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Use of microrespirometry to determine viability of immobilized *Burkholderia cepacia* G4

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Embedding of *Burkholderia cepacia* G4 cells in a polyurethane-based foam decreased their culturability by more than four orders of magnitude. However, respiration rates of immobilized cells were at least 33–41% of unimmobilized cells. Embedded cells also degraded trichloroethylene. Therefore, respirometry is a more reliable indicator of viability of polyurethane immobilized bacteria than culturing methods.

Introduction

The use of immobilized bacteria for *ex situ* bioremediation greatly depends on the retention and the viability of the microorganism after the embedding process (Cassidy *et al.*, 1996). Cell retention depends on such factors as polymer hydrophobicity and biomass concentration, while viability could be influenced by the toxicity of the polymer material and polymerization process. Although cell retention can be directly measured using microscopical methods (Hobbie *et al.*, 1977), viability is often determined via culturing techniques that only provide an estimate of microbial activity.

We have used polyurethane-based foams to immobilize algae for metal removal (Wilde *et al.*, 1997), and, more recently, to immobilize degradative bacteria (Radway *et al.*, 1996). The latter study demonstrated that biomass concentration as well as type and concentration of surfactant can influence cell retention. However, immobilized cells underwent a drastic decrease in culturability. In the present study, we have used microrespirometry to examine the effect of the embedding process on cell viability. This approach was based on the fact that respiration rates correlate directly with cell metabolic activity.

Materials and methods

Bacterial cultures and cell immobilization

Burkholderia cepacia G4 and *B. cepacia* PR131 were kindly provided by Malcolm Shields (University of West Florida). Axenic cultures of *B. cepacia* G4 and *B. cepacia* PR131 were grown by inoculating overnight cultures into 4 L Nalgene bottles containing 3 L yeast extract glucose medium (Shields and Reagin, 1992). Bottles were kept at room temperature (26 ± 2°C), and aerated through Teflon tubing and a 0.2 μm pore size filtering unit. Biomass was harvested via centrifugation at stationary phase (48–72 h). Prior to embedding, cells were resuspended in basal salts medium (BSM) (Shields *et al.*, 1989) to appropriate concentrations.

Foams consisted of 20 g hydrophilic polyurethane pre-polymer (Hermann, 1995), 13 g of biomass (wet weight), and 0.02% (final concentration) surfactant. Three surfactants were compared in the course of the study: HS-3 (lecithin-based), F-88 (ethylene- and propylene-oxide based), and DC198 (silicone-based). Sterile BSM was substituted for bacterial slurry in cell-free foam controls. Polyurethane prepolymers and surfactants were provided by Matrix R&D Corp. (Dover, NH). The foam was prepared by rapidly mixing all components, allowing polymerization to occur, and shredding the product in a Waring blender. Final biomass concentration was 8–17% (dry weight).

Degradation experiments

B. cepacia G4 cells were induced by adding phenol at 2 mM for 2 h prior to centrifugation (Shields *et al.*, 1989). Foam embedded bacteria (0.1 g) or equivalent mass of free cells (0.59 ml) were transferred to glass vials containing 10 ml of BSM broth or aquifer water collected from a monitoring well located at the Savannah River Site. Different concentrations (approximately 0.25, 1.0, 2.5, and 3.0 ppm) of trichloroethylene (TCE) were added to the vials as a methanol solution and vials immediately sealed using Teflon coated septa. TCE concentrations used represented typical contamination levels found in TCE polluted aquifers at this site. Headspace analysis

was performed after 3 days of incubation at 30°C by gas chromatography equipped with an automated headspace analyzer, an electron capture detector, and a 60 m, 0.32 mm ID, fused silica Vocol column (Supelco). Aquifer controls did not transform TCE during the experiment. TCE adsorption to the polyurethane foam was determined to be negligible.

