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I am submitting herewit a thesis written by Laurel Elizabeth O'Connor entitled "A Method for Encapsulating Viable and Metabolically Active Microorgansims in Polyurethane Foam". I have examined the final copy of the thesis for form and content and recommend that it be accepted in partial fulfilllment of the requirements for the degree of Master of Science, with a major in Chemical Engineering.

Paul Bienkowski, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor and
Dean of The Graduate School

A METHOD FOR ENCAPSULATION OF VIABLE AND METABOLICALLY ACTIVE MICROORGANISMS IN POLYURETHANE FOAM

A Thesis
Presented for the
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Degree
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Laurel Elizabeth O'Connor
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DEDICATION

This thesis is dedicated to Bill Key.

By his generousity I was encouraged to pursue this degree fulltime.

Without his insight into math and physics my path through this degree

would have been much harder.

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Lastly, I thank Bill Key who encouraged me to pursue this degree. It was my dream 15 years to complete a degree in Chemical Engineering but I didn't think I could take the time. Well, I finally did it.

Abstract

Polyurethane foam has several advantages over the hydrogels, alginate, carageenan and acrylamide, as an entrapment agent for live bacteria. These advantages include: 1) inert structure, 2) foam structure does not require ions for stability, 3) foam is semi-rigid and flexible, 4) the foam has better mechanical strength. The major disadvantage of polyurethane foam is toluene present in the foam hardening agent which results in low bacteria viability during encapsulation. In this study a procedure was developed to encapsulate *Pseudomonas putida* Idaho *and Burkholderia cepacia* G4 into polyurethane foam while maintaining cell viability.

A standard method for growth of each bacteria and encapsulation into the foam was developed. Experimental variables tested were, 1) formulation of the foam prepolymer, 2) use of protective agents during encapsulation, 3) bacterial growth medium and 4) temperature of the encapsulation. Bacteria viability was determined by performing plate counts (CFU) and comparing total counts of encapsulated vs. unencapsulated cells. Viability was also quantified by measuring encapsulated cell metabolic activity.

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Chapter 1

A Review of the Literature

1.1 Immobilization

Cell immobilization is defined as techniques that limit the free movement of microorganisms or cells (1). Cell immobilization prevents catalytically, or biologically active cells in reactor-like systems from entering the mobile phase, which carries the substrate and product, and prevents washout of the microorganisms from the reactor (2). Cell mobility can be restricted by confining microorganisms within a porous matrix or membrane, or by chemically or physically fixing them to a solid support. Immobilized microbial cell systems have been increasingly used in bioconversion processes such as the production of foods, beverages, pharmaceuticals, macromolecules (e.g. organic acids and amino acids), industrial chemicals and fuels (e.g. alcohol, methane and hydrogen) (2, 3). Another developing use of immobilized cells is treatment of biological wastewater and dilute chemical wastes (2,4). There are a number of notable advantages to immobilization.

- Higher culture densities can be attained with immobilized cells versus when the biocatalyst is immobilized, the reactor flowrates can be in excess of the washout dilution rate (1,6).
- The immobilizing or encapsulating material may have sorptive capacities which increase the surface concentration of the substrate, thus increasing reaction rates or reducing toxicity (1). For example Portier et. al. (7) used the sorptive properties of the aminopolysaccharide chitin to concentrate soluble PCB fractions at the material surface, increasing the substrate availability to PCB-adapted microbial populations. The concentration effect resulted in a doubling of the rate at which PCBs were removed from the system. Sorption can also protect the microorganisms from the toxic effects of high levels of contaminants. Removing the chemical from the mobile phase reduces the level of exposure of the microorganisms to the contaminant (8).
- Intrinsic catalytic activity can increase possibly due to physiological changes in the biocatalyst upon immobilization or conversely activity could decrease (1). Hu et. al. (9) showed that immobilized cells had a much higher degrading capacity and survived better than free cells. These

physiological changes could include denaturation of cellular enzymes that inhibit or activate a cell metabolic pathway (10). Also, immobilization could affect a change in the cell membrane, because of the chemicals used for polymerization, therefore affecting cell transport. The chemical composition of the immobilizing agent could also inactivate or inhibit cofactors that would be responsible for cellular enzyme activity (10).

- Active whole cells can be used thereby resulting in less costly production of "chemicals". The use of whole microbial systems can bypass the need to use costly purified enzymes and allows for replenishment of electron carriers, cofactors and enzymes (8).
- Immobilization may have a stabilizing effect on enzyme activity. For example Chibata and his colleagues (11) found the half-life of aspartase deactivation was 120 days for immobilized *E. coli* cells, compared to 11 days in suspended cells.
- The composition of the carrier support material may be used to effect both product yield and selectivity. Walsh et al found a marked increase in product yield when *Clostridium accetobutylicum* was immobilized (6).

Immobilization methods can be classified into three categories:

- adsorption to a support matrix
- covalent binding to a support matrix and
- entrapment within a support matrix.

1.2 Adsorption

Physical adsorption is the oldest and simplest method of immobilization (4). Adsorption is based on the contact of the cell with the surface of the support and involves the attachment of a biocatalyst by non-covalent forces at a phase boundary (usually liquid/solid) (Figure 1.1). Depending on the nature of the surface, the binding may be the result of ionic interactions, physical adsorption, hydrophobic bonding, or van der Waals attractive forces (or a combination of these interactions) (12). The specificity of the binding varies from general dipole or hydrophobic interactions, which involves the lining up of polar molecules sothat the positive and negative ends are close to each other, through interactions between proteins and phenols, such a ion chromatography using DEAE sephrose, to highly selective binding involving affinity between two biochemical species, as in antibody - antigen interactions (13). Affinity bonding is highly specific and makes use of the

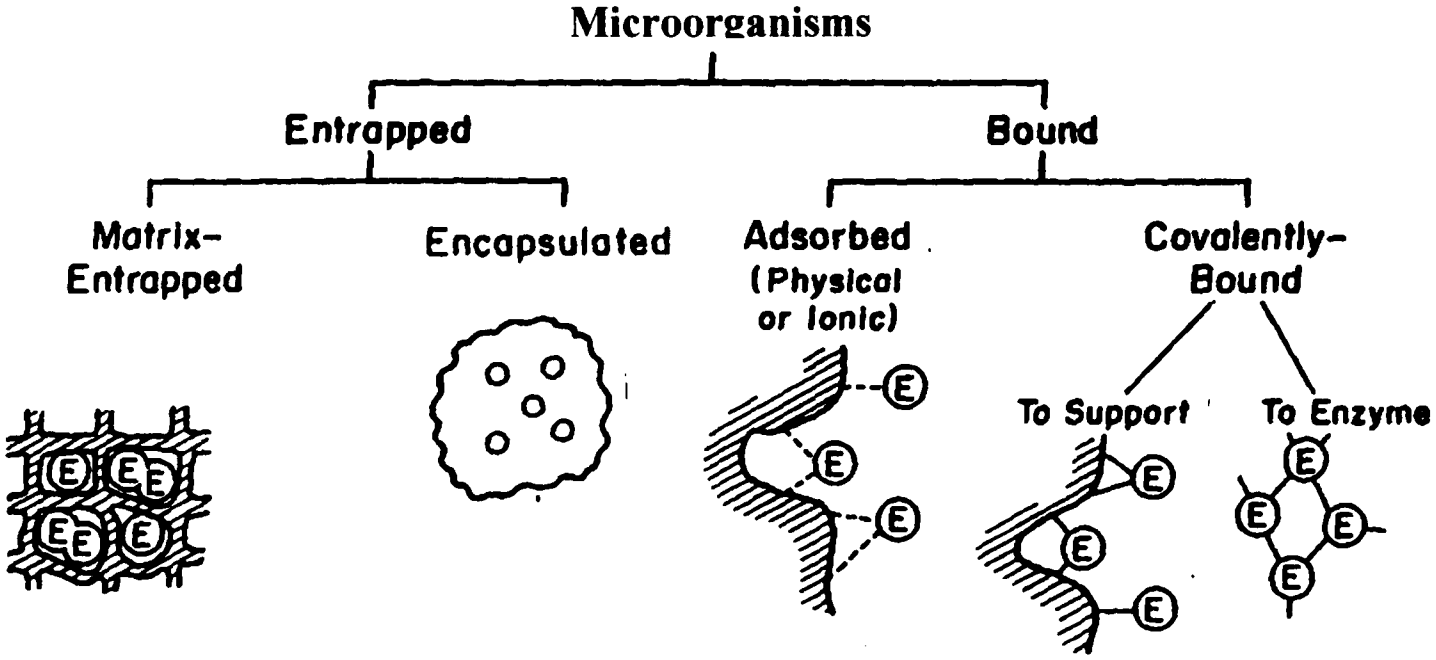


Figure 1.1 Major Immobilization Methods (5)

fact that a protein in its normal biological action will reversibly bind another specific molecule, called a ligand, very tightly to form a noncovalent protein-ligand complex. The adsorption method is usually very simple, mixing the cells and the support material under appropriate conditions and, following a period of interaction, separation of the bound cells/support (carrier) from the unbound cells by centrifugation or settling.

The natural tendency of many types of microorganisms to adhere to solid surfaces can sometimes cause problems, for example, material fouling or dental plaque, bacteria adsorb to surfaces forming a layer which can degrade the material. However this adherence also offers an opportunity for bioprocessing applications (4). Microbial films are effectively used in fixed film bioreactors to produce chemicals, food stuffs medicine and in wastewater treatment (4). According to Brouers et. al. (14), microbial biofilms accumulate as a consequence of the organisms ability to adsorb, replicate and metabolize on a physical support such as a pipe, tooth, or wall. During this nonspecific adsorption and subsequent cell growth, the cells remain viable and reactive.

The major advantage of using adsorption for cell immobilization is the gentleness of the preparation (13). The support is mixed with the bacteria, and generally no special reagents are necessary. After a period of contact nonadsorbed bacteria are removed either by centrifugation, settling or washing. The support is often regenerable since the binding is reversible (15).

The major disadvantage to adsorption is the sensitivity to changes in pH, ionic strength or temperature. According to separation theory, a compound is adsorbed to a support at its optimum pH, then by changing the pH, the bound compound is released into the mobile phase. If pH is poorly controlled the adsorbed species could be accidently released into the mobile phase and the biocatalyst washed out. Also another resident species, with a higher affinity to the binding surface could displace the desired bacteria from the surface. Another disadvantage, is the lack of control over the adsorption and growth processes. According to Brouers et.al. (14), slow adsorption and accumulation can prolong startup of a reactor system or excessive film formation can create unacceptable mass transfer limitations.

1.3 Covalent Bonding

The second method of immobilizing bacteria is by covalent or chemical bonding to a support. The introduction of covalent bonds requires use of reactive chemicals under severe conditions (eg. a wide range in pH). These conditions may result in loss of cell viability. The crosslinking or covalent bonding results from the formation of intermolecular bridges between cells by means of bifunctional or multifunctional reagents (Figure 1.1). Cross-linking agents used include gluteraldehyde, bisisocyanate, or disdiazobenzide (8). Covalent bonding can provide a bond that would not be broken easily under experimental conditions. However due to the severity of cross-linking conditions, pH extremes or uses of strong solvents, cell viability may suffer. Covalent or chemical bonding is almost exclusively used for enzyme or protein immobilization. Preactivation of hydroxyl supports (such as agarose and silica) with sulfonyl chlorides has been used to construct an analytical procedure for the determination of human proinsulin (15). Covalent coupling is also used in the purification of antibodies to be used for immunoassay development. Covalently coupling of the target antigen to a physical support allows antibody to be purified using very stringent conditions.

One advantage to covalent coupling is the stability of the support-agent after attachment. This coupling is resistant to changes in pH, temperature and ionic strength. A strong disadvantage to the use of covalent coupling is because strong agents are used for the coupling, the cells or proteins could be either inactivated to altered structurally.

1.4 Entrapment

A third means for immobilization of microorganisms is entrapment, the confinement of cells in the lattice of a polymer matrix or enclosure within porous spaces as illustrated in Figure 1.1. Physical entrapment of organisms inside a polymeric matrix is one of the most widely used techniques for cellular immobilization (4). Encapsulation is another category of entrapment, the complete surrounding of the microorganisms by a matrix. An advantage to encapsulation is the distribution of cells throughout a porous three dimensional structure allowing contact of the cells to the bulk fluid (4). Entrapment or encapsulation differs from adsorption or covalent bonding in that the cell itself is retained within the matrix and is not required to bond to the matrix or membrane. However some adsorption or chemical bonding can

occur. The following matrices are among those that have been employed (see Table 1.1): collagen, gelatin, agar, alginate, carrageenan, cellulose triacetate, polyacrylamide, epoxy resins, photo-cross-linkable resins, polyester, polystyrene and polyurethane. Among these matrices, polyacrylamide gel, calcium alginate, and carrageenan have been most extensively used for immobilization.

An advantage to entrapment of microorganisms is that it is unlikely to give rise to direct changes in the physical or chemical structure of the organisms (4). The microorganisms would maintain contact with nutrients and oxygen in the mobile phase while staying confined within polymeric three dimensional structure, like polyurethane or a hydrogel. O'Reilly et. al. (17) also reported that urethane surrounding the cells will adsorb some of the toxic organic contaminants. Thus, adsorption of the organic contaminant to the urethane matrix protects the cells within the matrix from concentrations that could kill the cells in the bulk phase.

