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Immobilization of *Burkholderia cepacia* in polyurethane-based foams: embedding efficiency and effect on bacterial activity

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Immobilization of the trichloroethylene-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; a lecithin-based compound retained more cells than pluronic- or silicone-based surfactants. Excessive amounts of surfactant led to increased washout of bacteria. Increasing the biomass concentration in the foam from 4.8 to 10.5% dry weight per wet weight of foam resulted in fewer cells being 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 L of water through columns containing 2 g of foam. All foam formulations tested reduced the culturability of embedded cells by several orders of magnitude, but O_2 consumption and CO_2 evolution rates of embedded cells were never less than 50% of those of free cells. Nutrient amendments stimulated an increase in cell volume and ribosomal activity in immobilized cells as indicated by hybridization studies using fluorescently labeled ribosomal probes. These results indicate that, although immobilized cells were mostly nonculturable, they were metabolically active and thus could be used for biodegradation of toxic compounds.

Keywords: polyurethane; immobilization; *Burkholderia cepacia*

Introduction

The use of immobilized degradative bacteria has been suggested as a possible approach in the *ex situ* treatment of hazardous chemicals [10,22]. Entrapment systems can provide bacteria capable of transforming many pollutants [20,24]. For example, phenol, *p*-nitrophenol, pentachlorophenol, and *p*-cresol can be transformed by immobilized microbial cells [3,11,12]. Immobilized bacteria may increase initial degradation rates of a compound in bioreactors by eliminating the need to wait for biofilm formation. Moreover, immobilized bacteria could be cost effective in bioremediation projects since they can potentially be used several times without significant loss of activity [13]. One advantage of this approach over bioreactors is the higher microbial biomass retention attainable in continuous bioconversion systems [17]. Additionally, the embedding material can offer the immobilized cells protection by sorption of toxic compounds. For example, a *Flavobacterium* sp transformed pentachlorophenol (PCP) while immobilized in agarose beads but not as free cells [12]. 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 appears 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, *B. cepacia* G4 is generally washed out from bioreactor systems. For example, G4 was replaced by native microbes within a few days after a trickling filter reactor was opened to water from a contaminated aquifer (Berry and Hazen, unpublished results). Therefore, despite many attempts to use *B. cepacia* 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 [23]. Immobilizing agents used in these techniques include polyacrylamide, agarose, alginate, carrageenan, clay, granular activated carbon, and polyurethane foams [5,10]. However, most studies involving immobilized degradative bacteria have used alginate and carrageenan beads [19]. Although alginate and carrageenan have potential to provide functional cells, they can cause a drastic reduction in viability and have poor resistance to natural degradation [10]. Other immobilization polymers, such as polyurethane-based foams, might circumvent some of these problems [18].

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 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. Conse-

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quently, 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 immobilization of degradative bacteria. The objective was to test the effects of various foam formulation parameters upon entrapment efficiency. The activity of embedded cells was 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

B. cepacia G4 and its constitutive mutant, strain PR131, were kindly provided by Dr Malcom Shields (University of West Florida, Pensacola, FL, USA). Bacterial strain 01-b and yeast No. 14 were provided by Dr Sydney Crow (Georgia State University, Atlanta, GA, USA). The latter microbial strains were originally isolated using phenol as the sole carbon source. The mixed community used in culturability experiments was isolated in our laboratory using minimal media plates and chlorobenzene vapors. Microbial isolates were normally grown on peptone-tryptone-yeast extract-glucose (PTYG) [2] plates prior to inoculation into liquid media. Axenic batch cultures were grown in *Pseudomonas* medium [1] or a yeast-glucose medium (YGM) [16], which consisted of basal salts medium (BSM) [15] plus 1 g L^{-1} glucose and 0.5 g L^{-1} yeast extract.