Viability estimates

A tissue grinder was used to release cells from foam (0.1 g) in phosphate buffered saline (pH 7.2). Culturable counts were determined by spreading appropriate dilutions onto PTYG plates (Balkwill *et al.*, 1989). Plates were incubated at 30°C for 3 to 5 days. Percent viability was determined by comparing the number of colony forming units to direct counts (Hobbie *et al.*, 1977). Viable and direct counts were done in duplicate or triplicate.

Respiration (CO_2 evolution and O_2 uptake) rates were determined using a Micro-Oxymax v5.12 indirect closed circuit microrespirometer (Columbus Instruments, Columbus, OH). The system consisted of CO_2, O_2, temperature, and pressure sensors, a sample pump, and expansion channels interfaced with a 386 Compac PC computer. Triplicate samples consisted of 8 g foam or the equivalent number of unembedded cells (5 ml slurry). Foams and bacterial slurries were transferred to glass chambers connected to the interface channels via teflon tubing. Each chamber was examined for leakage by running pressure tests. Controls consisted of autoclaved foam embedded bacteria and cell-free foam. Samples were incubated at 21.5 or 25.5°C with or without agitation (130 rpm). Temperature and agitation was controlled using a reciprocal shaking water bath. Respiration rates were measured at 2–3 hour intervals for approximately 1–3 days.

Results and discussion

Embedding of *B. cepacia* in polyurethane foam decreased its viability as determined by culturable counts by several orders of magnitude (Table 1). This suggested that most cells were killed by the embedding process. In contrast, CO_2 evolution and O_2 consumption rates indicated that embedded cells were as active as free cells (Fig. 1). Foams containing HS-3 surfactant (foam 10, Table 1) were used for these experiments due to their better cell retention (Radway *et al.*, 1996); however, similar results were obtained with bacteria embedded in other foam formulations. Since free cells could have suffered from poor oxygen transfer, bacterial slurries were agitated in a second experiment and respiration rates compared to those of embedded cells. Although agitation increased respiratory activity in bacterial slurries, respiration rates of immobilized cells were approximately 33–41% as high as those of free cells (Fig. 2). Thus, the polymerization process was not as damaging to bacterial activity as suggested by culturable counts.

The effect of temperature on the activity of embedded cells was examined using *B. cepacia* G4 and *B. cepacia* PR131 (Fig. 3). As previously observed, respiratory activity was higher in embedded cells than in unimmobilized bacteria when bacterial slurries were not agitated (Fig. 1). Embedded and unembedded cells responded similarly to temperature changes. Increasing temperature from 21.5°C to 25.5°C resulted in higher respiration rates, while temperature reductions caused respiration to decline. This correlates well with the assumption that the optimal growth temperature for these organisms is 28–30°C. These results further confirmed the viability of cells immobilized in the polyurethane based foam.

Table 1. Viability of embedded bacteria as determined by colony counts

Foam number	*Cells/g (X 10^{-10})*	*Surfactant type*[b]	*Surfactant concentration (%)*	*Embedding temperature*[a]	*% Viability*
1	3.7	F-88	0.016	RT	0.0004
2	8.5	F-88	0.016	RT	0.0004
3	3.7	DC198	0.016	RT	> 0.0001
4	3.7	HS-3	0.016	RT	0.0002
5	3.7	F-88	0.008	RT	> 0.0001
6	3.7	F-88	0.159	RT	> 0.0001
7	3.7	F-88	0.016	Cold	0.01
10	8.5	HS-3	0.016	RT	> 0.0001
11	3.7	HS-3	0.016	RT	0.005

[a]RT = room temperature (22 ± 2°C). Cold temperature was achieved by performing embedding in an iced water bath. [b]F-88 = ethylene- and propylene-based surfactant; DC198 = silicone-based surfactant; HS-3 = lecithin-based surfactant.