A disadvantage to encapsulation is potential mass transfer limitations. Because the cell is surrounded by a three dimensional structure, the

Table 1.1 Some Polymers Used for Entrapment of Cells (7)

Polymer	Principle of Gel Formation	Gel-forming Procedure
Synthetic		
Polyacrylamide	polymerization	addition of initiators to a solution of monomers
Epoxy resin	polycondensation	epoxy and polyamine precursors are cured
polyurethane	polycondensation	reaction of polyisocyanates with water
Carbohydrates		
Cellulose	precipitation	cellulose is dissolved in an organic solvent and precipitated in water
Agar, agarose	thermal gelation	cooling of heated polymer solution
χ-Carrageenan	thermal gelation + ionotropic stabilization	cooling of heated polymer solution
Alginate	ionotropic	dripping into a calcium chloride solution
Proteins		
Collagen	neutralization	neutralization of an acidic solution
Gelatin	thermal gelation	cooling of heated polymer solution
Fibrin	enzymatic	enzymatic conversion of fibrinogen to fibrin

movement of nutrients and oxygen to the cells on the inside of the matrix can be limited (4).

Physical entrapment of organisms inside a polymeric matrix is one of the most widely used techniques for cellular immobilization (4). The resulting material must have sufficient porosity to allow the transport of substrate in and product out while restraining the cells. See Table 1.1 for a list of the most commonly used gel materials. Natural gels such as alginate and carrageenan, which are also called hydrocolloidal gels or hydrogels, are most often used for this application. However, other polymeric networks (Table 1.1), including polyacryalmide, can also be used.

Alginate is a block copolymer of D-mannuronate and L-glucronate joined by α1,4-linkages. An aqueous solution of the polysaccharide forms a relatively stable gel in the presence of multivalent cations such as Ca^{+2}, Ba^{+2} or Al^{+3} (18). Spherical beads of alginate containing entrapped cells are obtained by dripping a cell/alginate suspension into a medium containing calcium chloride (18). A major disadvantage to this gel system is the requirement for multivalent cations for gel stability. The most commonly used cation is calcium, which must be present at a concentration of 5-10mM

in the medium (18). This may impose problems since microbial growth requires phosphate in the medium which binds with the calcium and forms a precipitate (18).

Carrageenaan is another material which contains a repeating disaccharide unit of D-galactose-4-sulfate and 3,4-anhydro-D-galatose. This strong gelling, anionic hydrocolloid can be used for the entrapment of cells under relatively mild condition in a manner similar to alginate (18). Beads are formed by dripping the cell/carrageenan suspension into a nontoxic hydrophobic phase. Carrageenan, while similar in physical structure to alginate, does not require ions for maintenance of its physical structure (18). Both hydrogels are mild encapsulating agents but lack the physical strength to withstand shear forces that can occur in a bioreactor. There can also be mass transfer resistances within the spherical bead, limiting access to nutrients and oxygen (4, 11, 18).

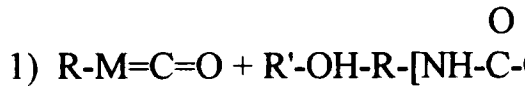
The most widely used monomer for encapsulation is acrylamide, and it is generally crosslinked with ethylene-bis-acrylamide (11). The resulting gel has a high water content (>90%), is very porous and the polymer is innocuous (11). The acrylamide physical structure is slightly stronger than the natural

gels. Unfortunately, the monomer itself is toxic to cells and the radical reaction can cause enzyme inactivation. Particulate materials such as cells are easily retained in polyacrylamide gels. However, the polymerization has been shown to cause changes in the ultrastructure of bacterial cell walls (11).

Natural gels and acrylamide are more fragile entrapping agents than the polyurethane products. Polyurethane prepolymers are used as immobilization agents for microorganisms due to their greater physical strength. In the early 1980's, O'Reilly et. al. (17) immobilized pentachlorophenol (PCP) degrading *Favobacterium* cells in polyurethane prepolymer. They found this polymer to be an improvement over alginate due to its greater mechanical strength and improved oxygen transfer characteristics. Also, adsorption of PCP to the polyurethane appeared to protect the cells from PCP degradation inhibition. The reversible binding of PCP to the polyurethane maintained the soluble PCP concentration below the toxic level while keeping the PCP available for degradation. Numerous authors (8,11,16,17,19,20,21,22,23,24) have reported on the use of urethane foams for encapsulation of toxin degrading organisms. For example urethane foams have been used for pentachloraphenol degradation by *Flavobacterium* O'Reilly et. al. (24), pentachlorophenol

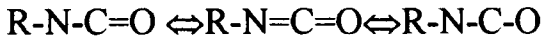
degradation by *Mycobacterium chlorophenicum* Karlson et. al.(25), chlorophenol degradation *by Rhodococcus chlorophenolicus* and *Rhodococcus* CP-2 Valo et. al. (26), polyaromatic hydrocarbons degradation by *Pseudomonas* CRE-7 Lin et. al. (27). Urethane encapsulated microorganisms have also been used for removal of nitrates from waste water, Urritia et. al. (20) and Cho et. al. (24), and to produce steroid precursors, Pinherio et. al. (27). Hu et. al. (9) investigated the adsorption and reaction kinetics of polyurethane encapsulated *Flavobacterium* and the biodegradation of pentachlorophenol. They found the polyurethane foam immobilized bacteria showed the ability to degrade PCP at concentrations 3 times greater than the level of cells immobilized in alginate. The adsorption of PCP to the foams also reduced the toxicity of PCP. Calcium alginate beads with bacteria were observed to be easily swollen and broken during operation in stirred reactors and fixed bed columns . The polyurethane was resistant to shear forces and did not collapse when placed under pressure. Adsorption and reaction kinetics equations were developed to compare alginate and urethane encapsulated organisms.

Polyurethane foams can be defined as plastic materials in which a proportion of the solid phase is replaced by gas in the form of numerous small bubbles (22). The gas may be in a continuous phase to give an open-cell material or it may be discontinuous to give noncommunicating cells (22). The general principle for preparing polyurethane is the dispersion of the gas phase in a liquid foam which will then be solidified to a solid cellular plastic. Gas is dispersed in the liquid prepolymer by chemical blowing from an *in situ* chemical reaction. Gas formation is achieved by the reaction between two species, one of which can be part of the polymer. The two important reactions in the preparation of the urethane foams are those between isocyanate and hydroxyl compounds (polyester or polyether polyols) and those between isocyanate and water. The first reaction for the formation of a urethane group is considered a chain propagation reaction (11).



1) R-M=C=O + R'-OH-R-[NH-C-O]-R'

The isocyanate group is considered to exist in the following resonance forms:



R-N-C=O ⇔R-N=C=O⇔R-N-C-O

The second reaction, water-isocyanate is responsible for foam formation by the liberation of carbon dioxide as an insitu blowing agent. The first step of

this reaction is formation of unstable carbonic acid which decomposes to form CO_2 and amine. The latter may react with an additional isocyanate to produce substituted urea (11).

2)a) $RNCO + H2O \Rightarrow R\underset{\underset{H}{|}}{N}\overset{\overset{O}{\|}}{C}OH$ (unstable carbamic acid)

b) $R\underset{\underset{H}{|}}{N}\overset{\overset{O}{\|}}{C}OH \Rightarrow RNH2 + CO_2 \Uparrow$ (amine formation, gas generation)

c) $RNH2 + RNCO \Rightarrow R\underset{\underset{H}{|}}{N}C\underset{\underset{H}{|}}{N}R$ (chain extension, cross-linking formation)

The main reactions which lead to branching and crosslinking are the isocyanate-urethane reaction producing allophanate linkages and the isocyanate-urea reaction which produces biuret.

Hardening agents in the liquid prepolymer can be toxic to some bacteria. However, the porous structure formed with the bacteria within lends physical strength to the system. Also the porous structure allows easy exchange of fluid and nutrients with the entrapped bacteria.

Chapter 2

Materials and Methods

2.1 Introduction

The purpose of this study was to determine if two different bacteria, *Burkholderia cepacia* G4 and *Pseudomonas putida* Idaho could be encapsulted into polyurethane foam while maintaining a high level of cell viability and metabolic activity.

Experiments were designed to determine the time necessary for each bacteria to reach late stationary phaseand the composition of bacterial growth media promoting higher cell viability. When the bacteria are at late stationary phase, the net growth rate is zero, therefor the maximum number of cells are present. Even though the net growth rate is zero during the stationary phase, cells are still metabolically active and produce secondary metabolites. The method of foam encapsulation was determined by detailing temperature of encapsultation, type of urethane prepolymer used, type of protective additive and sequence of foam making.

Three different methods were utilized to confirm cell viability or metabolic activity: 1) Viability was measured by comparing cells counts of encapsulated bacteria to nonencapsulated bacteria by performing plate counts (CFU, colony forming units) 2) metabolic activity was determined by measuring the conversion of glucose to CO_2 by encapsulated bacteria, and 3) degradation activity was determined by measuring loss of contaminant compounds, TCE by G4 and toluene by PPI.

2.2 Establishment of Standard Conditions

To establish standard conditions for subsequent experiments the time of growth to stationary phase, the necessity for use of an encapsulation additive, temperature conditions, growth medium components and polyurethane prepolymer type were determined. The first experiments performed were to establish the time necessary for *P. putida* and *B. cepacia* G4 to reach late stationary phase. In subsequent experiments bacteria were grown to late stationary phase prior to encapsulation.

2.3 Growth of Bacteria

Two different bacteria, *Pseudomonus putida* Idaho (PPI) and *Burkholderia cepecia* G-4 (G-4) were grown in appropriate medium, 1% PTYG or BS+G respectively following standard microbiological methods (30). The 1% PTYG medium contained (Difco Peptone 5.0g/L, Difco Tryptone 5g/L, Difco Yeast Extract 10g/L, Glucose 10g/L, Magnesium Sulfate - heptahydrate .6g/L, Calcium Chloride - di hydrate 0.07g/L) in Basal Salt Medium (BS+G) (18.6mM potassium phosphate - trihydrate, 7.2mM sodium phosphate - monohydrate, 37.4mM ammonium chloride, 0.65mM nitrilotriacetic acid - disodium salt, 0.81 mM magnesium sulfate - heptahydrate, 0.043mM ferrous sulfate - heptahydrate, 0.01mM zinc sulfate - heptahydrate, 0.018mM manganese sulfate monohydrate) + 20mM Glucose for PPI and Basal Salt Media (BS+G)+ 20mM Glucose for G-4.

Seed cultures of bacteria used in the experiment were started 2 days prior to batch growth by innoculating with colonies transfered from a plate into a 50 ml volume of 10% PTYG for PPI or BS+G for G-4. Seed cultures were incubated at 23°C for 2 days on a shaker. After 48 hours the total 50 ml volume was poured into 500 mls (1liter polycarbonate baffled culture

flask) of BS+G medium. Flasks were placed on a rotating shaker and allowed to mix. Samples were ascptically removed from each flask and the optical density was determined at 600 nm. The optical density was plotted on asemilog graph with the optical density on the y-axis and time in hours on the x-axis. Samples were collected until the growth ceased, stationary phase. The time necessary for each bacterium to reach late stationary phase, 7 to 8 days for PPI and 36 to 48 hours for G-4, was used to grow bacteria for subsequent experiments.

2.4 Preparation of Cell Paste

After cultures reached late stationary phase, a cell paste was prepared by decanting the flask contents into 2 x 250 ml sterile Nalgene centrifuge bottles and pelleting in a Sorvall centrifuge using a GSA rotor at 10,000 rpm (16,000 x g) for 20 minutes. The supernatent was decanted, the cell pellet in each bottle was resuspended into 5ml of phosphate buffered saline (PBS) (20mM sodium phosphate, 150mM sodium chloride, pH 7.0) and then combined.

2.5 Foam Encapsulation

One ml of cell paste was mixed with 9 ml of PBS (20mM sodium phosphate, 150mM sodium chloride, pH 7.0) in a 150 ml sterile beaker. To that mixture 5 ml of liquid prepolymer, Hypol 2000, 2002 or 3000, (Hampshire Chemical Company) was added. When a coating treatment was tested, 5 mls of the 9 mls of PBS was replaced with 5 mls of the experimental solution. The sample was mixed approximately 5 minutes by hand until polymerization began as indicated by the formation of foam. The foam mixture was allowed to harden for one hour, covered. After the foam had "hardened" the foam cylinder was trimmed to remove the outside surface, which is less porous (22).

2.6 Viability and Cell Numbers

Cell viability was determined using the CFU technique (49). Samples were plated using sequential dilution onto 10% PTYG + agar plates (10% PTYG medium + 17 grams Bacto Agar per liter). One gram of foam plus 10ml of PBS was homogenized in a Warring blender for 15 seconds. The sample was examined and if any "large" pieces were present the sample was

blended for an additional 15 seconds. For the control, "100% viable" sample, 1 ml of cell paste was added to 14 ml of PBS. This dilution approximated the volume ratio of the cell paste in the foam mixture. To 10 ml of PBS, 0.1 ml of this first dilution was added. Next a 1:10 dilution was performed into 0.1% sodium pyrophosphate. This dilution was approximately equal to a 10^{-4} dilution of cell paste. Sequential 1:10 dilutions in 0.1% sodium pyrophosphate were performed up to 10^{-9} dilution. For the treatment/foam encapsulated cells, 0.1 ml of homogenized foam encapsulated bacteria was added to 10 ml of PBS. This 1:100 dilution is equivalent to 10^{-4} dilution of the cell paste. Subsequent 1:10 dilutions in 0.1% sodium pyrophosphate were performed up to 10^{-9} dilution of sample. 10% PTYG + agar plates were prepared at least 48 hours prior to experiment. To triplicate plates, 0.1ml of sample was added and spread with a hockey stick. After plates were incubated at room temperature for three days colonies were counted. The colony (cell) number per dilution was determined for each sample or treatment. To get percent viability the total number of cells in the treatment were divided by the total number of cell in the control which was not trapped in the foam.