Small-scale (100–250 ml) batch cultures were grown in shake flasks (200 rpm, 30°C). Large-scale cultures 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 pore size filter to stimulate adequate growth. Cultures were routinely harvested for foam embedding after 3 days, at which time the biomass yield was approximately 0.5 g L^{-1} dry weight. Axenic status of the cultures was verified by streaking a loop of culture on a PTYG plate. For experiments requiring the induction of the toluene monooxygenase gene [15], cells of *B. cepacia* G4 were exposed to 2 mM phenol for 2 h prior to harvesting them by centrifugation ($15\,344 \times g$, 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 stored at 4°C prior to the embedding process. Cells were embedded in a proprietary hydrophilic polyurethane foam [7] within 2 h after slurry preparation. Foam samples were prepared by Frisby Technologies (Freeport, NY, USA) 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 foam. Three surfactants were compared: 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, and three other prepolymers of lower NCO values (Bipol 3, No. 350, No. 802) were used for comparison purposes to determine culturability. NCO indicates the number of sites in the prepolymer available for reaction with water. The addition of 0.02% (final concentration) silane as a binding agent was tested in certain foam formulations. Prepolymers, surfactants, and silane were provided by Matrix R&D Corp (Dover, NH, USA). Control (cell-free) foams were generated by substituting 20 g sterile medium for bacterial slurry. Foam samples were shredded in a Waring blender and stored at 4°C prior to use.

Washout experiments

Entrapment efficiency (ie, bacterial washout) was measured using 10 ml Poly-Prep chromatography columns (Bio-Rad Laboratories, Hercules, CA, USA) modified by 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 microscopic counts following staining with acridine orange. Formulations that released the fewest bacteria were retested using larger volumes of deionized water. In one experiment, 1 L of water was passed through duplicate columns in 50-ml aliquots, and 50-ml samples of effluent were preserved for analysis 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 2 L of water through duplicate columns, and the accumulated effluent was sampled when cumulative water addition reached 50, 1000, 1500, and 2000 ml.

Culturability and activity measurements

For estimates of culturability, cells were serially diluted in phosphate saline (pH 7.2, FA buffer; Difco Co, Detroit, MI, USA), spread on PTYG plates and incubated at 30°C for 3–5 days. Alternatively, we used the most probable number (MPN) technique [9] to measure culturability in liquid PTYG medium. Cells in the foam were released by vigorous vortexing for 30 s. The percent of viable cells was determined by comparing the number of colony forming units (CFU) or MPN to the direct microscopic counts. A modification of the acridine orange direct count (AODC) technique was used to determine direct microscopic counts [8]. Appropriate cell dilutions were spotted on heavy Teflon®-coated slides (Cel-Line Associates, Newfield, NJ, USA) and heat-fixed using a thermal block. Cells were stained with 0.01% acridine orange for 2 min at room temperature. Excess stain was removed by rinsing the preparation with 0.2-μm-filtered nanopure water. Slides were observed using a Zeiss Axioskop epifluorescent microscope (filter set 09; Carl Zeiss, Jena, Germany). Twenty microscopic fields were examined to determine total counts. Viable counts and direct microscopic 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, USA). 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 respiration rates of immobilized and free cells.

The physiological response to nutrient addition was also determined to evaluate the effect of embedding cells on their 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 $11\,000 \times g$ 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, USA) complementary to a highly conserved region of the 16S rRNA gene (positions 342–360 of *Escherichia coli*). This ribosomal probe targets the small ribosomal subunit (SSU) of eubacteria [6]. Hybridizations were performed in an Autoblot hybridization oven (Bellco Glass, Vineland, NJ, USA) following the procedure of Braun-Howland *et al* [4]. After hybridization and washing, cells were stained with DAPI (4′,6-diamidino-2-phenylindole) as described elsewhere [21] 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 formulations evaluated and the results from washout experiments are shown in Table 1. Surfactant F-88 was used to test the effects of temperature, surfactant concentration, and silane on 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.

An apparent reduction in bacterial washout from *ca* 36% to *ca* 25% was achieved when the embedding was performed in an ice bath (Table 1), but this decrease was not statistically significant. Adding silane further reduced bacterial washout to 13%. However, the greatest reduction in washout (<8%) occurred when the 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 were DC198 and F-88, although F-88 achieved a similar reduction in washout when bacterial biomass was 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 (at the 0.016% level) in subsequent experiments.

