IN SITU QUANTIFICATION OF HYDROGEL ENTRAPPED MICROBIAL CELLS

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

ABSTRACT

Wadhawan, Tanush, M.S., Environmental and Conservation Sciences Program, College of Graduate and Interdisciplinary Studies, North Dakota State University, May 2010. In situ Quantification of Hydrogel Entrapped Microbial Cells. Major Professors: Dr. Eakalak Khan and Dr. John McEvoy.

Entrapped bacteria are used in several applications including food and beverage production, antibiotic production, and wastewater treatment. To date in order to determine the viability of entrapped bacteria, they have to be de-entrapped from the matrix first. However, cell de-entrapment procedures, such as matrix dissolution by acid or heating at high temperatures, may affect the viability of the cells. In this study, three viability assays were assessed for in situ estimation of the number of entrapped cells. Also, a new method was developed to determine the effect of entrapment procedures on bacterial cell viability using one of the three assays, LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit. The other two quantitative assays used in this study were the bioreducible tetrazolium salt (XTT) assay and the adenosine triphosphate (ATP) based assay. The applications of the assays and the new method were performed on two cell entrapment techniques widely used in environmental applications, phosphorylated-polyvinyl alcohol (PPVA) and calcium alginate (CA).

The data from the XTT and ATP assays showed linearity and strong correlations between the viability signals and number of beads in which each bead contained a similar number of live cells. An application of the XTT assay on the PPVA entrapped bacterial beads was an exception to these results. Effects of the acid and heat dissolution de-entrapment procedures on cell viability were also evaluated by using both assays and a traditional plate count method. The heating process showed the greatest reduction in bacterial viability when compared to the other de-entrapment procedures. The ATP assay is

a more sensitive and less time consuming approach for viability estimation when compared to the XTT assay and traditional plate count method. Both XTT and ATP assays have potential for use in quantifying the viability of entrapped bacteria.

The new method developed for determining the effect of entrapment procedures on bacterial cell viability involved entrapping bacteria directly onto glass slides. This new method was compared with traditional approaches which require dissolution of the entrapment matrix using chelating agents and heat. Both the developed and traditional methods require labeling with fluorescent dyes from the LIVE/DEAD® assay and observing and quantifying live and dead cells under fluorescence illumination. The viability of entrapped cells was compared to the viability of free cells prior to the entrapment. The developed method was applicable to both PPVA and CA entrapped cells. Both methods indicated that the entrapment procedures resulted in reductions in cell viability, but the new method showed less viability reduction than the previously used method. This suggests that the matrix dissolution prescribed in the traditional method negatively affected cell viability and the new method is therefore more reliable. The percent of live bacterial cells before the entrapment ranged from 54 to 74%, while the percent of live cells following the entrapment based on the new method was 39 to 62%. The approach used in the method could potentially be adopted for other cell entrapment techniques.

ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

PVA	Polyvinyl alcohol
PPVA	Phosphorylated polyvinyl alcohol
ATP	Adenosine tri phosphate
XTT	Sodium 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate
EPS	Extracellular polymeric substance
AS	Activated Sludge
LB	Lightly bound
TB	Tightly bound
E. coli	*Escherichia Coli*
M blocks	Poly-mannuronic acid chain
G blocks	Poly-guluronic acid chain
GS	Guluronic acid
3-D	Three dimensional
UV	Ultraviolet
PVA-B	Boric acid cross linked PVA
PVA-N	Sodium nitrate cross linked PVA
PVA-P	Orthophosphate cross linked PVA
PVA-B-P	Sodium dihydrogen phosphate cross linked PVA
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
MTT	3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide

WST-1	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt
INT	(2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride)
FDA	Fluorescein diacetate
LDH	Lactate dehydrogenase
GFP	Green fluorescent proteins
BCA	Bicinchoninic acid
DH	Dehydrogenase
ETS	Electron transport system
PMS	Phenazine methylsufate
CTC	(5-cyano-2,3-ditolyl tetrazolium chloride)
XO	Xanthine–xanthine oxidase
SOD	Superoxide dismutase
PI	Propidium iodide
EDTA	Ethylenediaminetetraacetic acid
AMP	Adenosine monophosphate
PPi	Inorganic phosphate
LB	Luria-Bertani
DW	Deionized water
RLUs	Relative luminescence units
Cfu/ml	Colony forming units per milliliter
RCBD	Randomized complete block design
PBS	Phosphate buffered saline
JM109	*E. coli* K-12 JM109

J14a	*Agrobacterium radiobacter* J14a
ADMC	Atrazine-degrading mixed culture
DNMC	Denitrifying mixed bacterial culture

