Immobilization of Degradative Bacteria in Polyurethane-Based Foams: Embedding Efficiency and Effect on Bacterial Activity

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Immobilization of degradative bacteria in polyurethane-based foams: embedding efficiency and effect on bacterial activity

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ABSTRACT

The immobilization of the TCE-degrading bacterium Burkholderia cepacia was evaluated using hydrophilic polyurethane foam. The influence of several foam formulation parameters upon cell retention was examined. Surfactant type was a major determinant of retention, with a lecithin-based compound retaining more cells than pluronic or silicone based surfactants. Excessive amounts of surfactant led to increased washout of bacteria. Increasing the biomass concentration from 4.8 to 10.5% caused fewer cells to be washed out. Embedding at reduced temperature did not significantly affect retention, while the use of a silane binding agent gave inconsistent results. The optimal formulation retained all but 0.2% of total embedded cells during passage of 2 liters of water through columns containing 2 g of foam. All foam formulations tested reduced the culturability of embedded cells by several orders of magnitude. However, O_2 and CO_2 evolution rates of embedded cells were never less than 50% of unembedded cells. Nutrient amendments stimulated an increase in cell volume and ribosomal activity as indicated by hybridization studies using fluorescently labeled ribosomal probes. These results indicated that, although immobilized cells were nonculturable, they were metabolically active and thus could be used for biodegradation of toxic compounds.

Introduction

The use of immobilized degradative bacteria has recently been suggested as a possible approach in the *ex situ* treatment of hazardous chemicals (Levinson et al., 1994; Wilde et al., 1996). Several studies have shown that entrapment systems can deliver bacteria capable of transforming many pollutants (Weir et al., 1995; Xu et al., 1996). For example, the transformation of phenol, p-nitrophenol, pentachlorophenol, and p-cresol by immobilized microbial cells has recently been documented (Bettmann and Rehm, 1984; O'Reilly and Crawford, 1989a). Immobilized bacteria might increase initial degradation rates of a compound in bioreactors by eliminating the need to wait for biofilm formation. Moreover, immobilized bacteria could prove to be cost effective in bioremediation projects since they can potentially be used several times without significant loss of activity (Rhee et al., 1996). One advantage of this approach over bioreactors is the higher microbial biomass retention attainable in continuous bioconversion systems (Tanaka et al., 1986). Additionally, the embedding material can offer protection to the immobilized cells against chemical toxicity by sorption of toxic compounds. For example, a Flavobacterium sp. was capable of transforming pentachlorophenol (PCP) while immobilized in agarose beads but could not as free cells (O'Reilly and Crawford, 1989b). It was concluded that the initial high concentrations in the batch reactors were toxic to free cells, thus preventing PCP degradation.

Immobilization by entrapment or encapsulation would appear particularly advantageous in cases where the adhesion properties of the degradative microorganisms make them unsuitable for use in bioreactors. One example is Burkholderia cepacia (formerly Pseudomonas cepacia) G4, a bacterium capable of transforming several chemicals, including trichloroethylene (TCE). Due to its poor attachment to surfaces, G4 is generally washed out from bioreactor systems. For example, G4 was replaced by native microbes within a few days after a trickled filter reactor was opened

to water from a contaminated aquifer (Berry, unpublished results). Therefore, despite many attempts to use G4 in the *ex situ* treatment of TCE, this bacterium does not normally form part of mature biofilm communities in bioreactors.

Numerous techniques for immobilizing bacteria have been evaluated, most of which could be classified as entrapment or adsorption methods (Woodward, 1988). Some of the immobilizing agents used in these techniques include polyacrilamide, agarose, alginate, carrageenan, clay, granular activated carbon, and polyurethane foams (Levinson et al., 1994; Casidy et al., 1996). However, most studies involving immobilized degradative bacteria have used alginate and carragenan beads (Trevors et al., 1992). Although some studies have shown the potential of alginate and carragenan in delivering functional cells, they can cause a drastic reduction in viability and have poor resistance to natural degradation (Levinson et al, 1994). Other immobilization polymers, such as polyurethane based foams, might circumvent some of these problems (Thomas and Macaskie, 1996).

Although several factors might affect the success of an immobilization technique in a biotechnological application, in most cases the immobilization or entrapment efficiency and the effect on microbial activity are of utmost importance. For example, the capability of an immobilization technique to prevent the washout of microbial cells will undoubtedly affect the practical duration or functional half life of the process in question. In addition, if viable microbial biomass is needed in the process the immobilization technique should not impair enzymatic or metabolic activities required for the desired application. Consequently, a careful examination of these factors is critical when evaluating an immobilization technique.