The effect of silane and increased biomass concentrations were re-evaluated in formulations containing HS-3, since both factors had reduced washout in the presence of F-88. Results are summarized in Figure 1. Increasing bacterial biomass to 10.5% 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 culturability experiments.

Bacterial washout was further examined by adding larger volumes of water to these foams. In the experiment shown in Figure 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 with subsequent aliquots of water. Similar decline in cell washout after the first 50 ml are

Table 1 Washout of immobilized *B. cepacia* PR131

Foam no.	% Bacteria in foam (dw/ww)[a]	Type of surfactant	Surfactant concentration (v/v)	Embedding temperature[b]	Silane addition	% Washout[c]
1	4.8	F-88	0.016%	RT	No	36.38 (7.09)
2	10.5	F-88	0.016%	RT	No	7.24 (2.12)
3	4.8	DC198	0.016%	RT	No	19.21 (5.93)
4	4.8	HS-3	0.016%	RT	No	7.82 (3.14)
5	4.8	F-88	0.008%	RT	No	37.02 (8.45)
6	4.8	F-88	0.159%	RT	No	80.21 (18.34)
7	4.8	F-88	0.016%	Cold	No	25.13 (7.75)
8	4.8	F-88	0.016%	RT	Yes	13.07 (3.19)

[a]The per cent of bacterial dry weight per total wet weight of foam.
[b]Temperature at which the embedding process was performed. RT = room temperature (22 ± 2°C); Cold = iced water bath.
[c]Percentage of total immobilized cells that were detached from the foam using 50 ml of sterile water. Cell densities were determined using the acridine orange direct count method. Numbers of immobilized cells were determined from the amount of bacterial slurry used for the embedding. Numbers in parentheses represent standard errors.

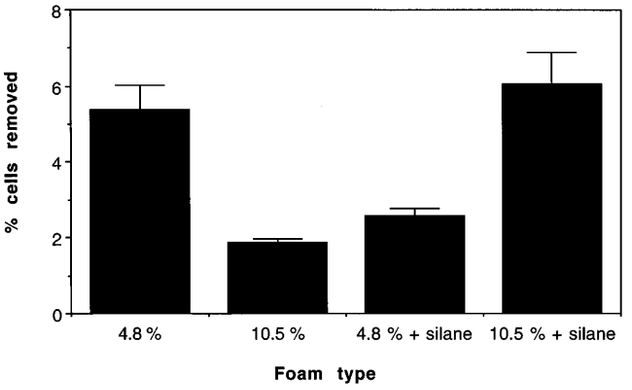

Figure 1 Effect of biomass concentration and silane in the washout of *B. cepacia* PR131 cells embedded in foam formulations containing the 1% surfactant HS-3. Silane was added at a final concentration of 0.02%. The final biomass concentration (dry wt/wet wt) used was 4.8 or 10.5. Values represent mean percentages of total embedded *B. cepacia* PR131 cells removed from duplicate columns containing 2 g of foam during passage of 50 ml of filter sterilized water. Error bars indicate standard deviation of means.

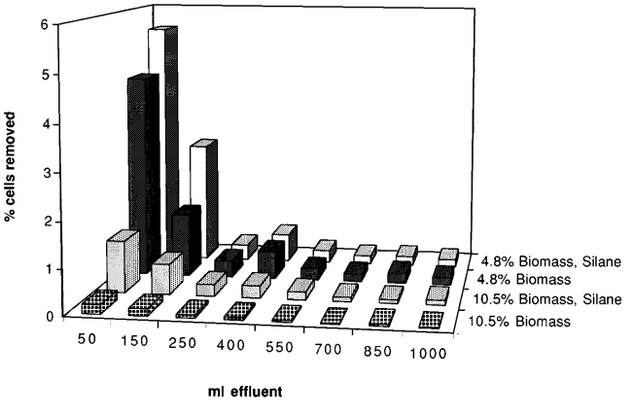

Figure 2 Sequential washout of embedded cells in foams containing different concentrations of biomass and with or without silane as binding agent. Foams used are described in Figure 1. A liter of water was passed through columns in 50-ml aliquots. Total cell numbers were determined in 50-ml effluent samples collected at the intervals indicated. Numbers represent means of duplicate columns.