CHAPTER 1

INTRODUCTION

1.1. Background

Microbial biotechnology, from fermentation to metabolite production to vaccine production to recombinant deoxyribonucleic acid technology, has been around for thousands of years and is successfully applied at the commercial level (Demain 2000). A great number of industries are based on bioconversion processes that are carried out by cells adhered to surfaces. Metabolic activity of cells can change with surface properties to which they are attached (van Loosdrecht *et al.* 1990). Cell immobilization is a phenomenon which confines or attaches cells to a matrix and cell entrapment is one of its type. The entrapment procedure uses hydrogels and/or other polymers to restrict movement of the cells. Calcium alginate, polyvinyl alcohol (PVA) and carrageenan are examples of common hydrogels used to entrap cells (Jen *et al.* 1996). Entrapment sometimes increases cellular abilities to survive in harsh environment and to degrade organic substrates.

To study effects of different environmental conditions (pH and temperatures) and entrapping gels on the viability of entrapped cells, it is necessary to be able to quantify viable and/or metabolically active entrapped cells. This will also provide a better understanding of the biotechnological processes associated with the applications of entrapped cells. A number of viability quantifying assays for free cells are commercially available but none of the assays have been proven for entrapped cells. For this reason, it is important to identify and evaluate the applicability of major assays on entrapped cells.

1.2. Research problem statement

Methods to determine the viability of entrapped bacteria are available but almost all of them involve dissolving the hydrogel matrices (to release bacteria). Entrapment hydrogels evaluated for cell viability without dissolution are usually in a bead like structure with diameters in a range of few hundred microns. This size range is too small for the actual industrial applications. The de-entrapment requires additions of acids or chelating agents or heating or slicing of the bead into thin sheets (for microscopic quantification) which may affect cell viability. These de-entrapment methods can lead to inaccurate measurements of cell viability. This is a major drawback which researchers face while quantifying the viability of entrapped cells using existing techniques (bead diameter $\geq$ 1 mm). To date, there is no reliable procedure for probing the viability of entrapped bacteria without de-entrapping them (bead diameter $\geq$ 1 mm).

1.3. Research objective

The major objectives of this study were to modify existing viability assays for free cells for estimating the viability of hydrogel entrapped bacterial cells without dissolving the hydrogel matrix and to study the effect of entrapment on microbial viability. Calcium alginate and phosphorylated polyvinyl alcohol (PPVA) were used as the entrapment media because of their extensive applications in industries. Reviewing the literature helped to identify the candidate assays for assessing the viability of entrapped bacteria. The research focused on evaluating three candidate viability assays and entrapment effect of two hydrogels on microbial viability. The assays and corresponding cellular parameters measured are:

a. Microbial adenosine triphosphate (ATP) content - BacTiter Glo assay

b. Microbial activity - Sodium 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6- nitro)benzene-sulfonic acid hydrate (XTT) assay

c. Microbial membrane integrity - Live/Dead BacLight assay

The data from all the experiments were statistically evaluated to determine the sensitivity of each assay.

1.4. Benefits of research

Entrapped cells are successfully being used in a number of industrial applications. Estimating the viability of entrapped cells allows us to understand their physiological and biochemical behaviors during a biotechnological process. As hydrogel based entrapments are widely studied, assessing the viability of cells entrapped in these matrices is of great importance. Assays based on four different viability parameters were identified. Each parameter has been previously established as a successful viability indicator. Quantifying cell viability without having to dissolve the matrix will be a valuable tool for studying and understanding the biotechnological processes associated with entrapped cells. The dissolution of the matrix is time consuming and can affect cell viability. The assays used to assess cell viability without hydrogel dissolution can be used to determine a hydrogel that is the most beneficial to cell viability and related characteristics such as growth and metabolic activity.

1.5. Thesis organization

This thesis is divided into five chapters. Chapter 1 is the introduction which includes background, research problem statement, research objectives and thesis organization. Chapter 2 contains literature review and Chapter 3 is made up of work entitled "Assessing tetrazolium and ATP assays for rapid in situ viability quantification of

bacterial cells entrapped in hydrogel beads" which is modified from an accepted journal manuscript which will appear in *Enzyme and Microbial Technology*. Chapter 4 consists of work entitled "A method to determine the effect of entrapment procedures on bacterial cell viability using fluorescent nucleic acid staining" which soon will be submitted to a journal. Chapter 5 describes conclusions and recommendations for future work.