2.7 Effect of Protective Agents

PPI cells were encapsulated into prepolymer as described above. Three coatings were tested for their effect on improving cell viability during encapsulation. One ml of cell 5% nonfat dry milk (NFDM), 2% gelatin (bloom 75), or 100% glycerol. After the cell paste, PBS and protective agent were combined and mixed, the prepolymer was added and solution was mixed until polymerization began. Beakers were covered and allowed to harden for 1 hour. After foam had hardened samples were processed as described above. All treatments and controls were diluted and plated in triplicate. Percent viability was determined by counting colonies.

2.8 Effect of Prepolymer Type

The hardening agent, toluene diisocyanate (TDI), used in Hypol prepolymer (Hampshire Chemical Company), was suspect in reducing the cell viability of urethane encapsulated bacteria (11). TDI is necessary for the formation of the foam structure (21), thus 3 levels of prepolymer, Hypol 3000, 2002, and 2000, with varying levels of this agent were tested to determine the effect of this agent on cell viability. Explicit concentrations of

the hardening agent, TDI, were not available, due to proprietary formulation, however, the concentration of TDI decreased with the decrease in product identification number (21). As described above, PPI bacteria cells paste was combined with 5 ml of 2% gelatin (bloom 75) as the protective agent, 4 ml of PBS and 5 ml of one of the above prepolymer formulations. After the foam had hardened samples were processed as described. Percent viability was determined by counting colonies.

2.9 Temperature Effects

PPI was grown and encapsulated as described above. One ml of cell paste was mixed with 5 ml of 2% gelatin (bloom 75) and 4 ml of PBS. Five ml of Hypol 2000 prepolymer was added and solution was mixed, allowed to foam and hardened. Beakers with the foam/cell mixture was hardened either at room temperature or on ice (4°C). Foam encapsulated samples were processed as described previously. Percent viability was determined by counting colonies.

2.10 Effects of Media Dilution

Experiments were designed to test for possible stabilizing effects related to the use of dilute media on cell viability of the PPI bacteria, when encapsulated into polyurethane foam. Seed cultures, 1 x 50 ml bottle for each treatment, were grown for 2 days in medium containing different levels of PTYG components. Starter cultures (50 ml) were begun in 10%, 1%, 0.5%, and 0.1% PTYG. These differing levels of PTYG were prepared by making an initial stock medium of 10% PTYG, according to the formula described earlier, and then making appropriate dilutions with distilled/deionized water to produce the levels of PTYG for the initial starter cultures. A control culture (no PTYG) was incubated in 50 ml (BS+G) medium. After 2 days, these seed cultures were transfered into 450 ml of BS+G which diluted the PTYG components 10-fold to 1%, 0.1%, 0.05% or 0.01%. Bacteria were grown to late stationary phase, about 8 days. Bacteria were harvested and encapsulated as described earlier using 2% gelatin and Hypol 2002 prepolymer. Percent viability was determined as previously described.

2.11 Best Standard Method

Results from each of the previous experiments described above were evaluated and the best combination of medium, growth time, encapsulation options was developed and used for the metabolic studies described below. The standard procedure was: Bacteria were grown to late stationary phase, 7 to 8 days for *P. putida* Idaho and 36 hours for *B. cepacia* G-4 in .01% PTYG in BS+G or BS+G respectively. Cell paste was prepared as described. A 1 ml portion of the cell paste was resuspended in 4 ml of PBS plus 5 ml of 2% gelatin (bloom 75). Cells were encapsulated into Hypol 2002 at room temperature. Specifices on preparation of cell paste, manipulation of foam and determination of viability was as described earlier.

2.12 Respiration

Gross bacteria activity was measured by determining the rate of respiration by foam encapsulated microorganisms. A 500 ml culture of G-4 and PPI bacteria was grown as described earlier. Foam encapsulated bacteria were prepared as previously described. Foam was cut into small pieces, about $1mm^3$ to $5mm^3$ in size. Controls included (1) killed bacteria, (2) killed

bacteria in foam, (3) PBS without bacteria or foam (blank) and (4) foam with no bacteria. Killed bacteria were prepared by autoclaving a portion of the cell paste for 45 minutes. Triplicate samples were established with either 1 gram of foam + bacteria or 1 ml of bacteria (not encapsulated). The incubation medium used was 10ml of PBS with 20mM glucose in 150ml serum bottles. Nonfoam controls contained 1 ml of cell paste, live or killed, that was diluted 1:14 in PBS.

Respiration was measured at zero time, 1day, 3day and 7 day. The reaction was stopped by the addition of 1ml of 1M NaOH and then inverting the bottle. To maximize the amount of CO_2 in the gas phase, 1ml of 6N HCL was added to each bottle about 1 hour prior to analysis. After acidification, respiration was quantified by measuring the CO_2 present in the gas phase in each bottle using a Schimatzu GC-8A gas chromatograph with a 2.74 m, 3.2mm diameter Carbosieve 8000 packed column and a thermal conductivity detector (31).

2.13 TCE Degradation by G4

Burkholderia cepacia G4 $PR1_{301}$, a non-genetically engineered constitutive trichloroethylene (TCE) degrader espresses an enzyme, a monooxygenase, which is responsible for TCE degradation (32). To produce enzymatically active G4, a starter culture was initiated by taking cells from a non-selective agar plate of R2A growth media (Difco Laboratories, Detroit MI) with 20 mM glucose (33). The bacteria was resuspended in 10 ml of BS+G medium and incubated for 2 days on a rotary shaker (250 rpm) at ambient temperature. An aliquot was then added to 100 ml of BS+G, in a 250 ml sterile Erlenmeyer flask, and incubated until the OD at 600nm was in excess of 2.0 OD (33).

The monooxygenase enzyme, was measured using the TFMP (triflouromethyl phenol or m-hydroxy benzotrifluoride) ocidation assay (8). The rate of production of THFA (7,7,7-trifluoro-2-hydroxy-6-oxo-2,4-heptadienoic acid), a yellow product, from TFMP correlates to the potential rate of TCE degradation by the monooxygenase (33). When enzyme activity was detected, the entire 100 ml sample was transfered to an air lift reactor which contained 450ml of BSM+ 20mM glucose. House air was connected

to the lower portion of the reactor and air flow was adjusted such that total fluid phase was being aerated without excessive foaming. The reactor was harvested after 36 hours. Foam encapsulation on harvested cells was performed as described using Hypol 2002 prepolymer. Percent viability of encapsulated bacteria was determined by counting colonies after 3 to 4 days. For the TCE degradation study, 0.5 gram foam encapsulated bacteria samples were used.

Extensive controls were used to distinquish effects of the foam on TCE degradation by the encapsulated bacteria and the level of TCE adsorption to the cell or foam structure. Live cells, 1ml of the 1:15dilution, were used as a baseline to define the maximum amount of TCE that would be degraded. To test for possible adsorption of the TCE to the bacteria structure, 5ml of the bacteial suspersion was autoclaved for 15 minutes at 120°C. One ml of the "killed" bacteria was added to 14ml of PBS. Also, one ml of killed bacteria was encapsulated into the urethane prepolymer as described to examine adsorption to the mixture. To distinquish the source of adsorption foam was also made that did not contain bacteria. One extra ml of PBS was added to the foam mixture in place of the cell suspension.

TCE degradation studies were modeled after those of Palumbo et al. (34). To triplicate 40ml EPA vials fitted with lids and teflon septums was added either 0.5 grams of foam, (with or without bacteria) or 1 ml of bacteria (live or killed, 1:15 dilution) and 10 mls of PBS (see Table for outline of samples). 2ppm of a saturated TCE solution (1100 mg/l) was added to each sample using a Hamilton syringe. Vials were incubated, inverted, on a shaker and TCE concentration was measured at 24 hours and 48 hours. Samples were analyzed with a Hewlett Packard 5890 gas chromatography (GC) and separations were achieved by using a J&W Scientific DB624 megabore capillary column (30m x 0.53mm) coated with fused silica. TCE was separated using a 4 minute temperature ramp from 70°C to 110°C with detection by electron capture detection (ECD). A standard curve was run with TCE concentrations of 0,1,2,5 and 10 ppm and 5 ul injections from triplicate vials were used.

The ability of foam encapsulated G-4 to degrade 10 ppm TCE was also investigated. Samples were prepared and tested in the same manner as the 2 ppm TCE study.

2.14 Toluene Degradation by PPI

Toluene degradation was used as a measurement of metabolic activity for PPI. PPI cells were scraped off a plate and transfered into 50 mls of 10% PTYG medium. The culture was allowed to incubate, with aggitation, for several days, until growth was visible. This 50 ml aliquot was then poured into 500 ml of BS+G medium in a baffle flask and allowed to grow to late stationary phase, about 1 week. Cells were harvested by centrifugation as previously described and viability was tested by measuring CFUs. Foam encapsulated bacteria were used for the PPI/toluene study in the same manner as in the G-4/TCE study. After encapsulation, triplicate 40ml EPA vials with 0.5g of foam were used. The experimental scheme was similar to that of the G-4/TCE study. 5 ppm of a saturated toluene solution (515 mg/l) was added to each sample using a Hamilton syringe. Toluene concentrations were measured at 24hr, 48hr and 5 days. Vials were incubated inverted on a shaker. Samples were analyzed with a Hewlett Packard 5890 gas chromatograph (GC) and separations were achieved by using a HP-5 capillary column (30m x 0.32nm) with a 0.25μm film of 5% phenyl methyl silicone. Samples were analyzed using a temperature ramp program with a 2.8 min.

range of 40°C to 75°C. Toluene was quantitated by use of flame ionization gas chromotography. A toluene standard curve was before each sample run, with toluene concentrations at 0, 2, 5, 10 and 25 ppm with 5 ul injections from triplicate vials.

Polyurethane foam cubes were also test for the ability to adsorb toluene to their structure. Foam was prepared as described but without bacteria. Toluene vials were prepared in triplicate with either 0.25 grams or 0.5 grams of foam. Two sets of samples were prepared one set having 5 ppm toluene and the other set having 25 ppm toluene. Samples were inverted and incubated for 48 hours. Toluene was measured as described above. The adsorbed samples were compared to samples with toluene, 5 or 25 ppm, without foam.

Chapter 3

Results

3.1 Establishment of Standard Conditions

The first experiment established that under the growth conditions tested *P. putida* took longer to reach stationary phase than *B. cepacia* G4. *P. putida* reached late stationary phase after 7 to 8 days of incubation in a culture flask (Figure 3.1). *B. cepacia* G4 reached late stationary phase after 36 hours using an airlift reactor (Figure 3.2). Late stationary phase was chosen because the cells have grown to their maximum density, therefore growth has ceased but the cells maintain their metabolic activity. Also, the enzyme responsible for TCE degradation by the G-4 bacteria, TCE orthomonooxygenase, is not induced, or active, until the bacteria are in stationary phase. These times were used as guides in subsequent experiments where bacteria were grown to late stationary phase prior to encapsulation.