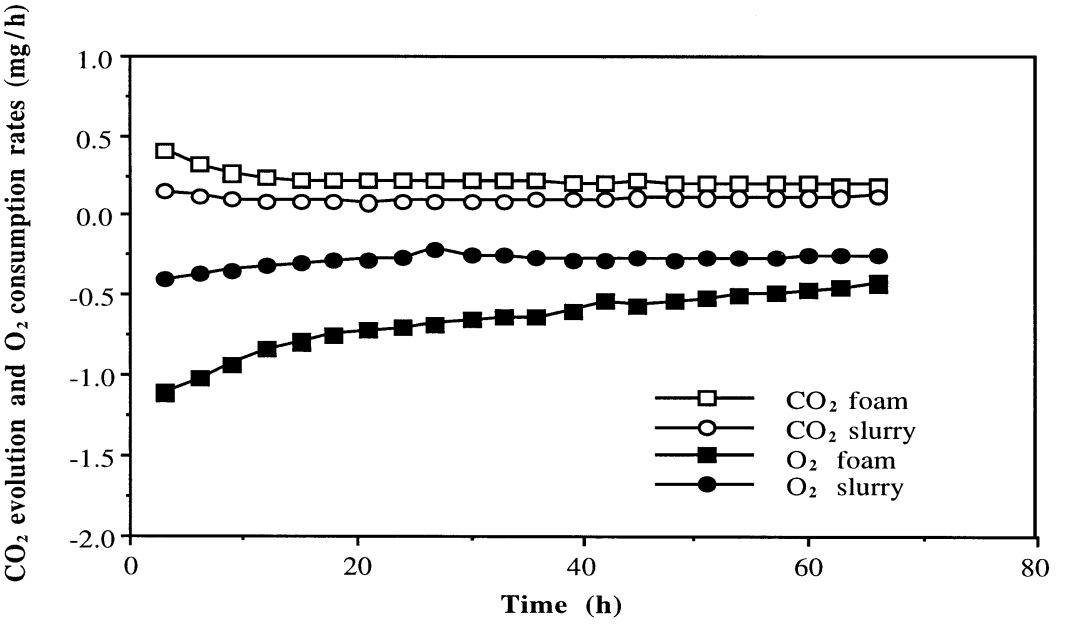

Figure 1. Effect of immobilization on the respiration rates of embedded *B. cepacia* G4. Bacterial slurries and foam contained yeast extract (1 g/L) and glucose (2 g/L).