CHAPTER 2

LITERATURE REVIEW

2.1. Cell immobilization

Cell immobilization is a process that restricts free cellular movement. Cells can be attached at an interface, to each other, and inside a barrier or a matrix. Whole cell immobilization has been defined as "the physical confinement or localization of intact cells to a certain defined region of space with preservation of some desired catalytic activity" (Karel *et al.* 1985). Types of immobilization systems available are biofilm, self segregation, barrier confinement, and matrix entrapment (described below in detail). These systems are either naturally occurring or artificially created. For an ideal immobilization system, cell viability and activity should not be affected. In addition, supporting material should be compatible for cells to attach, easy to handle, insoluble, not biodegradable, mechanically stable, cost effective and able to achieve high cell density and substrate diffusion (Leenen *et al.* 1996; Kourkoutas *et al.* 2004).

2.1.1. Microbial aggregates.

Microbial aggregates have been known to have large number of biotechnological applications; wastewater treatment is one example. Microbial aggregates have been effectively used for organics, nitrogen (N) and phosphorous (P) removal from wastewater. These aggregates are capable of carrying out simultaneous nitrification, denitrification and phosphorous removal (Meyer *et al.* 2005).

There are two types of microbial aggregates: cell flocculant and biofilm. Both, cell flocculant and biofilm have two basic structures: cells and an extracellular polymeric

substance (EPS) (Figure 1). The EPS keeps all the cells intact and gives a rigid structure to the aggregate (Characklis 1990; Wingender *et al.* 1999). Few microbial aggregate characteristics studied are the amount and time of biomass retention, substrate diffusion into the core of the aggregate, settling of biomass and the growth rate gradient. Depending on the characteristics being studied or application, biofilm and flocculants have different advantages and disadvantages. One basic advantage of using biofilm over flocculants is that the interface/particulate media used for biofilm attachment gives high surface area for the cells to grow and the extra weight of the media helps biomass settle faster, while flocculants have comparatively low surface area and slow settling capacity (Nicolella *et al.* 2000a).

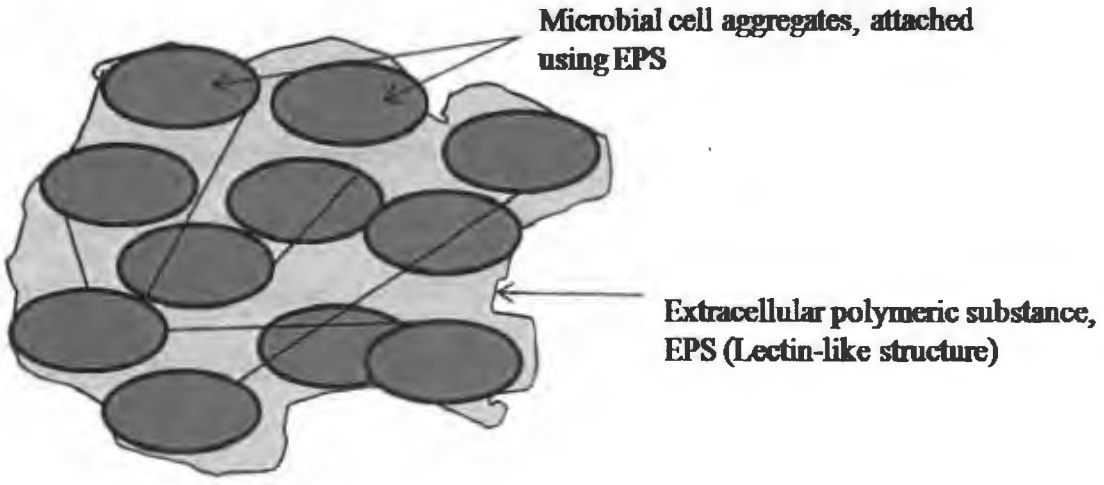

Figure 1: Microbial aggregate structure

2.1.1.1. Biofilm.

Biofilm is formed by a community of microbes that proliferate to produce a sessile colony on a surface or an interface. This interface can be any two combinations of phases such as liquid:air, liquid:solid, solid:air or even air:air and liquid:liquid interfaces. Different types of biofilms can be grown using different kinds of reactors. Few main types of

particulate biofilm reactors used for industrial applications are upflow sludge blanket, biofilm fluidized bed, expanded granular sludge blanket, biofilm airlift suspension, and internal circulation reactors (Nicolella *et al.* 2000a; Nicolella *et al.* 2000b).