PPI and G-4 encapsulated into polyurethane foam exhibited very low cell viability (Figure 3.3). For the PPI viability was <1% and was

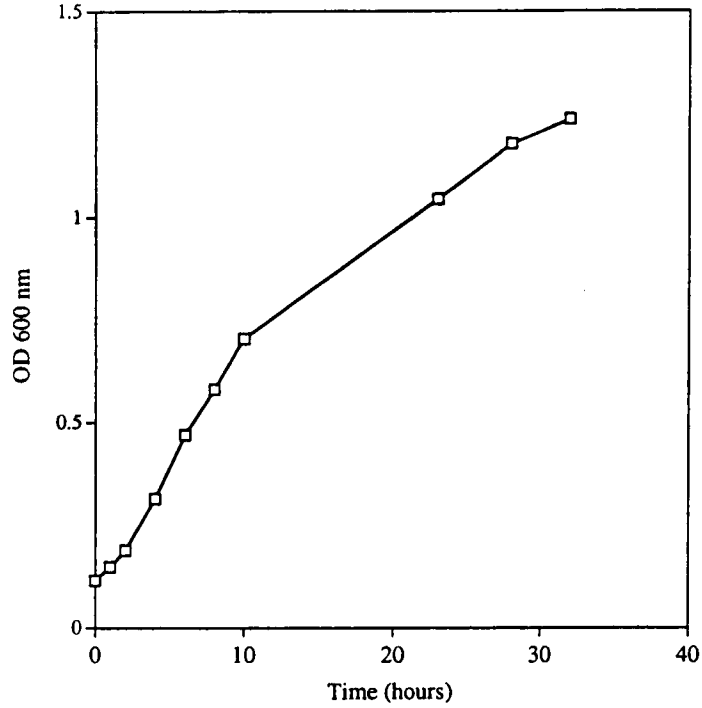


Figure 3.1 Growth Curve for *Pseudomonas putida* Idaho

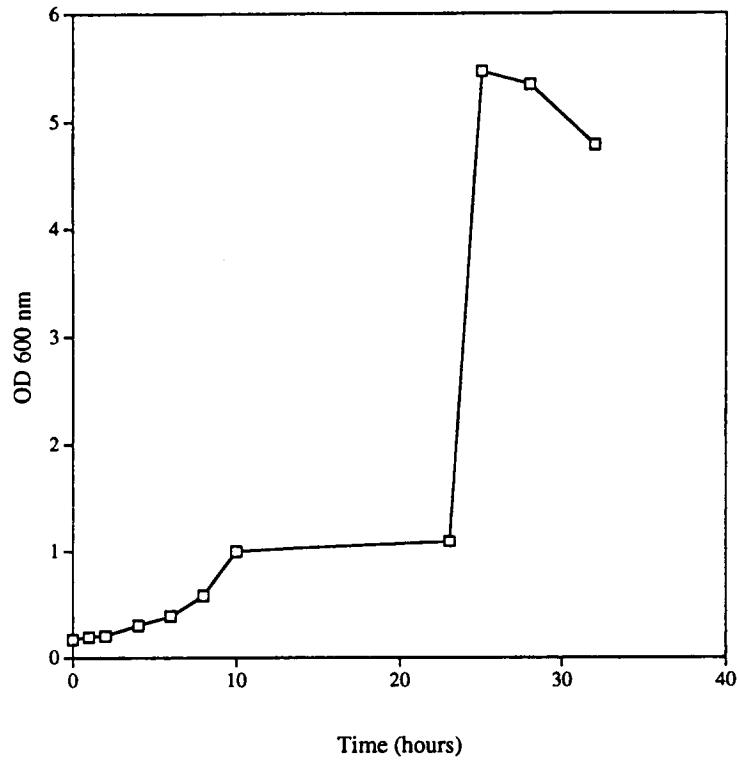


Figure 3.2 Growth Curve for *Burkholderia cepacia* G-4

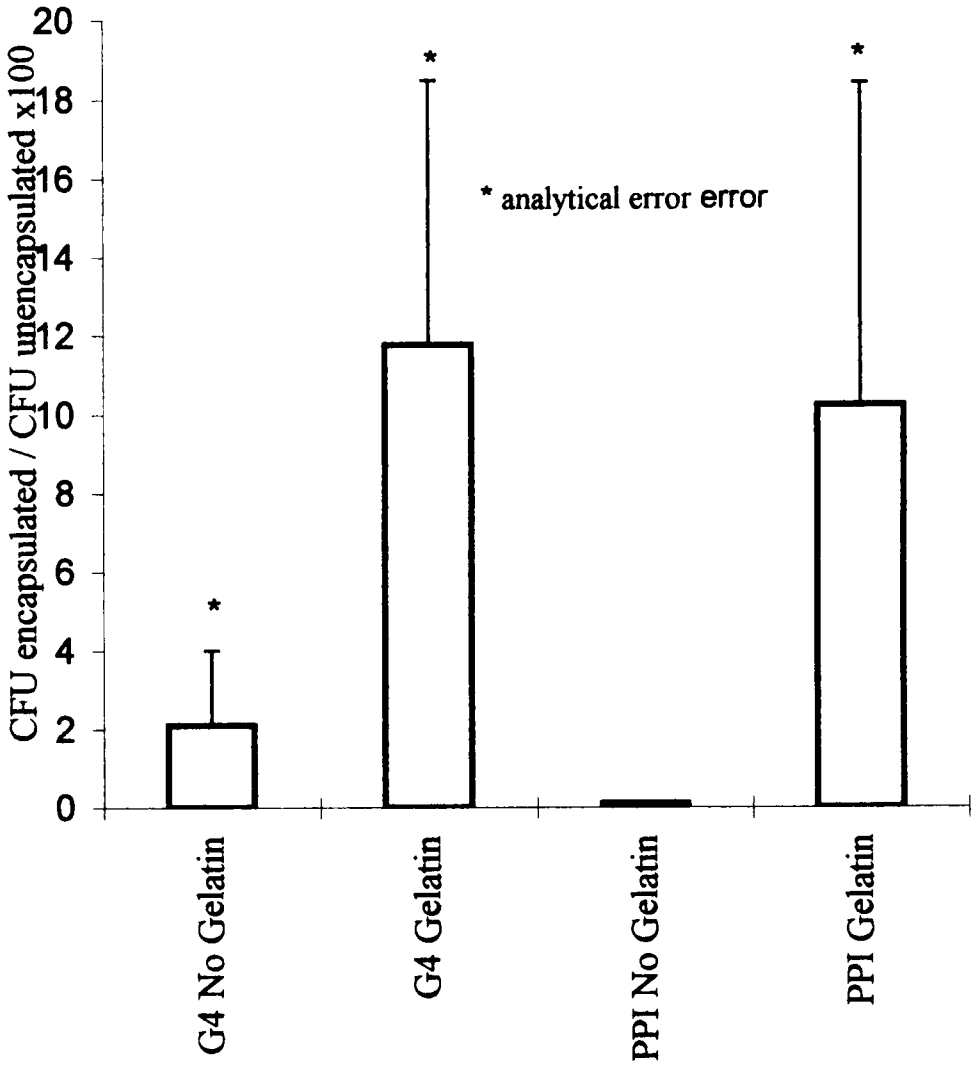


Figure 3.3 Effect of Gelatin on Bacteria Viability

* (Bars indicate standard deviation. Where bars are not present, standard deviation was done but not discernable.)

approximately 2% for the G-4 bacteria. It was thought that the addition of proteins or glycerol might protect the cells from the solvent in the foam hardening agent. Also since the PPI bacteria is grown in a much richer medium than the G-4, possibly the more defined medium of the G-4 contributed to the slightly improved viability. The effect of protective agents and next we investigated the effect of medium additives on encapsulated cell viability were subsequently tested in an effort to improve the viability. Two protein sources, 5% non fat dry milk (NFDM) and 2% gelatin (Bloom 75) improved percent viability of the bacteria to 5% and 7% respectively (Figure 3.4). These proteins are commonly used in ELIZA assay design as stabilizers or protectants (35) and appeared to improve viability upon addition of 5 ml of 5% nonfat dry milk or 2% gelatin to the polymerization cocktail. The levels of the skim milk and gelatin were chosen from general methods used in immunoassays (35). The addition of 5 ml of 100% glycerol to the encapsulation cocktail did not improve viability. In fact the glycerol cause the hardened foam to compress and become very sticky.

In a separate experiment, the effect of 2% gelatin on viability of G-4 and PPI was determined. The addition of 2% gelatin to the encapsulation

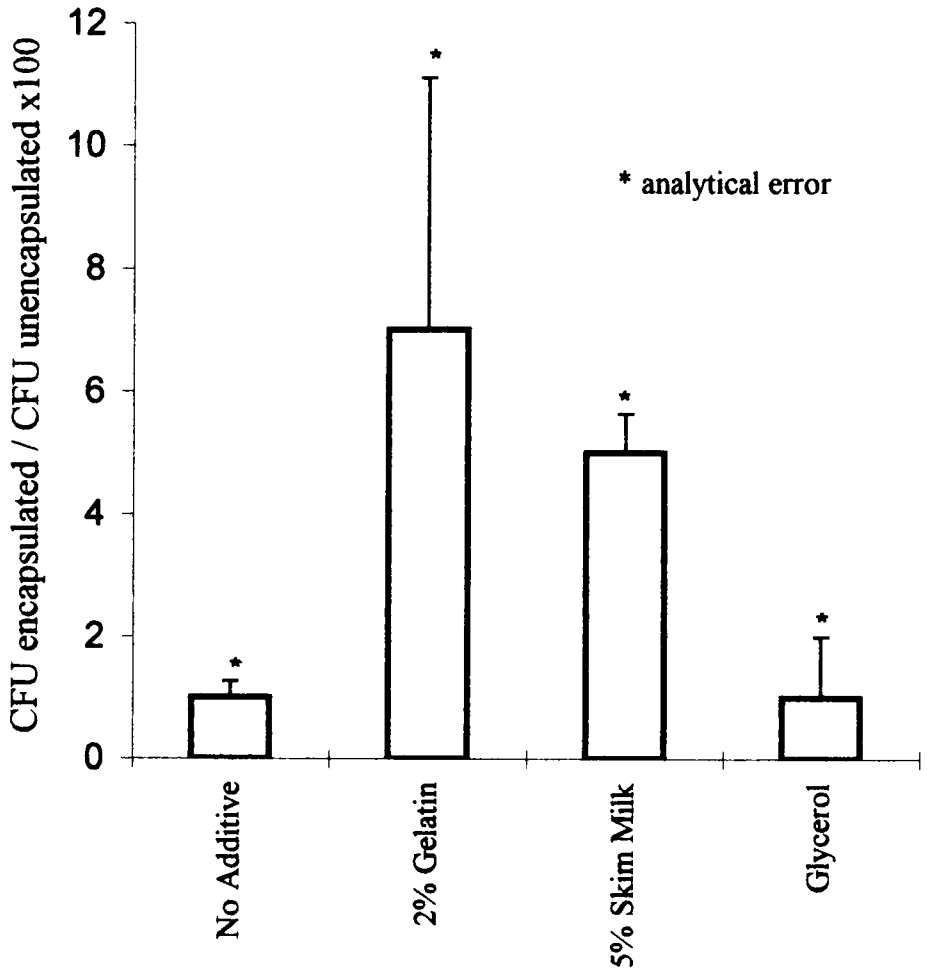


Figure 3.4 Effect of Protective Agents on Viability of PPI

* (Bars indicate standard deviation. Where bars are not present, standard deviation was done but not discernable.)

cocktail as the protective/coating agent, improved cell viability approximately 5-fold (Figure 3.4), from 2% to >10%, for the G4 and 100 fold, from <0.01 to >10%, for PPI. There was wide variability of viability between experiments. Based upon these results, 2% gelatin (Bloom 75) was included in the standard method of encapsulation.

The richness of the media also appeared to have an effect on viability. In unoptimized media the G-4 bacteria exhibited a higher viability (2% vs <0.1%) (Figure 3.3) than the PPI. One potenitally significant difference between the two encapsulation methods was that the G-4 bacteria were grown in BS+G medium and the PPI bacteria were grown in the 10% PTYG medium. To determine if the media differences caused the differences in viability the G-4 bacteria was grown in 10% PTYG medium and encapsulated into polyurethane foam using gelatin as the protective agent, and cell viability fell to <0.1%, (Figure 3.5). When G-4 bacteria were grown in BS+G medium and encapsulted with gelatin a protective agent, cell viability improved dramatically to > 16% (Figure 3.5). Dilution of the undefined PTYG media increased viability of PPI after encapsulation. When PPI was grown in medium containing 0.01% PTYG, cell viability was approximately

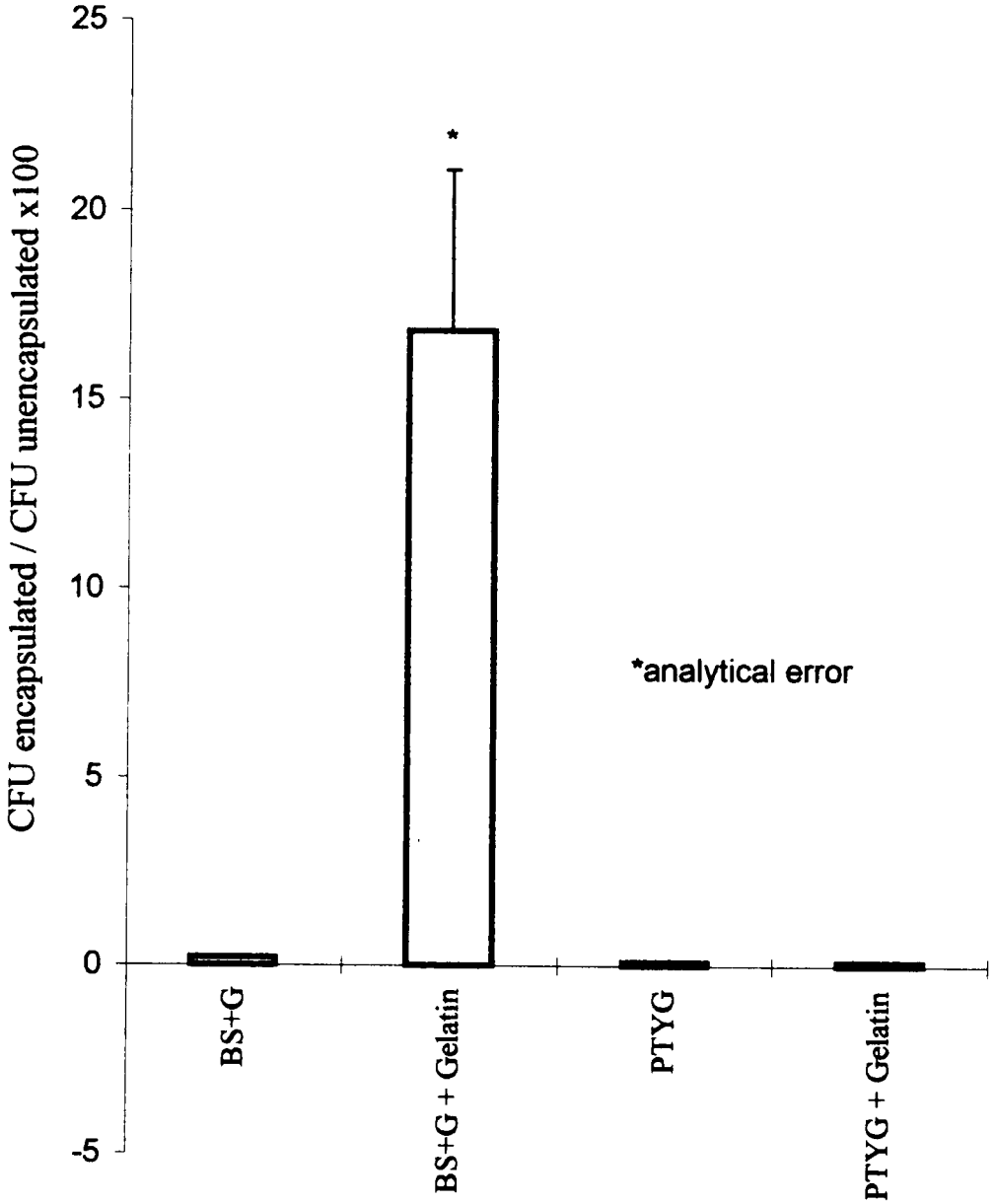


Figure 3.5 Effect of Media Composition and Gelatin on Viability of G-4

* (Bars indicate standard deviation. Where bars are not present, standard deviation was done but not discernable.)