In this study, we evaluated the use of a novel hydrophilic polyurethane based foam for the immobilization of degradative bacteria. The objective was to test the effects of various foam formulation parameters upon entrapment efficiency. The viability and activity of embedded cells were also

determined using plate counts, most probable number analysis, respirometry, and hybridization using 16S rRNA targeting oligonucleotide probes.

Materials and Methods

Bacterial strains and growth conditions. The strain B. cepacia G4 and its constitutive mutant, strain PR131 were kindly provided by M. Shields (University of West Florida). Bacterial strain 01-b and yeast #14 were provided by S. Crow (Georgia State University). The latter microbial strains were originally isolated using phenol as the sole carbon source. The mixed community culture used in viability experiments was isolated in our laboratory using minimal media plates and chlorobenzene vapors. Microbial isolates were normally grown on PTYG (10 g glucose, 10 g yeast extract, 5 g peptone, 5 g tryptone, 0.6 $MgSO_4 \cdot 7H_2O$, and 0.07 $CaCl_2 \cdot 2H_2O$) plates prior to inoculation into liquid media. Axenic batch cultures were grown in Pseudomonas medium (Atlas, 1993) or a yeast-glucose medium (YGM) (Shields and Reagin, 1992), which consisted of basal salts medium (BSM) (Shields et al., 1989) plus 1 g/l glucose and 0.5 g/l yeast extract.

Small-scale (100 - 250 ml) batch cultures were grown in shake flasks (200 rpm, 30°C). Large-scale cells were grown by inoculating 20 ml of a batch culture into 4 L polycarbonate bottles containing 3 L YGM or Pseudomonas medium. These cultures were maintained at 26 ± 2°C and aerated through a sterile 0.2 µm filter. Cultures were routinely harvested for foam embedding after 3 d, at which time biomass yield was approximately 0.5 g/l dry weight. Axenic status of the cultures was verified by streaking on PTYG plates. For experiments requiring the induction of the toluene monooxygenase gene (Shields et al., 1989), G4 cells were exposed to 2 mM phenol for 2 h prior to harvesting. Harvesting was by centrifugation (10,000 rpm, 10 min, 15°C). Cell pellets were resuspended in BSM or Pseudomonas medium to a density of 2.2 - 17.7% dry weight.

Immobilization of bacterial cells. Bacterial slurries were routinely maintained at 4°C prior to the embedding process. Cells were embedded in

hydrophilic polyurethane foam (U. S. Patent No., 5,405,648) within 2 h after slurry preparation. Foam samples were prepared by Frisby Technologies (Freeport, NY) at their Aiken, SC facility. Ingredients of each foam sample were: slurry, 20 g; prepolymer, 13.33 g; surfactant, 0.54 g. A 5% (dry wt) slurry yielded approximately 3 g dry wt bacteria per 100 g wet wt foam. Three surfactants were compared in the course of the study. These were HS-3 (lecithin-based), F-88 (ethylene oxide and propylene oxide based), and DC198 (silicone-based). The prepolymer Bipol 6B (NCO=6) was routinely used, with three other prepolymers of lower NCO values (Bipol 3, #350, #802) being used for comparison purposes in viability studies. NCO indicates the number of reactive sites available in prepolymer that reacts with water in aqueous phase. The addition of silane as a binding agent was tested in certain foam formulations. Prepolymers, surfactants, and silane were provided by Matrix R&D Corp. (Dover, NH). Control (cell - free) foams were generated by substituting 20 g sterile medium for bacterial slurry. Foam samples were granulated by means of a Waring blender and stored at 4°C prior to use.

Washout experiments. Entrapment efficiency (i.e., bacterial washout) was measured using 10 ml Poly-Prep chromatography columns (Bio-Rad Laboratories, Hercules, CA) modified by the replacement of the stock fritted disk with 75-80 mg glass wool. Duplicate columns were loosely packed with 2.0 ± 0.01 g (wet wt) of each foam type. Cell retention by various foam types was routinely compared by passing 50 ml of autoclaved, 0.2 µm filtered deionized water through each column (gravity feed) and collecting the effluent. Effluents were preserved with 0.2 µm filtered formaldehyde (3.7% final concentration) and their bacterial content was determined by direct microscopical counts following staining with Acridine Orange (described below). Formulations that released the fewest bacteria were retested using larger volumes of deionized water. In one experiment, 1000 ml water was passed through duplicate columns in 50 ml aliquots, with 50 ml samples of effluent being collected when cumulative water addition had reached 50, 150,

400, 550, 700, 850, and 1000 ml (intervening effluent aliquots were discarded). A second experiment involved passage of 2000 ml through duplicate columns, with the accumulated effluent being sampled when cumulative water addition reached 50, 1000, 1500, and 2000 ml.

