance of obtaining estimates for both K_m and K_i is readily evident. When $K_i > K_m$, the system can approach a maximum rate at $S = (K_m K_i)^{0.5}$, but when $K_i \approx K_m$, substrate inhibition occurs at low concentrations, thus preventing the system from reaching its apparent V_{max}.

This model can serve as a framework for understanding the effects of immobilization on consortia which utilize inhibitory substrates. Process stability for continuous culture systems should be increased both by virtue of the higher K_i obtained by immobilization and by prevention of cell washout at dilution rates higher than maximum growth rates. While our modified inhibition model may not be directly applicable to all types of substrate-inhibited systems (1, 3), it is important to note that substrate inhibition can be modeled for immobilized cells and estimates can be obtained for the kinetic parameters of substrate degradation. The substrate

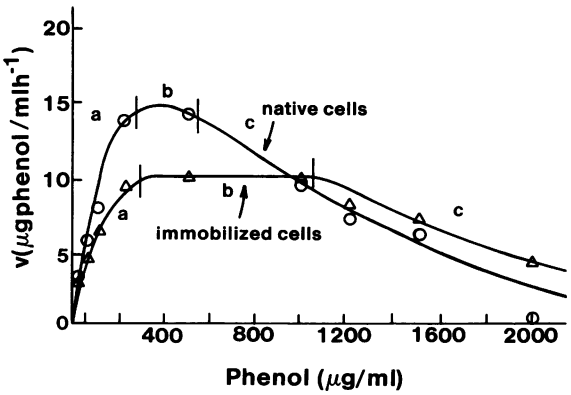

FIG. 6. Simulated curves of phenol degradation versus phenol concentration for both native and immobilized cells. Experimental data for native (○) and immobilized (△) cells are presented in the same plot to show their fit with the model-derived curves. Each datum point is a mean of triplicate determinations. Plots indicate areas of substrate stimulation (a), saturation (b), and inhibition (c).

limitation parameter is both easily understood and quantifiable, which makes it a useful concept for other models as well.

ACKNOWLEDGMENTS

We thank John Weeg for helping design the model program, Genevieve Macomber of the Center of Electron Optics for the scanning electron microscopy work, Stuart Pankratz of the Department of Microbiology and Public Health for the transmission electron microscopy work, Frank Dazzo for photomicrography, Mark Mikesell for the gift of the phenol-degrading enrichment, and Steve Simkins and Joe Robinson for helpful discussions.

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