8% (Table 3.1). However, the growth of PPI bacteria in 10% PTYG resulted in <0.001% viability. Growth of PPI in BS+G resulted in cell viability of 3% (Table 3.1). Therefore, as the PTYG medium components were reduced the cell viability after encapsulation improved.

Prepolymer formulation also had a significant effect on cell viability (Figure 3.6). PPI cells encapsulated using the 2000 and 2002 prepolymers, with gelatin as the protective agent, survive much better than those in the 3000 product. There was approximately 100 fold improved viability of the encapsulated bacteria in the 2000 and 2002 vs. the 3000.

Glucose and temperature also had significant effects on survival (Figure 3.7). Keeping the PPI bacteria for one hour at room temperature after mixing with the prepolymer and allowing to harden improved cell viability 1000-fold from less than 0.01% for bacteria kept on ice during hardening to over 10%. Exclusion of glucose from the incubation medium of the PPI bacteria, reduced viability of the encapsulated cell from 10% to approximately 5% when hardened at room temperature. Addition of glucose to the growth medium improved the cell viability from <0.01% to 1% when

Table 3.1 EFFECT OF MEDIA COMPOSITION ON VIABILITY OF FOAM ENCAPSULATED *PSEUDOMONAS PUTIDA* IDAHO

MEDIA COMPOSITION (SEED CULTURE / FINAL DILUTION)	CFU ENCAP / CFU UNENCAP x100	CFU ENCAP	CFU UNENCAP
10% PTYG / 10% PTYG	<0.001	2.5×10^2	2.7×10^7
10% PTYG / 1% PTYG	1.0	1.3×10^5	2.7×10^7
1% PTYG / 0.1% PTYG	3.0	1.9×10^6	6.7×10^7
0.5% PTYG / 0.05% PTYG	1.0	1.4×10^6	1.6×10^8
0.1% PTYG / 0.01% PTYG	8.0	2.9×10^6	3.6×10^7
0 PTYG	3.0	9.3×10^5	2.9×10^7

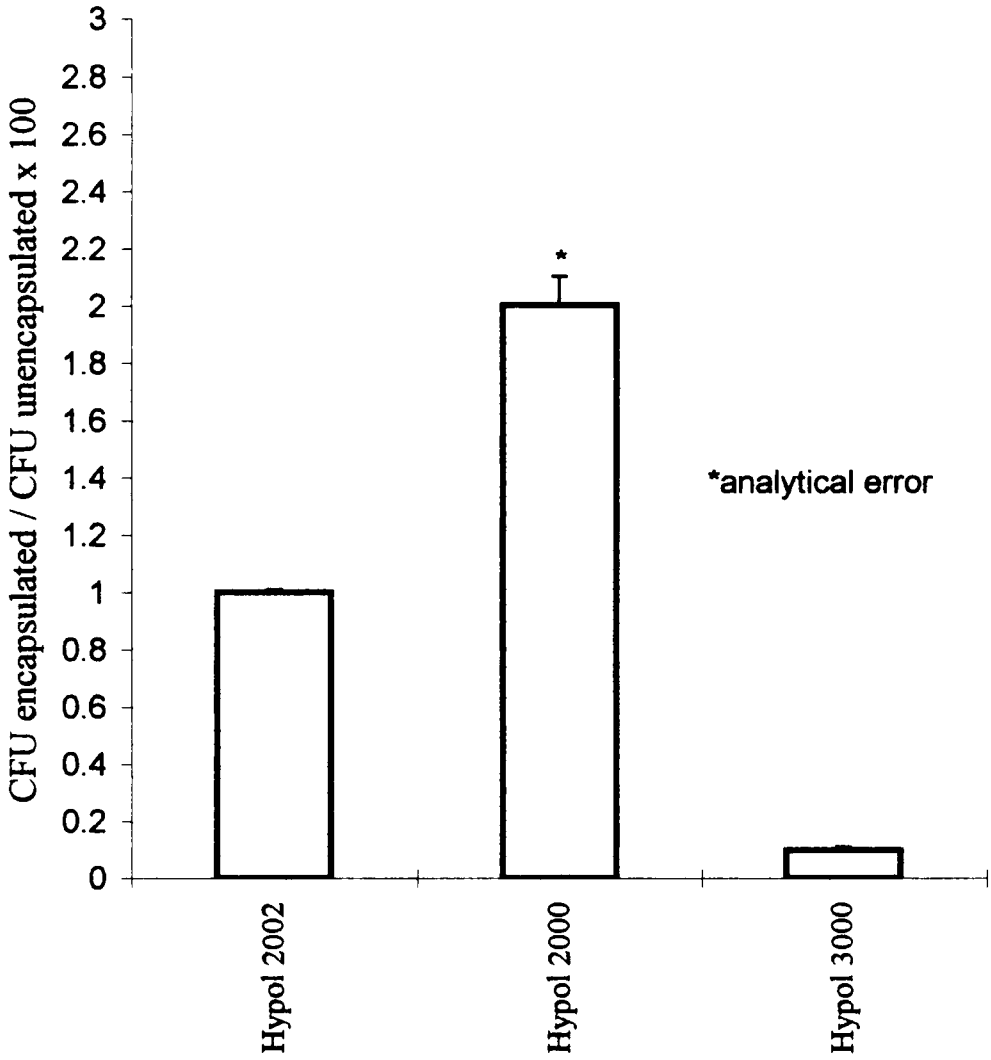


Figure 3.6 Effect of Polymer Type on Viability of PPI

* (Bars indicate standard deviation. Where bars are not present, standard deviation was done but not discernable.)

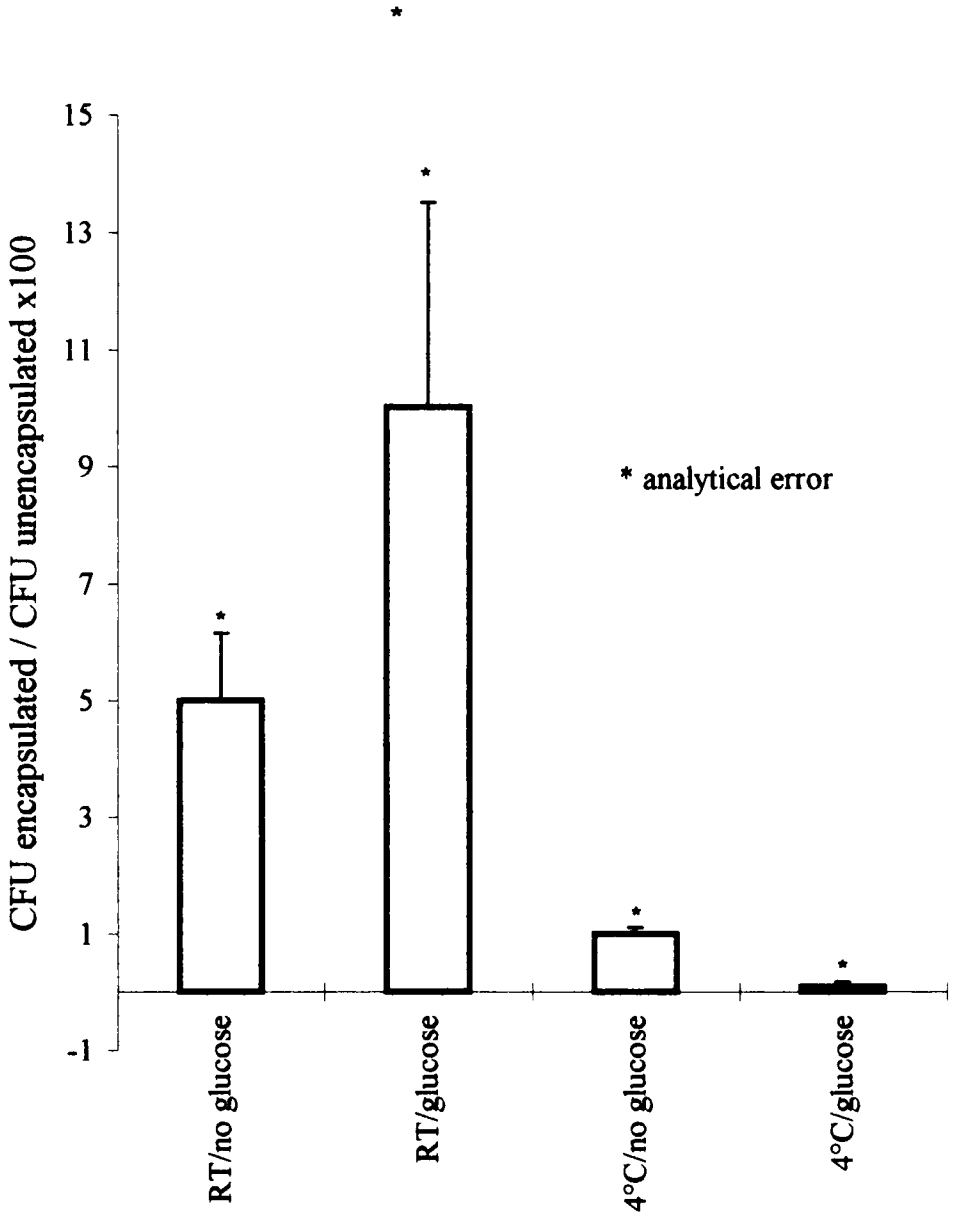


Figure 3.7 Effect of Temperature and Glucose on Viability of PPI

* (Bars indicate standard deviation. Where bars are not present, Standard deviation was done but not discernable.)

encapsulation mixture was hardened at 4°C. Thus, the maximum viability results from incubation with glucose and hardening at room temperature.

The previous experiments established the standard method for growth of each bacteria and foam encapsulation for subsequent experiments. PPI was grown in medium containing 0.1% PTYG in BS+G to late stationary phase, 7 to 8 days. G-4 bacteria were grown in BS+G medium to late stationary phase, 36 to 48 hours. Cells were encapsulated in Hypol 2002 foam at room temperature with 2% gelatin (bloom) as the protective agent. Viability of PPI and G-4 using this standard method approached 10% (Figure 3.8)

3.2 Survival of Respiratory Activity

Encapsulated G4 produced approximately equivalent amounts of CO_2 when compared to nonencapsulated live bacteria (Figure 3.9). All controls, either with killed bacteria or no bacteria produced approximately the same amount of CO_2, 50 μmoles, over 7 days, establishing a background level. In contrast, live cells produced > 300 μmoles in 3 days. The encapsulated G4 appeared to convert glucose to CO_2 slightly better than the nonencapsulated