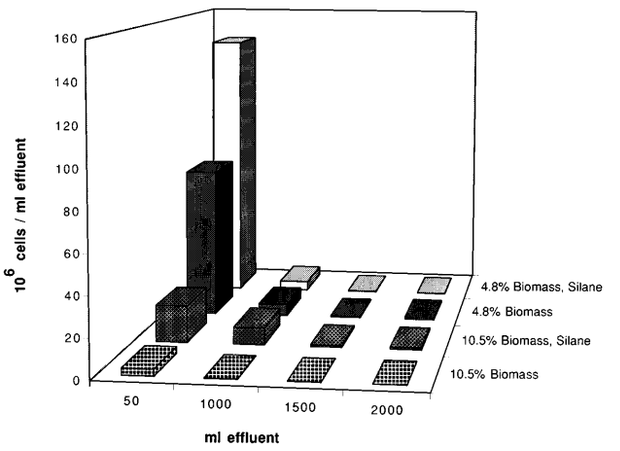

Figure 3 Cumulative washout of embedded *B. cepacia* PR131 bacteria in foams used in Figure 1. A total of 2 L of water was passed through foam columns. The first 50 ml were collected, followed by 950 ml, 500 ml, and 500 ml. Values represent mean cell density in each sample.

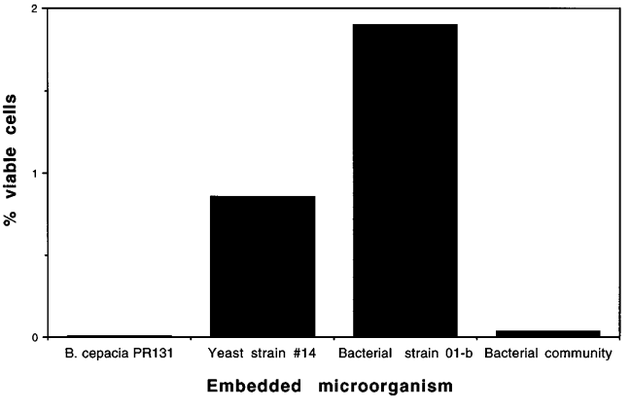

Figure 4 Culturability of embedded microorganisms. Two bacterial strains (*B. cepacia* PR131 and strain O1-b), a bacterial community, and a yeast (*Candida* sp) were embedded in polyurethane foams containing surfactant HS-3. Colony and microscopic counts were performed on samples of cells removed from foam by vortexing. Values represent means of duplicate or triplicate samples.

shown in Figure 3. In these experiments (Figure 3), 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 2 L of water. Passage of 2 L water liberated 0.2%, 13.9%, 16.2%, and 6.0% of the total embedded cells. More than 90% of the bacteria released in 2 L were removed after the first liter. In fact, Figure 2 suggests that most washout 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.

Culturability

A drastic reduction in the number of culturable cells was observed after *B. cepacia* PR131 was immobilized in the polyurethane-based foam as evidenced by a decrease in CFU to 0.006% of total cell numbers (Figure 4). This represented a decline of more than five orders of magnitude. 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 medium (Figure 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, the 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 culturability loss, 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, culturability 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 cells was also varied to determine whether this factor affected culturability. However, culturability of the embedded bacteria was extremely low (ie, <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 culturability 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 used in the manufacture of polyurethane foams and suspected to be carcinogenic. The use of prepolymers containing reduced free TDI levels did not increase the culturability of embedded bacteria, even when TDI was reduced below detection levels (data not shown). These results suggested that the polymerization process inactivated or killed a large percentage of the immobilized bacteria in an undetermined manner.