Viability and activity measurements. For viability estimates, cells were serially diluted in FA buffer (Difco Co., Detroit, MI), spread on PTYG plates and incubated at 30°C for 3 to 5 days. Alternatively, we used the Most Probable Number (MPN) technique (Koch, 1994) to measure their culturability in liquid PTYG medium. Cells in the foam were released by vigorous vortexing (30 sec). The percent of viable cells was determined by comparing the number of colony forming units (CFU) or MPN to the direct microscopical counts. A modification of the acridine orange direct count (AODC) technique was used to determine direct microscopical counts (Hobbie et al., 1977). Appropriate cell dilutions were spotted on heavy teflon coated slides (Cél-Line Associates, Inc., Newfield, NJ) and heat fixed at approximately 50° C using a thermal block or a hybridization oven. Cells were stained with 0.01% of acridine orange for 2 min at room temperature. Excess stain was removed with 0.2 µm filtered nanopure water. Slides were allowed to air dry and immediately observed under a Zeiss Axioskop epifluorescent microscope (filter set 09; Carl Zeiss, Inc., Jena, Germany). Twenty microscopic fields were examined to determine total counts. Viable counts and direct microscopical counts were done in duplicate or triplicate.

CO_2 evolution and O_2 uptake were measured using a Micro-Oxymax v5.12 indirect closed circuit respirometer (Columbus Instruments, Columbus OH). Triplicate samples consisting of 8 g of foam containing embedded bacteria or the equivalent number of unembedded cells (5 ml slurry) were used in the respiration experiments. Samples were incubated at two different temperatures (approximately 20°C or 25°C) with or without agitation (130 rpm) to compare the effect of temperature and oxygen availability on the respiration rates of immobilized cells and unimmobilized cells.

The physiological response to the addition of a carbon source was also determined to evaluate the effect of embedding on bacterial activity. Immobilized and slurry cells were transferred to mineral media containing 0.2% glucose and incubated at room temperature for 24 h. Aliquots were taken after 2, 4, 6, and 24 h, fixed with 3.7% formaldehyde (final concentration), and stored at 4°C for 24 h. Cells were centrifuged at 11,000 x *g* to remove formaldehyde and then resuspended in 0.2 µm filtered nanopure water. Aliquots were fixed on slides as described above and hybridized with a tetramethylrhodamine labeled oligonucleotide probe (Genosys, The Woodlands, TX) complementary to a highly conserved region of the 16S rRNA gene (positions 342-360 of Escherichia coli). This ribosomal probe has been shown to target the small ribosomal subunit (SSU) of eubacteria (DeLong et al., 1989). Hybridizations were performed in an Autoblot hybridization oven (Bellco Glass, Inc., Vineland, NJ) following the procedure of Braun-Howland et at. (1992). After hybridization and washing, cells were stained with DAPI (4',6-diamidino-2-phenylindole) as described in Weiss et al. (1996) to estimate total bacteria per field. Fluorescing cells were observed by epifluorescence microscopy using a Zeiss Axioskop and filter sets 2 and 15.

Results

Washout experiments. A list of the different formulations evaluated and the results from washout experiments is shown in Table 1. Surfactant F-88, was used to test the effect of temperature, surfactant concentration, and presence of silane in bacterial washout. As shown in Table 1, decreasing the concentration of surfactant F-88 from 0.016 % to 0.008 % or increasing it to 0.16% did not reduce bacterial washout. In fact, increasing the concentration of surfactant considerably increased the number of bacteria removed from the foam, arguably due to an increase in the number and size of interconnected pores in the matrix.

A reduction in bacterial washout from ca. 36% to ca. 25% was achieved when the embedding was performed in the cold, although it was not statistically significant (Table 1). Adding silane further reduced bacterial washout to 13%. However, the greatest reduction in washout (to < 8%) occurred when the concentration of bacterial biomass in the slurry used for embedding was increased from 4.8% to 10.5% final concentration. Surfactant type also had a noticeable effect on retention of bacterial cells. Surfactant HS-3 was more effective in preventing washout than DC198 and F-88, although F-88 can achieve a similar reduction in washout when bacterial biomass is 10.5%. Since HS-3 was the most effective of the three surfactants in preventing washout when biomass content was held constant, it was used in all subsequent experiments.