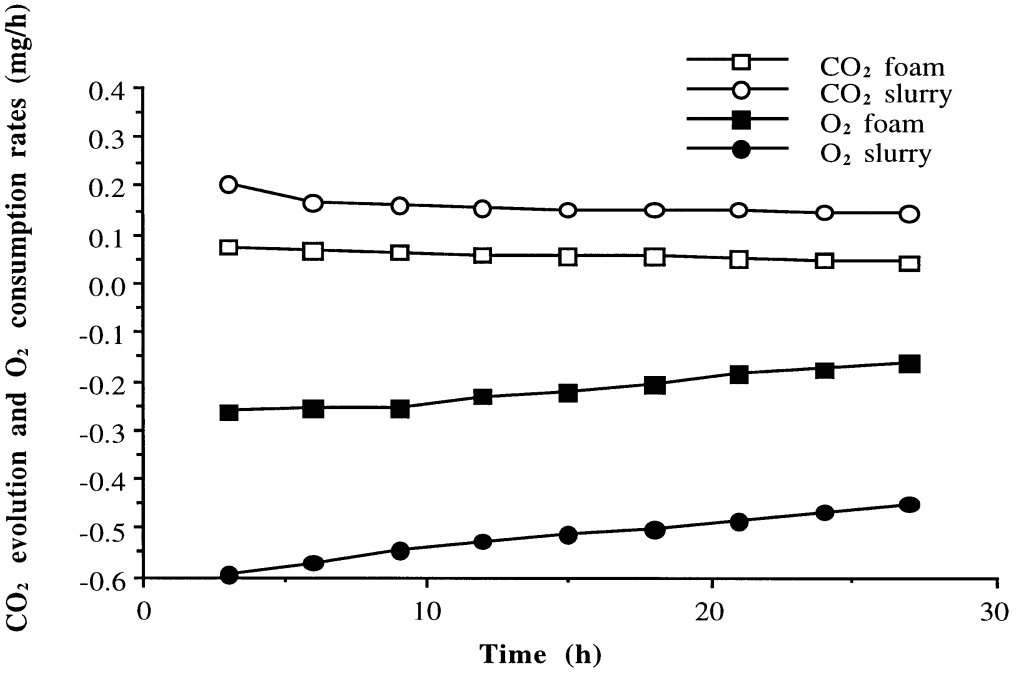

Figure 2. Effect of aeration on the respiration rates of *B. cepacia* G4 embedded cells and bacterial sluries. Experiment was performed with foam and slurries from Figure 1, but slurries were agitated at 130 rpm.

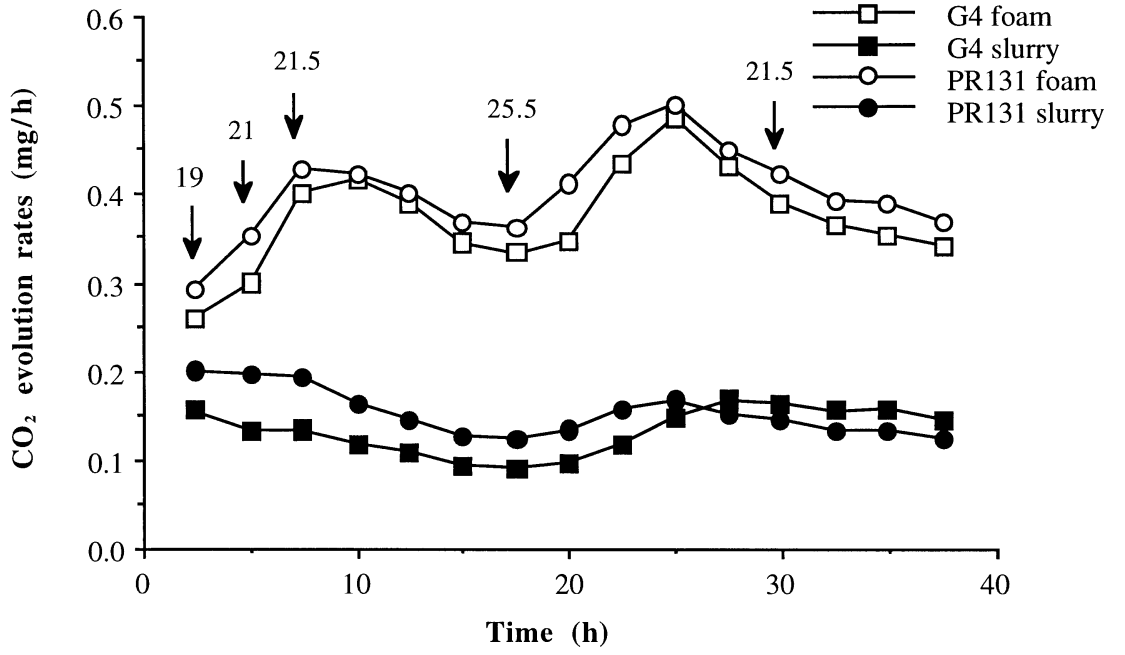

Figure 3. Effect of temperature changes in the respiration rates of *B. cepacia* G4 and *B. cepacia* PR131. Bacterial slurries were not agitated during this experiment. Arrows indicate the changes in temperature (°C). Values represent mean of triplicates.