Respiration

Since bacteria can remain active despite their inability to form colonies on laboratory media, we investigated the effect of embedding cells on their metabolic activity using respirometry. Respiration rates of embedded *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 bacterial slurries when both populations were incubated at room temperature (approximately 20°C; Figure 5a). When temperature was increased to 25°C and aeration augmented by shaking at 130 rpm the slurries showed higher respiration activity than embedded cells (Figure 5b). Nevertheless, immobilized cells retained more than 50% of the respiration activity shown by the cells in the slurry suspension, suggesting that the polymerization process had not altered the physiological ability 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 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 nutrient additions with an increase in signal intensity after hybridization with ribosomal probes. Approximately 2% and 4% of the total embedded and free cells, respectively, hybridized to the ribosomal probe after 4 h of nutrient amendments (Figure 6). This represented an increase of nearly five orders of magnitude compared to the number of active cells detected prior to nutrient addition. After 6 h, the number of hybridizing cells increased for both embedded and free cells to nearly 8%. A further increase in the percentage of hybridizing cells was noted after 24 h, although the response was more dramatic for free than embedded cells (ie, 65% and 23%, respectively). Many of the embedded bacteria became elongated and showed intense hybridization signals, suggesting that they could grow but were not capable of cell division. This was in contrast with free cells which seemed to be actively dividing. In fact, the higher percent of free cells which hybridized 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, embedded and free cells did not differ significantly in their response to nutrient addition. Together with respirometric data, these findings suggest that the embedding process did not have as significant an effect on the metabolic potential as was suggested by the culturability data.

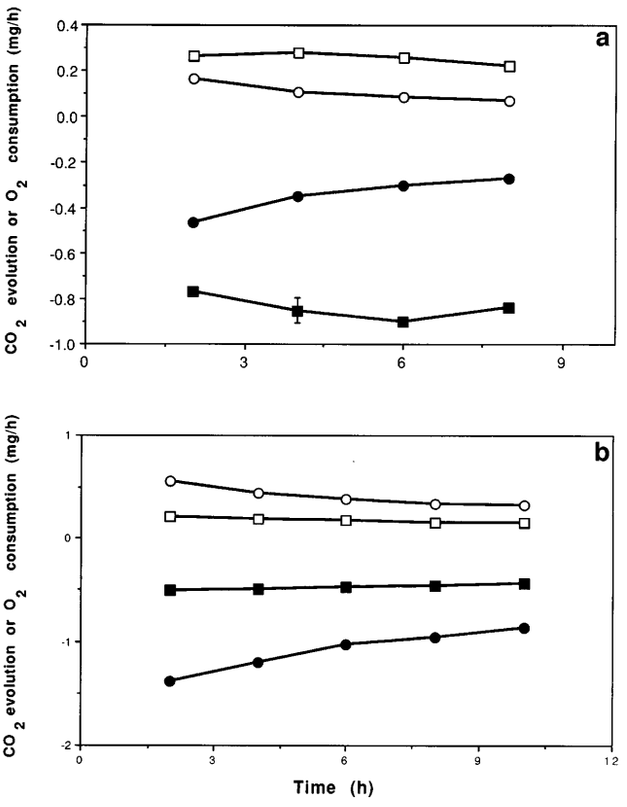

Figure 5 Respiration rates of embedded and free *B. cepacia* G4. Open and solid squares represent the CO_2 evolution and O_2 consumption rates, respectively, of immobilized bacteria. Open and solid circles represent the CO_2 evolution and O_2 consumption rates, respectively, of unembedded bacterial slurries. Cultures were exposed to 2 mM phenol prior to embedding. (a) Foam and bacterial slurries were maintained at room temperature (20 ± 2°C). (b) Foams and slurries used in (a) were then incubated at 25°C and shaken at 130 rpm. Values represent mean of triplicates and error bars represent standard deviation of means.