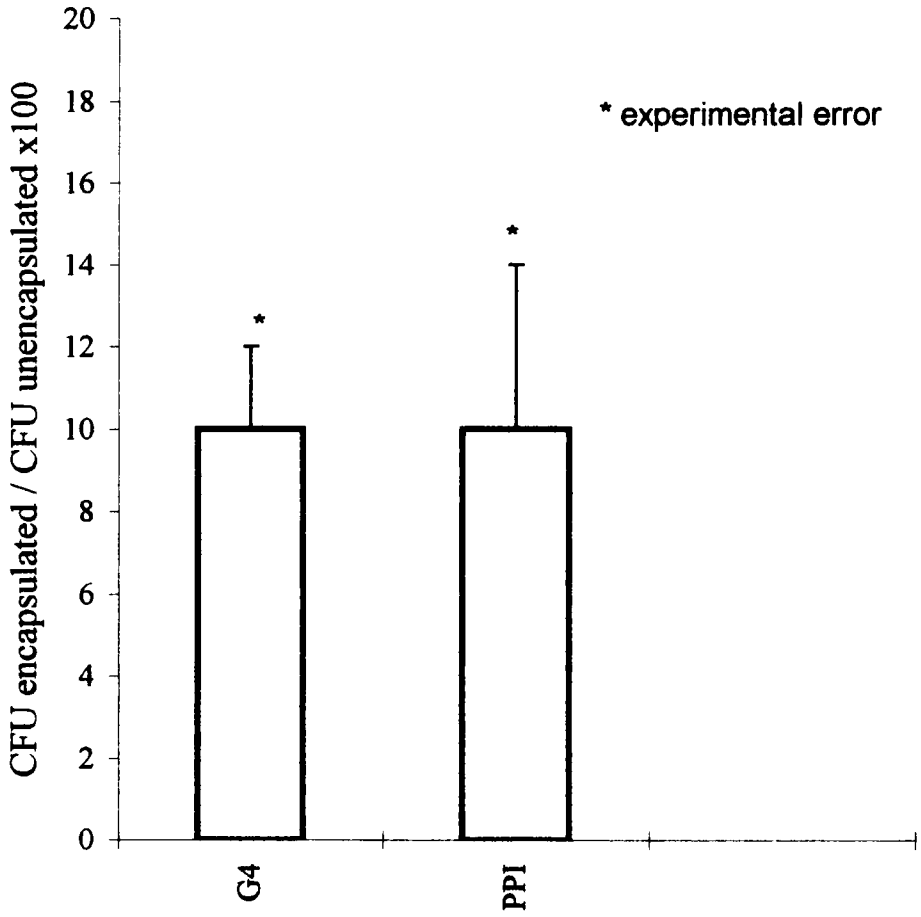
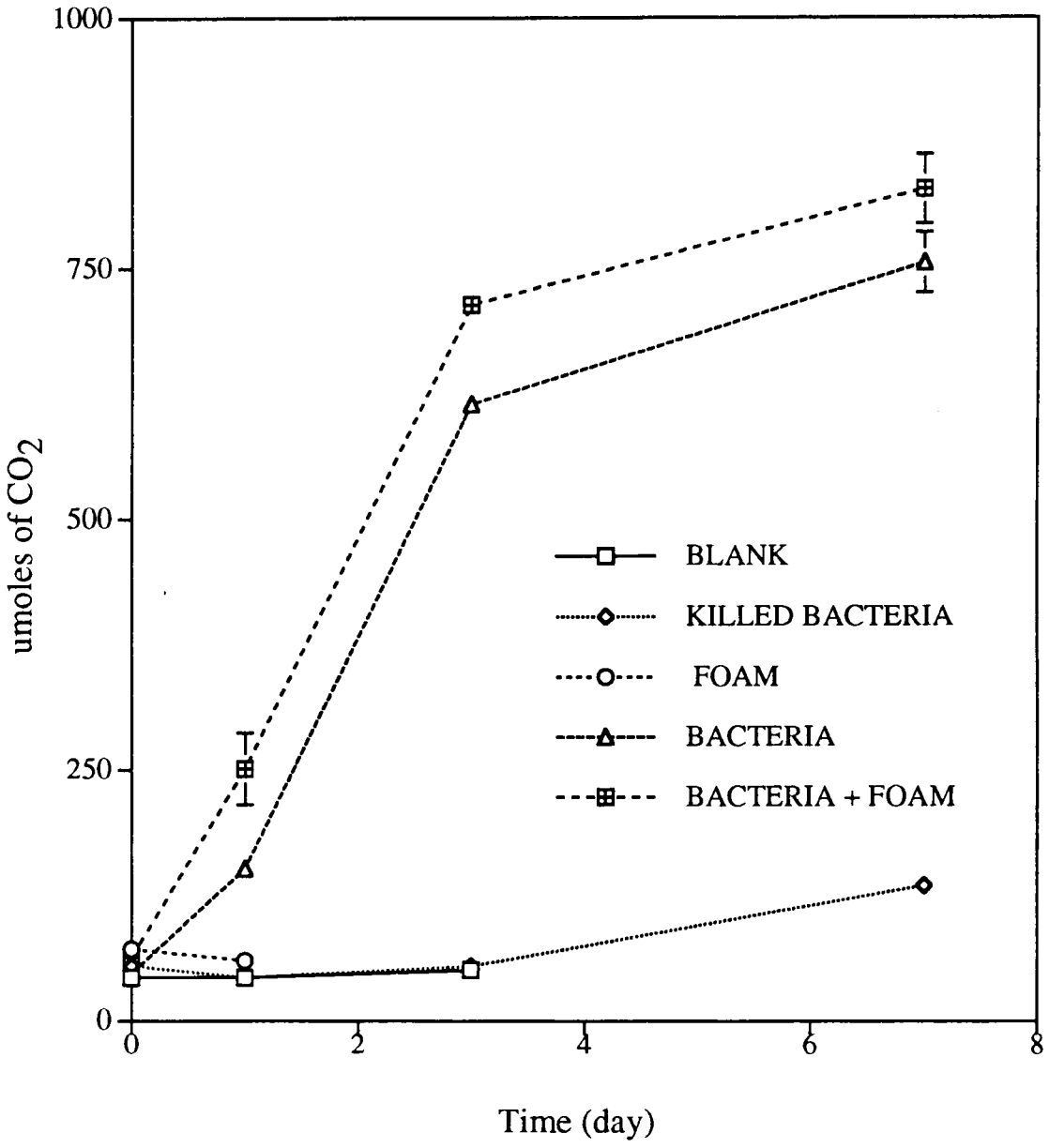


Figure 3.8 Viability of Microorganisms Encapsulated by the Standard Method

* (Bars indicate standard deviation. Where bars are not present, standardivation was done but not discernable.)



* (Bars indicate standard deviation. Where bars are not present, standard deviation was done but not discernable.)

Figure 3.9 Effect of Foam Encapsulation on CO_2 Production by *Burkholderia cepacia* G-4. *

bacteria. The steep rise in CO_2 between day 1 and day 3 indicates that there was no lag in CO_2 production at the start of the experiment. The CO_2 production leveled off between day 3 and day 7.

As with the encapsulated G4, the encapsulated PPI bacteria showed conversion of glucose to CO_2 at about the same level as nonencapsulated PPI (Figure3.10). The production of CO_2 was at least 10-fold over the background level of 50 μmoles. At day one and day three the encapsulated bacteria appeared to convert the glucose to CO_2 at a faster rate as shown by a steeper and higher curve. There appears to be a slight lag in glucose conversion in the nonencapsulated bacteria. These results suggest that the encapsulated bacteria, PPI and G4 have maintained high metabolic activity and appear to convert the liquid phase glucose to CO_2 at a slightly higher level than the nonencapsulated bacteria.

3.3 TCE Degradation in Encapsulated G4

Encapsulated G4 bacteria incubated with 2 ppm TCE degraded >90 % of the available TCE and the unencapsulated live bacteria degraded 99% (Figure 3.11). When comparing TCE degradation at the 48 hour time point

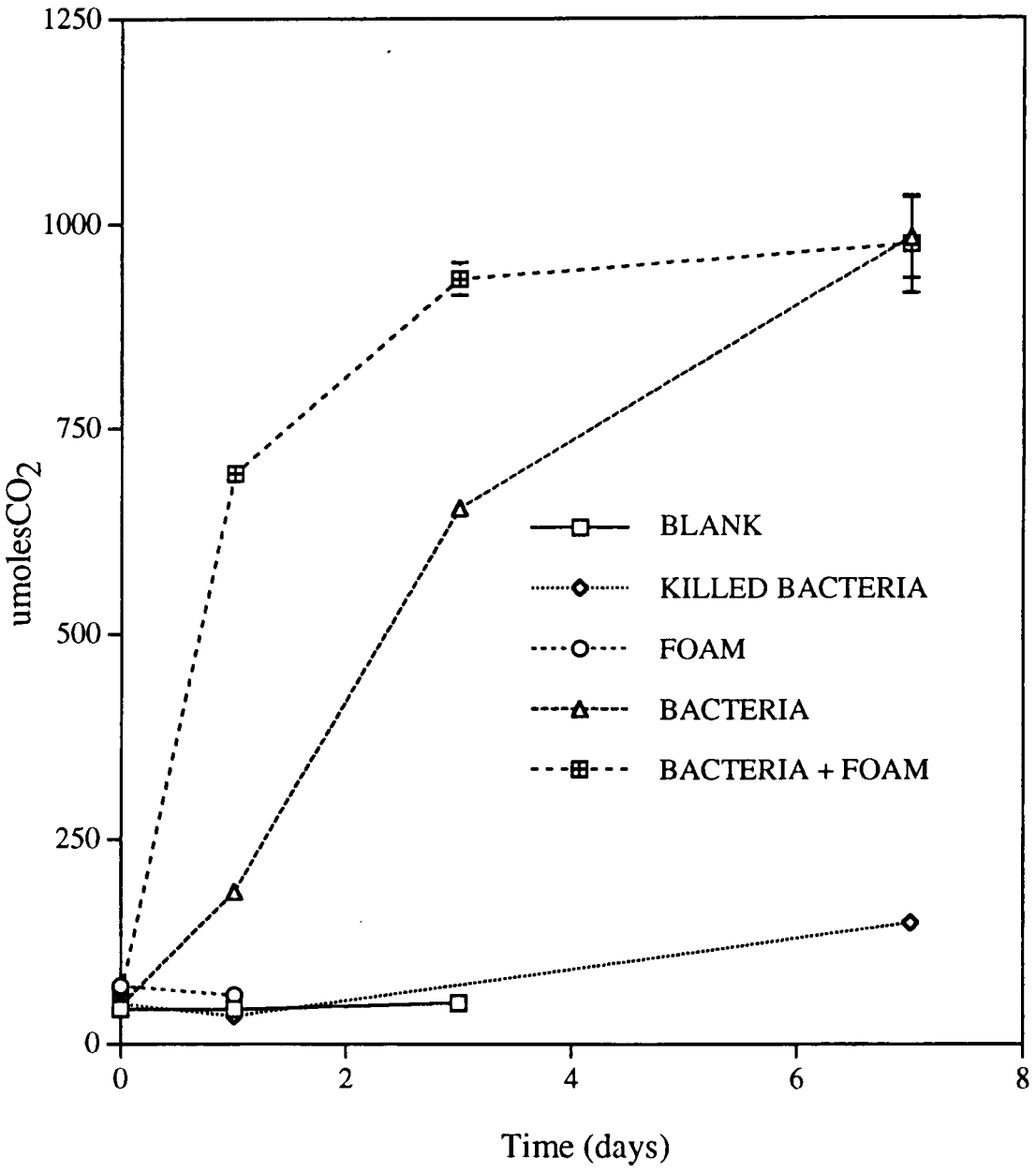


Figure 3.10 Effect of Foam Encapsulation on CO_2 Production by *Pseudomonas putida* Idaho. *

* (Bars indicate standard deviation. Where bars are not present, standard deviation done but not discernable.)

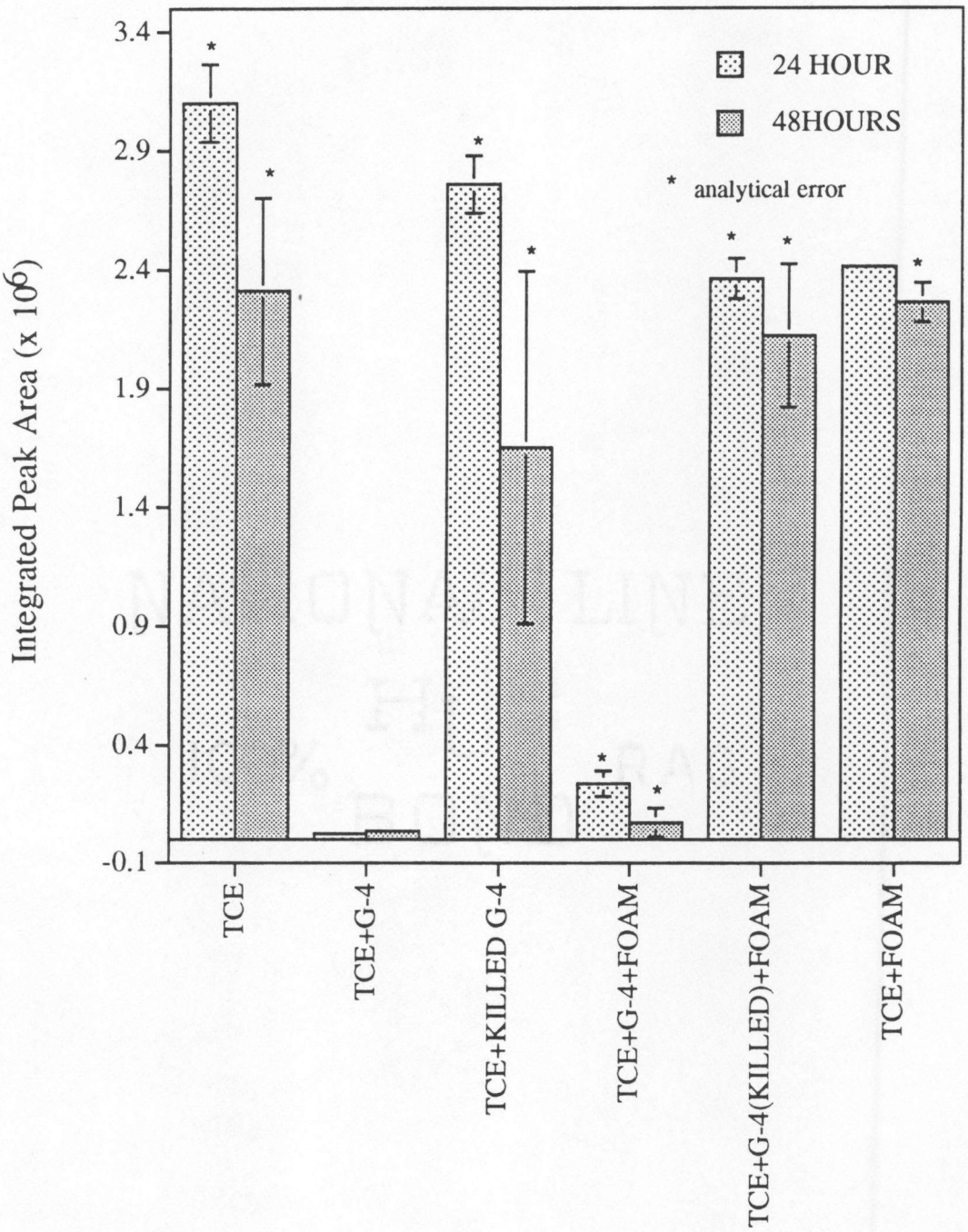


Figure 3.11 Effect of Foam Encapsulation on TCE Degradation by *Burkholderia cepacia* G-4