The effect of silane and increased biomass concentrations were re-evaluated in formulations containing HS-3, since both parameters reduced washout in the presence of F-88. Results are summarized in Fig. 1. Increasing bacterial biomass to approximately 11% reduced bacterial washout from nearly 6 % to less than 2 %. The combination of 8% bacterial slurry and silane reduced washout to approximately 2%. However, in contrast with previous results (Table 1), increasing biomass concentration resulted in increased

washout when silane was included in the formulation. Thus, silane was not added to foam formulations used in subsequent viability experiments.

Bacterial washout was further examined by adding larger volumes of water to these foams. In the experiment shown in Fig. 2, the first 50 ml of water liberated 0.2%, 2.0%, 1.2%, and 0.7 % of the total embedded cells from foams containing 10.5% bacterial biomass, 10.5 % bacterial biomass with silane, 4.8% bacterial biomass, and 4.8% bacterial biomass with 1% silane, respectively. These initially low values declined even further with subsequent aliquots of water. Similar decline in cell washout after the first 50 ml are shown in Figure 3. In these experiments, the above mentioned foams released 0.1%, 0.6%, 5.3%, and 9.7% of total embedded cells into the first 50 ml of water, representing 40.5, 37.6, 71.8, and 9.3 %, respectively, of the total cells removed by 2000 ml of water. Passage of 2000 ml water liberated 0.2%, 13.9%, 16.2%, and 6.0% of the total embedded cells. More than ninety per cent of the bacteria released in 2000 ml were removed after the first liter. In fact, data shown in Fig. 2, it is plausible that most of the washout in the first liter occurred in the first 150 ml. Results of tests using small water volumes thus provide a reasonable means of predicting the comparative performance of foams exposed to larger volumes of water.

Viability experiments. A drastic reduction in apparent viability was observed after B. cepacia PR131 was immobilized in polyurethane based foam as evidenced by a decrease in CFU to 0.006 % of total cell numbers. This represented a decline of more than five orders of magnitude (Fig. 4). Dramatic decreases in viable counts were also observed for other environmental isolates as well as for a mixed community previously enriched on chlorobenzene minimal salts media (Fig. 4). Colony counts performed using other foams shown in Table 1 indicated that culturability was severely impaired by all the foam formulations (data not shown).

During the polymerization process, initial pH of the reaction mixture is approximately 7; the pH then briefly drops to ca. 5 before returning to ca. pH 7

(Hermann, unpublished results). We also observed a brief temperature increase to ≤ 42°C during polymerization. To test whether these factors caused viability loss to embedded cells, we subjected B. cepacia PR131 slurries to heat shock (using a 42°C water bath) and a pH 7→ 5 → 7 shift (accomplished by adding dilute HCl, then dilute NaOH). A control slurry was maintained at constant (room) temperature and pH 7. Total and culturable cell numbers were then determined for all three preparations. In addition, the effect of temperature on culturability was also tested by carrying out the polymerization reaction in an ice bath to prevent temperature increase. In general, viability of the slurries underwent relatively little change as a result of temperature or pH shock (data not shown). Biomass concentration in slurries used for embedding was also varied to determine whether this factor affected viability. However, culturability of the embedded bacteria was extremely low (i. e., < 2%) under all conditions tested (data not shown).

To test whether embedding simply caused B. cepacia PR 131 to become unable to grow on agar-solidified media, we compared colony counts with results obtained using the MPN technique. Both methods yielded exceedingly low viability estimates (data not shown), indicating that previously embedded cells were unable to grow on either liquid or solid media. We also investigated the potential toxic effect of free toluene 2,4-diisocyanate (TDI), a chemical use in the manufacture of polyurethane foams and suspected to be carcinogenic. Reducing the concentration of free TDI did not increase the culturability of embedded bacteria, even when the prepolymer used contained undetectable levels of TDI (data not shown). These results suggested that the polymerization process was inactivating or killing a significant percentage of the immobilized bacteria in an undetermined manner.

Respiration studies. Since it is possible for bacteria to remain active despite their inability to form colonies on artificial media, we investigated the effect of embedding on the metabolic activity of immobilized bacteria using respirometry. Respiration rates of embedded bacteria (B. cepacia G4) were

compared with those of bacterial slurries by measuring CO_2 evolution and O_2 consumption rates. Surprisingly, embedded cells showed higher respiration activity than the bacterial slurries when both populations were incubated at room temperature (approximately 20°C; Fig. 5A). When temperature and aeration were increased (25°C) the slurries showed higher respiration activity than embedded cells (Fig. 5B). Nevertheless, immobilized cells retained > 50% of the respiration activity shown by the cells in the slurry suspension, suggesting that the polymerization process had not altered the physiological activity of embedded bacteria as severely as the culturability results indicated. Similar results were obtained with other environmental isolates immobilized in polyurethane foams (data not shown). We also compared the effect of immobilization on the respiration rates of B. cepacia G4 and B. cepacia PR131. No meaningful differences were observed between the mutant and the parental strain (data not shown). These data, together with the absence of microscopically visible changes in cell morphology, suggest that immobilization did not severely impair the overall metabolic capacity of the microorganisms.