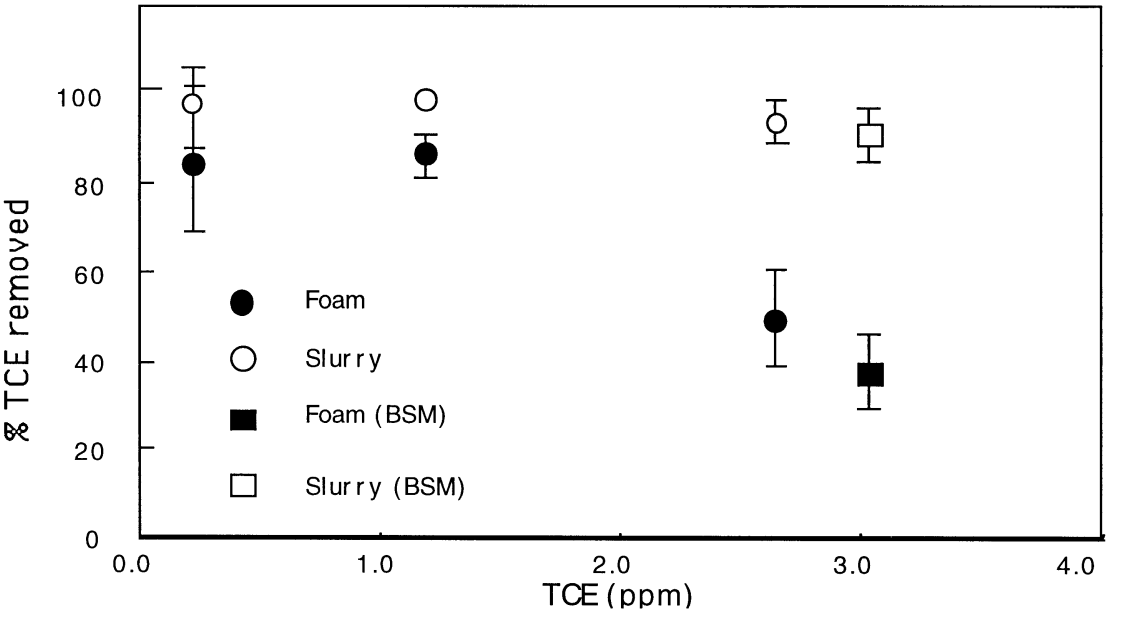

Figure 4. TCE degradation of embedded and unimmobilized *B. cepacia* G4. Circles represent experiments performed in aquifer samples while experiments using BSM broth are represented by squares. Bars represent standard deviation of the mean.

The degradative capability of immobilized *B. cepacia* G4 was investigated using TCE degradation as an indicator of transformation potential. This organism was been shown to degrade TCE via toluene monooxygenase after induction with phenol (Shields *et al.*, 1989). As shown in Figure 4, immobilized cells were capable of transforming approximately 40–85% of TCE in TCE-amended aquifer water, strongly indicating that the polymerization process did not impair the degradative activity of *B. cepacia* G4. Embedded cells showed considerably lower degradation activity when TCE concentrations exceeded 2.5 ppm in both aquifer water and BSM broth.

The present study indicates that culturing techniques can significantly underestimate viability of immobilized degradative bacteria. Similar losses of culturability after immobilization in polyurethane foams were also observed for other microorganisms (data not shown). However, respirometry data indicated that cells remained metabolically active. Thus, respiratory activity appears to be a better indicator of cell viability and degradative potential than the ability of the embedded organism to grow on artificial media. The effect of the embedding process on culturability could not be attributed to changes in pH and temperature during embedding, nor to the presence of toluene diisocyanate, a product liberated during polymerization process (Santo Domingo and Radway, unpublished results). Thus, polymerization might have influenced processes involved in cell division, for example, de novo synthesis of cell wall or cell membrane components. Nonetheless, other metabolic activities were not severely impaired by the embedding process as shown by the respiration and the TCE degradation data. These results are relevant in light of recent improvements in bacterial cell retention achieved with polyurethane based foams (Radway *et al.*, 1996).

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