* (Bars indicate standard deviation. Where bars are not present, standard deviation was done but not discernable.)

the TCE was below the level of detection (2 ppb) for the live bacteria and >99% degraded for the foam encapsulated bacteria. There was negligible adsorption of the TCE to the foam or to the killed bacteria as shown by a less than 10% reduction in the headspace TCE. At higher TCE concentrations (10 ppm for 24 h) the encapsulated G-4 degraded slightly less TCE than the unencapsulated (Figure 3.12). There was a >40% reduction in headspace TCE compared to a 50% reduction in headspace TCE with nonencapsulated bacteria. At this concentration there was detectable adsorption of the TCE to the foam encapsulated killed bacteria + foam, but that is probably because of a larger amount of TCE being present and is was not degraded a quickly. These results suggest that the bacteria survived the encapsulation procedure and were metabolically active.

3.4 Toluene Degradation

When bacteria were incubated with 5ppm toluene, there was a >90% reduction in headspace toluene by the nonencapsulated cells and a >50% reduction in the foam encapsulated bacteria at 24 hours when compared to the toluene standard (Figure 3.13). At 48 hours the there was no detectable

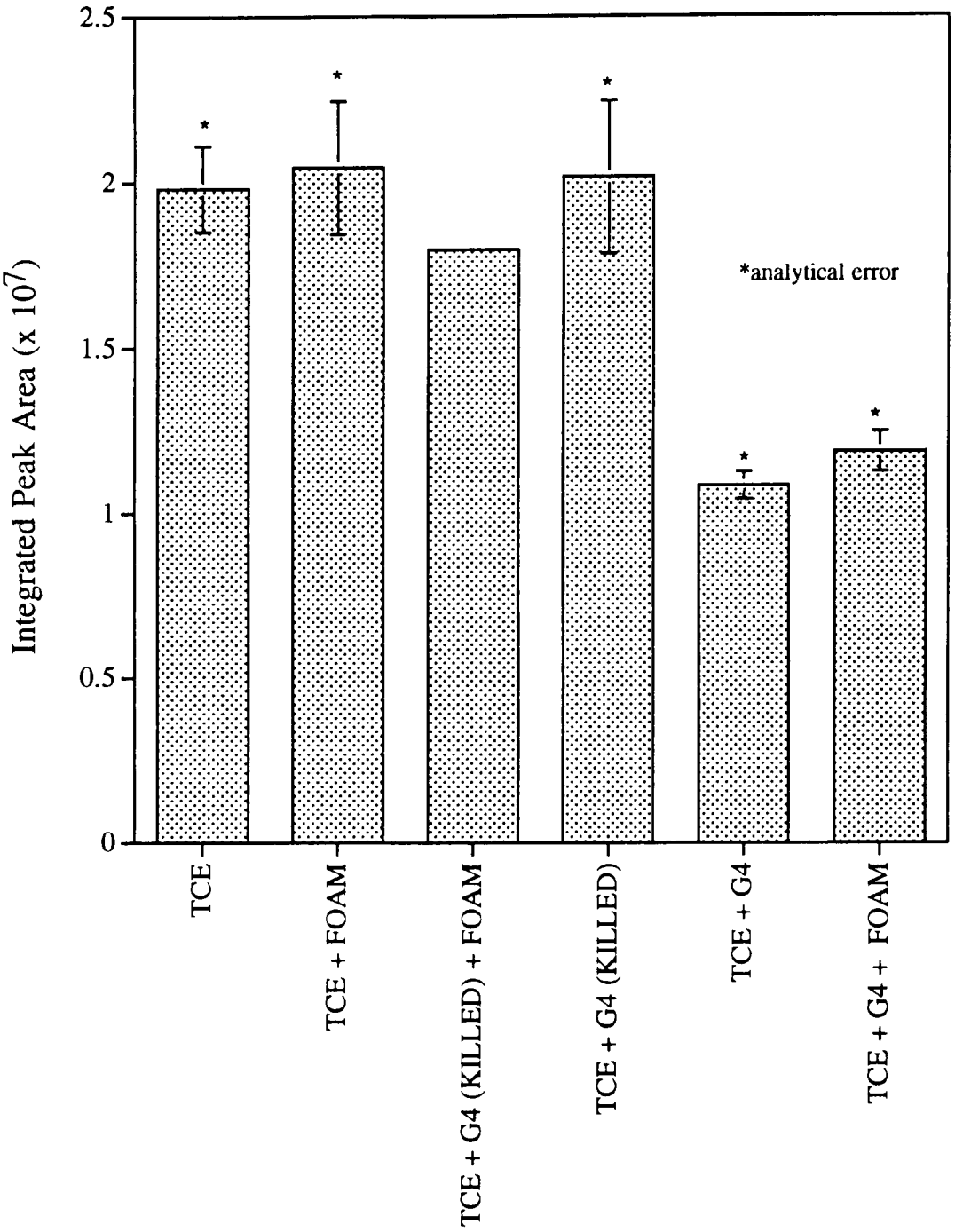


Figure 3.12 Effect of Foam Encapsulation on 10 ppm TCE Degradation by *Burkholderia cepacia* G-4

* (Bars indicate standard deviation. Where bars are not present, standard deviation was done but not discernable.)

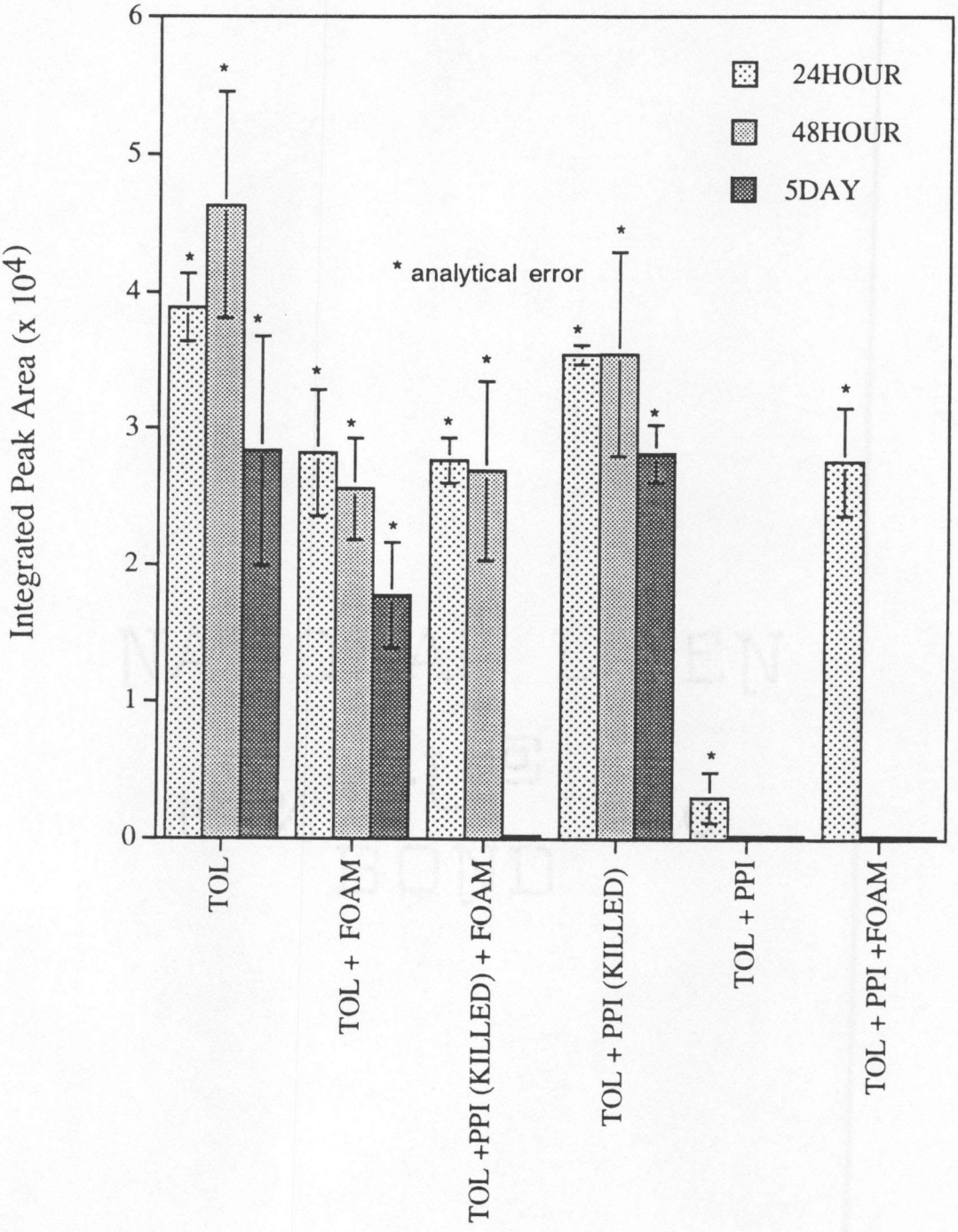


Figure 3.13 Effect of Foam Encapsulation on Toluene Degradation by *Pseudomonas putida* Idaho

* (Bars indicate standard deviations. Where bars are not present, standard deviation was done but not discernable.

toluene in the headspace with the foam encapsulated bacteria vials or in the live bacteria vials. At 24 and 48 hours there appears to be some adsorption of the toluene to the foam matrix and to the cells as illustrated by an approximate 50% decrease in the headspace toluene in the killed bacteria, encapsulated killed bacteria and the foam without bacteria. Adsorbance conutinues over time and by day 5 there is no detectable toluene in the encapsulated killed bacteria samples.

When foam samples were tested for their toluene adsorption potential, there was no difference in the headspace toluene between samples that contained foam and the toluene standard (Figure 3.14).

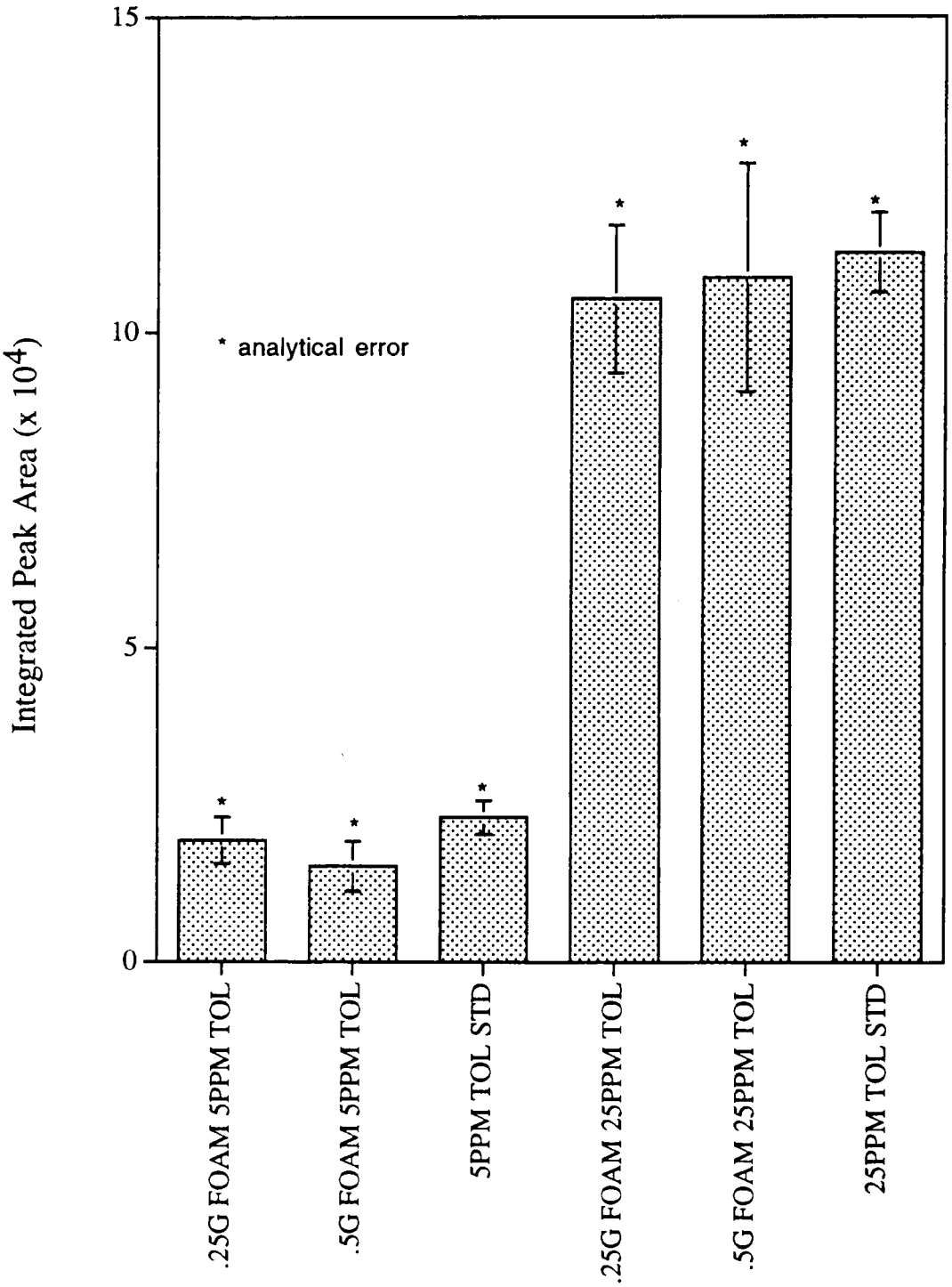


Figure 3.14 Adsorption of Toluene to Polyurethane Foam

* (Bars indicate standard deviation.)