Nutrient amendments. Immobilized cells and bacterial slurries responded to the addition of nutrients by increasing the signal intensity after hybridization with ribosomal probes. Approximately 2 % and 4 % of the total embedded and unembedded cells, respectively, hybridized to the ribosomal probe after 4 hours of nutrient amendments (Fig. 6). This represented nearly five orders of magnitude increase in cell activity previous to glucose addition. The number of hybridizing cells increase for both embedded and unembedded cells after 6 h to nearly 8 %. A further increase in the percentage of hybridizing cells was noted for both types of cells after 24 h, although the response was more dramatic for the unembedded cells than for the embedded cells (i.e., 65 % and 23 %, respectively). Many of the embedded bacteria became elongated and showed very intense hybridization signals, suggesting that they could grow but were not capable of cell division. This

was in contrast with unembedded cells which seem to be active in cell division. In fact, the higher percent of hybridizing cells for slurry cells after nutrient addition could be attributed to multiple rounds of cell division during the 24 h period. However, based on the number of hybridizing cells after 4 and 6 h, differences in response to nutrient addition were not significant between both cell types, further suggesting that the embedding process did not have the significant effect in the metabolic potential of embedded cells as suggested by the culturability data.

Discussion

The aim of this study was to optimize polyurethane based formulations for the entrapment of degradative bacteria. Our main criterion was the capacity of each formulation to prevent the release of immobilized cells. This criterion is of great importance regarding the functional longevity of this type of carrier system in bioremediation applications. Moreover, in applications involving the use of genetically engineered microbes, the ability to retain cells would seem critical in view of public concern regarding the release of such microorganisms into the environment. This aspect has received little attention by researchers evaluating immobilization agents, polyurethane foams in particular.

Our results indicate that bacterial biomass concentration and the type and concentration of surfactant are major determinants of cell retention. The effect of surfactants could be related to the observation that large numbers of embedded cells are actually located in fluid-filled pores and hence are readily released after the foam is torn or cut. Increasing the surfactant concentration visibly increased total pore volume of the resulting foam and led to higher washout rates. The chemical composition of the surfactant also influences the size, total volume, and interconnectedness of pores due to differences in surface tension, and hence will affect cell retention. The reason for the decrease in washout observed at higher biomass concentrations is unclear, but may be related to the lower water content of foams made with denser slurries. Based on our results, foam made with 1% HS-3 and a 10.5% dry weight bacterial slurry was most effective in retaining embedded B. cepacia. It remains to be seen whether this formulation is equally effective with other organisms.

The dramatic decrease in culturability as a result of immobilization suggested that the embedding processes was detrimental to the physiological status of the bacteria. However, we found no evidence that cells were killed

due to the temperature and pH changes during embedding. Culturability was severely reduced in the presence of all three surfactants, and was not affected by the free TDI content of prepolymers used in foam manufacture. These findings suggested that some unknown factor inherent in the polymerization process was lethal to microorganisms, and led us to question the ability of polyurethane-based formulations to deliver functional bacterial cells. However, respirometry data demonstrated that, although incapable of forming colonies, embedded B. cepacia remained metabolically active. Indeed, respiration rates of embedded bacteria compared favorably to those of free cells. This is consistent with the physiological response observed for both cell types (i.e., increase in cell volume and ribosomal content) and with of considerable increase in the number of embedded cells hybridizing to the ribosomal probe shortly after nutrient addition. The fact that several B. cepacia cells produced elongated forms after nutrient additions suggests that cells were active but could not divide, probably due to a malfunction of membrane or cell wall synthesis. This explains their inability to reproduce in artificial media. Other studies have reported significant reductions in bacterial viability using different embedding matrices (Levinson et al., 1994). However, most of these studies used culturing techniques to assess bacterial viability, and thus might have overestimated the lethal effect of the immobilization process on the metabolic activity of the immobilized bacteria.

The results in our study indicate that the polymerization process caused the embedded bacteria to become viable but nonculturable, a phenomenon previously reported by Roszak et al. (1984). Thus, it appears that culturing techniques are not reliable indicators of the metabolic status of polyurethane embedded cells. In light of these findings, it is likely that embedded B. cepacia will retain its degradative capabilities and potential for bioremediation. Future studies will address this issue in bioreactor systems.

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