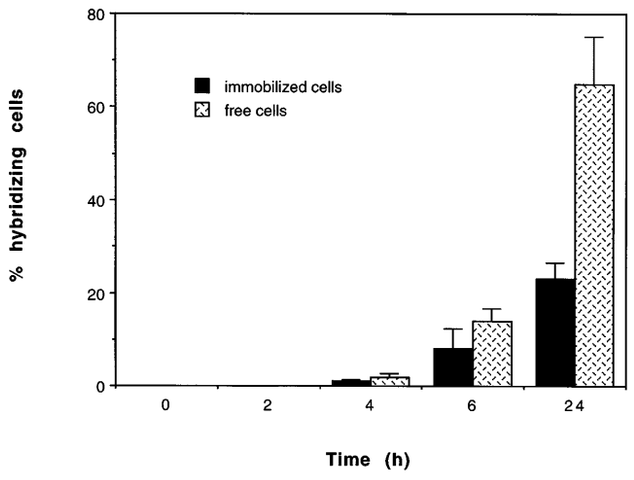

Figure 6 Effect of nutrient amendments on the activity of immobilized *B. cepacia* G4 cells. Samples of embedded and free cells were exposed to 0.2% of glucose for 4 and 24 h. At each interval, aliquots were fixed with formaldehyde and spotted on gelatin-coated slides for *in situ* hybridizations. A universal probe labeled with tetramethylrhodamine and targeting the 16S rRNA was used to determine the number of hybridizing cells. Values represent mean of triplicates and bars indicate standard deviation of means.

Discussion

The aim of this study was to optimize polyurethane-based formulations for the entrapment of degradative bacteria. The formulations used were hydrophilic and provided efficient permeability to oxygen and chlorinated aliphatics (eg, TCE). Our main criterion for optimization was the capacity of each formulation to prevent the release of immobilized cells. This criterion is of great importance in determining the functional longevity of this type of carrier system in bioremediation applications. Moreover, in applications involving the use of genetically engineered microbes (GEM), 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. Although we showed that cell retention in the optimal formulation represented a significant improvement over the rest of the foams tested, additional modifications will be needed to prevent cell release completely. While the loss of culturability observed after immobilization could further reduce the persistence of a GEM in any environment, complete immobilization will be necessary in cases where the potential of detrimental horizontal gene transfer might exist.

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 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% bacterial slurry (dry wt/wet wt) was most effective in retaining embedded *B. cepacia*. It remains to be seen whether this formulation is equally effective with other microorganisms.

The dramatic decrease in culturability as a result of immobilization suggested that the embedding process 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 suggest 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 (ie, increase in cell volume and ribosomal RNA content) and with the considerable increase in the number of embedded cells hybridizing to the ribosomal probe shortly after nutrient addition. The fact that some *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 could explain their inability to reproduce in artificial media. Others have reported significant reductions in bacterial viability using different embedding matrices [10]. However, most of these studies used culture techniques to assess viability, and thus might have overestimated the lethal effect of the immobilization process.

Our results indicate that the polymerization process caused the embedded bacteria to remain viable but to be nonculturable, a phenomenon documented by Roszak *et al* [14]. Thus, it appears that culturing techniques are not reliable indicators of the metabolic status of polyurethane-embedded cells. In the 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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References