Chapter 4

Discussion

4.1 Introduction

Many laboratory-identified microorganisms, both in pure culture and mixed culture, have been shown to be effective for degrading recalcitrant organic compounds. Development of biocatalysts using these organisms is considered one of the promising approaches to enhancing biodegradation of toxic organic compounds in the environment (18). Current methods of entrapment using alginate, carrageenan or acrylamide while successful as an immobilization medium do not have the physical strength or shear resistance to be use on a large field scale, where the ability to control the environment is limited.

4.2 Establishment of Standard Conditions

The purpose of this thesis was to determine if, *Pseudomonas putida* Idaho or *Burkholderia cepecia* G-4, could be encapsulated in polyurethane foam while maintaining viable cells. The first set of experiments were designed to

develop a standard encapsulation method that resulted in an acceptable level of viability using procedures (CFU methods) that were easy to follow. The standard method included:

- Encapsulation of bacteria at room temperature
- Use of Bloom 75 gelatin as a protective agent
- Use of Hypol 2002 polyurethane prepolymer as the encapsulating agent.
- Growth of bacteria in defined medium with glucose and minimal yeast extract as additives.
- Cell viability determination using the colony forming units (CFU) method.

Viability was shown to improve when this standard method was used (Figure 3.8), however other measures were used to confirm that the encapsulated cells were metabolically active and that the encapsulated cells were able to perform their degradation activities.

According to Lin et. al. (36), when PAH degrading bacteria were immobilized in polyurethane foam the viability was probably high when compared to another method of entrapment but they were not able measure cell numbers in the foam. Hu et. al. (8) showed lower activity with polyurethane encapsulated PCP degrading bacteria when compared to

alginate or free cells but there is no data reported that show cell counts. When one looks at the degradation data for their experiments one can hypothesize that the reason their activity was so low was because their encapsulation resulted in a high rate of cell death therefore fewer cells.

When either bacteria, *Pseudomonas putida* Idaho or *Burkholderia cepecia* G-4, was encapsulated in Hypol 2002 at room temperature and with gelatin as an additive the cell viability was consistently 8 % or better. The viability of the encapsulated PPI bacteria varied considerably. Since the PPI bacteria was a very slow grower, 8 days to stationary phase, there was difficulty in getting a dense cell paste. Therefore the counts used for the viability were at the limit of detection. Also it is possible the when there is a larger cell number present, that there is safety in numbers. If you think of a thick culture as one cell surrounded by many cells, you would have a community surrounding each cell. The outside layer of the community would protect the inside cell or cells from the toxic compound. We were able to grow a very dense culture of the G-4 bacteria using the airlift reactor and the viability of the encapsulated G-4 was consistently higher that the PPI bacteria.

Many chemicals, especially proteins are used as stabilizing agents in laboratory methods. The proteins include: albumin, gelatin, and non fat dry milk. The reason why these agents stabilize proteins is somewhat unclear but could involve nonspecific binding of the coating protein to the surface of the cell therefore providing a protective layer. Gelatin might act as a layer surround the bacteria there fore forming a barrier to the TDI in the foam hardening agent. Also, the TDI might adsorb to the gelatin therefore removing the toluene from the medium surrounding the cells.

There appears, from these results, that growing these cultures in media that has minimal protein additives in the form of yeast extract, results in better encapsulated cell viability in the PPI bacteria. Possibly due to the restriction in crude nutrients there is a change in the cell membrane that makes the PPI bacteria more resistant to the toluene. Morita et al. (37) stated that bacteria kept on low-nutrient media will have a change in their membrane structure, specifically the phoshpolipid composition. The PPI bacteria grown in the 0.01% yeast extract containing medium also had a higher cell number, when you compare the total unencapsulated bacteria cell counts (Table 3.1).

When glucose is absent from the growth medium of either bacteria, the encapsulate viability was reduced. Growth, without glucose could be considered starvation, since the glucose provides the majority of carbon for energy. According to Morita et al. (37) when bacteria are in a starvation phase, that even though the cell number increases over time the viabile count will decrease.

The effect of the different Hypol prepolymers on encapsulated cell viability is probably due to the amount of TDI (toluenediisocyanate) present. TDI is the hardening agent in the polyurethane prepolymer. There was not a great difference between Hypol 2000 and 2002. The greater difference was between the 3000 and 2000 series prepolymers. According to Kenji et.al. (19), the higher the identification number the more TDI present in the liquid prepolymer.

4.4 Survival of Respiratory Activity

The encapsulated bacteria, G4, converted bulk phase glucose to CO_2 at a level greater than the nonimmobilized cells throughout the 7 day incubation period. The PPI bacteria while initially converting glucose to CO2 at a higher

rate, after 7 days the encapsulated bacteria reached the same endpoint. O'Reilly and Crawford (7) found the urethane immobilized *Flavobacaterium*, when degrading PCP, 70 to 80% of the substrate was converted to CO_2. Also they showed the at higher PCP concentrations, the presence of polyurethane led to a decrease in the lag time prior to PCP degradation. These results show a decrease in the lag time for conversion of glucose to CO2. The foam could possibly improve the movement of the glucose to the bacteria because the bacteria are individually isolated, therefore are bathed on all sides by the liquid. According to Hu et al. (8) the mass transfer of PCP diffusing through the liquid-filled polyurethane foam matrix was enhanced at least 10 times. With convective flow through the pores of the foam particle matrix, the foam was impregnated uniformly with PCP medium, and thus the degradative capacity of the entrapped cells was enhanced (8).

4.5 TCE and Toluene Degradation in Encapsulated Bacteria

Encapsulated PPI and G-4 were able to degrade their respective organic compounds to the level of the encapsulated cells. According to O'Reilly and Crawford (7) urethane encapsulated PCP cells were actually

able to degraded higher levels of organic compounds that the nonencapsulated cells. The urethane adsorbed the excess PCP in the reaction medium, therefore protecting the cells. There was some adsorption of toluene to the foam matrix but that adsorption would not account for the total amount of toluene degraded.

Chapter 5
Conclusions

This thesis involved development of a method to encapsulated microorganisms in polyurethane foam while maintaining high cell viability and metabolic activity. Several authors had stated that they were not able to determine the viability of their polyurethane encapsulated cell. By using the CFU (colony forming units) method, we were able to not only count the foam encapsulated cells but use these counts to determine the effects of several experimental parameters on encapsulated cell viability.

Growth of G-4 bacteria in a rich medium, 10% PTYG resulted in a very low encapsulated viability. PPI grown in 10% PTYG exhibited poor viability when encapsulated. When PPI and G-4 were grown in a nutrient poor, chemically defined medium the viability after encapsulation improved. More work is needed to attempt to define what aspect of the medium is effecting the difference in viability. Possibly a medium could be developed to maximize viability with any bacteria encapsulated in polyurethane foam.

Other procedure variations also had significant impacts on cell viability and a general method for encapsulation of the PPI and G4 bacteria was developed. This method included encapsulation of both bacteria using the Hypol 2002 urethane prepolymer with gelatin (Bloom 75) as an encapsulation protective additive. The urethane foam / cell mixture was allowed to harden at room temperature. Both bacteria were grown to late stationary phase in a defined medium with minimal yeast extract. The use of gelatin in the foam encapsulation procedure and hardening the foam at room temperature improved the cell viability of the PPI and G-4 bacteria when encapsulated. Use of the Hypol 2000 or 2002 polymer, which have a lower toluene content, did improve cell viability. Only one concentration of the three potential protective agents were tested and a more complete matrix, testing other possible protective agents could be tested. Other potential protective agents include, polyvinyl alcohol, polyethylene glycol, other blooms of gelatin, bovine serum albumin, and the list goes on. A more complete list of possible protective agent can be seen in Tijssen (35).

Measurement of metabolic activity confirmed that viable cells were encapsulated in the polyurethane foam. The two experimental methods used were the meaurement of respiratory activity (production of CO2) of the encapsulated bacteria and quantitation of the amount of either TCE (G4) or toluene (PPI) that was degraded by the bacteria.

Future experiments could include adding increasing levels of TCE or toluene to the degradation experiments to test for a protective effect. The level of either organic required to kill nonencapsulated cells compared to that required to kill encapsulated cells.

Additional studies could involve reactor design. The foam has been used in a DVR (dififferential volume reactor) (8). A plunger is used to compress and expand the foam in order to bathe the entrapped cells in fresh medium. Experiments could be designed to look at degradation under these conditions. Regeneration of cells could be examined by feeding them fresh medium after they have been used for a degradation experiment and to follow the recovery and subsequent degradation rate. This would help determine the lifetime of the system and long term

stability. Also studies could be done to follow cell growth and death of the bacteria on the foam structure.

Chapter 6

Engineering Applications

6.1 Introduction

Of the various immobilization methods presently available, entrapment of biocatalyst within a porous polymeric matrix is superior in its wide applicability for the immobilization of microbial cells. The entrapmetn media most ofter used have been gels such as agar, alginate, κ-carrageenan, and polyacrylamide. However the insufficient mechanical and chemical stability of gel immobilization systems often limit the application and lifetime of the immobilization biocatalyst. As an alternative to the gels, porous polyurethane foams have been used successfully used as immobilization matrices or carriers for different biocatalysts (8, 9, 17).

6.2 Advantages of Polyurethane as a Support for Biocatalysts

The foams produced for polyurethane have advantages as supports for bacteria including a wide porosity range, good mechanical properties (flexibility, variable sizes and shapes), resistance to dilute solvents, a high

additive capacity and high water adsorbance, and resistance to enzymatic attack.

Using immobilized cells or bacteria for a process avoids the necessity of isolation or purification of a single enzyme which is a costly process. Also, since the desired enzymes are in their native environment, operational denaturation of the enzymes would be minimized (18). Weighing in favor of cells vs purified enzymes as a biocatalyst, is the fact that co-factor regeneration can take place under suitable conditions (18), avoiding the addition of costly chemicals during a process. Besides the economy of using immobilized cells as a biocatalyst vs immobilized enzymes, their use in reactors has advantages which include:

- higher biocatalyst densities can be attained with immobilization
- because the cells are immobilized, the reactor flow rates can be in excess of the washout dilution rate
- intrinsic catalytic activity has been shown to impove with immobilization (1)
- conditions of the system can be designed to regenerate the cells therefore regenerating the catalyst

- cells are more economical the purified enzymes
- separation of cells from the product is easy and regeneration is simplified

6.3 Polyurethane as a Reactor Support

Use in a fluidized bed reactor would be a good application of polyurethane encapsulated bacterial cells. Because the live cells are surrounded by a matrix, though porous, there is possible mass transfer limitation for substrates and the necessary oxygen to keep the cells alive. Also it is necessary to continually remove products to prevent inhibition of the substrate conversion process.

Fluidization is the operation by which solids are transformed into a fluidlike state through contact with a gas or liquid. At a fairly high velocity of the suspending liquid, a point is reached when the particles are all just suspended in the upward flowing liquid. In the fluidized bed reactor, liquid flows upward through a long vertical cylinder. The biocatalyst particles are suspended by the drag forced exerted by the rising liquid. Entrained catalyst pellets are released at the top of the tower by the reduced liquid drag at the expanding cross section and fed back into the column.

The advantages of the fluid ized bed include:

- Because of the turbulant flow in the upward movement of the medium, there would be better transfer of nutrients and substrate into the cell.
- The active mixing of the immobilized cells, would improve contact of the biocatalyst with the oxygen contained in the medium. Also oxygen via air could be bubbled up through the bed to improve gas exchange.
- With foam immobilization high cells density would increase the yield of the chemical process.
- Fluidized bed reactors are easy to build and have a high capacity therefore better product yield.
- Foam immobilized cells would be easy to remove inorder to regenerated the catalyst or to add new catalyst.
- Because the foam has good mechanical strength, the reactor could be run at high flow rates.

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Vita

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