1 Atlas R. 1993. Handbook of Microbiological Media. CRC Press, Boca Raton, FL.
2 Balkwill DL, JK Fredrickson and JM Thomas. 1989. Vertical and horizontal variations in the physiological diversity of the aerobic chemoheterotrophic bacterial microflora in deep south-east coastal plain subsurface sediments. Appl Environ Microbiol 55: 1058–1065.
3 Bettman H and HJ Rehm. 1984. Degradation of phenol by polymer entrapped microorganisms. Appl Microbiol Biotechnol 20: 285–290.
4 Braun-Howland EB, SA Danielsen and SA Nierzwicki-Bauer. 1992. Development of a rapid method for detecting bacterial cells *in situ* using 16S rRNA-targeted probes. Biotechniques 13: 928–932.
5 Cassidy MB, H Lee and JT Trevors. 1996. Environmental applications of immobilized microbial cells: a review. J Ind Microbiol 16: 79–101.
6 DeLong EF, GS Wickham and NR Pace. 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single microbial cells. Science 259: 803–806.
7 Hermann P. 1995. Coating particulate material with a polymer film. United States Patent No. 5405648.
8 Hobbie JE, RJ Daley and S Jasper. 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl Environ Microbiol 33: 1225–1228.
9 Koch A. 1994. Growth measurement. In: Methods for General and Molecular Bacteriology (P Gerhardt, RGE Murray, WA Wood and NR Krieg, eds), American Society for Microbiology, Washington, DC.
10 Levinson WE, KE Stormo, H-L Tao and RL Crawford. 1994. Hazardous waste cleanup and treatment with encapsulated or entrapped microorganisms. In: Biological Degradation and Bioremediation of Toxic Chemicals (RS Chaudhry, eds), Dioscorides Press, Portland, OR.
11 O'Reilly KT, and RL Crawford. 1989. Kinetics of *p*-cresol by an immobilized *Pseudomonas* sp. Appl Environ Microbiol 55: 866–870.
12 O'Reilly KT and RL Crawford. 1989. Degradation of pentachlorophenol by polyurethane immobilized *Flavobacterium* cells. Appl Environ Microbiol 55: 2113–2118.
13 Rhee S-K, GM Lee and S-T Lee. 1996. Influence of a supplementary carbon source on biodegradation of pyridine by freely suspended and immobilized *Pimelobacter* sp. Appl Microbiol Biotechnol 44: 816–822.
14 Roszak DB, DJ Grimes and RR Colwell. 1984. Viable but nonrecoverable stage of *Salmonella enteritidis* in aquatic systems. Can J Microbiol 30: 334–338.
15 Shields MS, SO Montgomery, PJ Chapman, SM Cuskey and PH Pritchard. 1989. Novel pathway of toluene catabolism in the trichloroethylene-degrading bacterium G4. Appl Environ Microbiol 55: 1624–1629.
16 Shields MS and MJ Reagin. 1992. Selection of a *Pseudomonas cepacia* strain constitutive for the degradation of trichloroethylene. Appl Environ Microbiol 58: 3911–3983.
17 Tanaka H, H Kurosawa and H Murakami. 1986. Ethanol production from starch by a coimmobilized mixed culture of *Aspergillus awamori* and *Zymomonas mobilis*. Biotechnol Bioeng 28: 1761–1768.
18 Thomas RAP and LE Macaskie. 1996. Biodegradation of tributyl phosphate by naturally occurring microbial isolates and coupling to the removal of uranium from aqueous solution. Environ Sci Technol 30: 2371–2375.
19 Trevors JT, JD van Elsas, H Lee and LS Overbeek. 1992. Use of alginate and other carriers for encapsulation of microbial cells for use in soil. Microb Rel 1: 61–69.
20 Weir SC, SP Dupuis, MA Proventi, H Lee and JT Trevors. 1995. Nutrient-enhanced survival of and phenanthrene mineralization by alginate-encapsulated and free *Pseudomonas* sp. UG14r cells in creosote-contaminated soil slurries. Appl Microbiol Biotechnol 43: 946–951.
21 Weiss P, B Shweitzer, R Amann and M Simon. 1996. Identification in situ and dynamics of bacteria on limnetic organic aggregates (lake snow). Appl Environ Microbiol 62: 1998–2005.
22 Wilde EW, JC Radway and JR Benemann. 1997. Bioremoval of heavy metals by microalgae. In: Recent Advances in Marine Biotechnology (R Nagablushanam, M Thomson and M Fingerman, eds), Oxford and IBH Publishing Co, New Delhi.
23 Woodward J. 1988. Methods of immobilization of microbial cells. J Microb Meth 8: 91–102.
24 Xu P, XM Qian, YX Wang and YB Xu. 1996. Modeling for waste water treatment by *Rhodopseudomonas palustris* Y6 immobilized on fiber in a columnar bioreactor. Appl Microbiol Biotechnol 44: 676–682.