Biofilms are very unique, complex and are controlled by complex transcriptional network (Prüß *et al.* 2006). They are known for their architecture, which resembles a city, where the individual bacteria form blocks of the long towers, which constitute a mature biofilm. Between these towers or micro-communities are the streets which are technically called the water channels that fill with fluids containing all essential nutrients, which are necessary for the microbial survival (Watnick and Kolter 2000). Usually, microbial cells are considered to be physically adsorbed onto the surface, either due to electrostatic force or covalent bonding between the cellular membrane, or organelles and the interface. A planktonic cell attaches onto the surface, intermediately forms a microcolony, and ultimately forms a mature biofilm. As the biofilm is exposed directly to the environment, the microbes on the upper most layer detach and either grow in the suspension or find other surface to attach (Characklis 1990) (Figure 2). This is a major drawback in the biofilm processes, especially, where cell free effluent is desired.

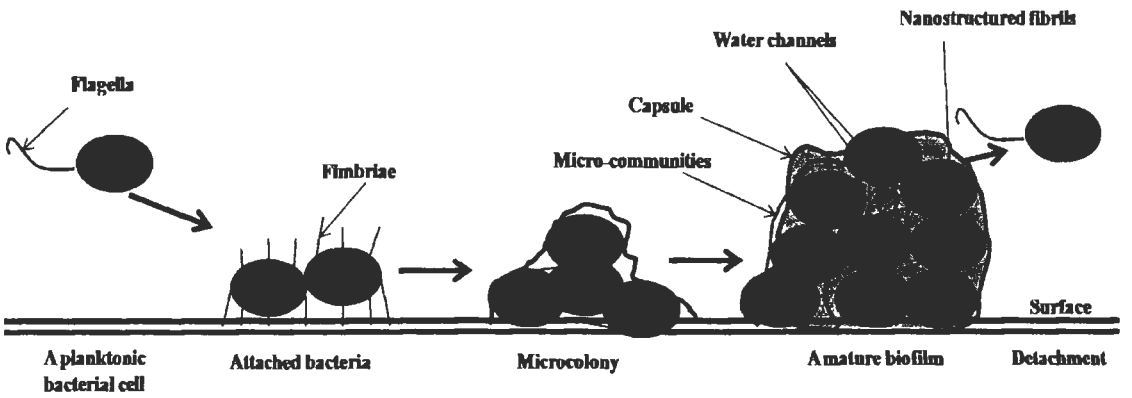

Figure 2: Process of biofilm formation

2.1.1.2. Self cell aggregation or flocculation.

Cell flocculation is defined as an ability of cells to adhere to each other using some form of EPS, lectins, to form larger units (Frolund *et al.* 1996; Higgins 1995). Major application of flocculants is in the wastewater treatment. The activated sludge (AS) is used in biological wastewater treatment and cell aggregates constitutes a large part of it. The basic principle behind the biological wastewater treatment is that organic contaminants are degraded by microorganisms present in the AS to form more biomass, which settles down and is removed (Figure 3). This biomass or the sludge constitutes both cells and organic matter exterior to the cells (Wingender *et al.* 1999).

Different sludge retention time and carbon sources affect abundances of EPS and result in different flocculation and separation characteristics. Lengthening the sludge retention time improves sludge flocculation and separation. Cells aggregate can have two types of EPS, lightly bound (LB) and tightly bound (TB), the LB-EPS has a negative effect on bioflocculation and sludge separation. Both types of EPS are found at the same time but their amount might vary. Type of EPS affects the ability of cells to attach to each other, if TB-EPS is more and LB-EPS is less, then resulting floc is a good bioflocculant, it undergoes less cell detachment and increases separation of sludge (Li and Yang 2007).

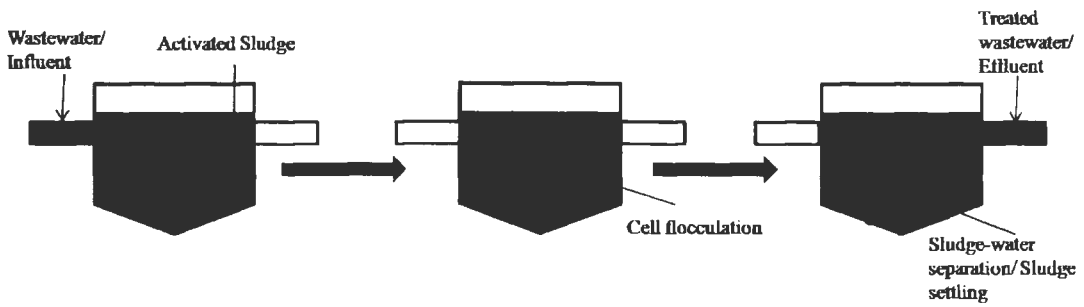

Figure 3: Principle behind biological wastewater treatment

2.1.2. Barrier contained cell system.

Main strategies for mechanically confining cells behind barriers are encapsulation and microporous filter membranes (Figure 4). These semi-permeable barriers are designed to allow diffusion of oxygen, nutrients and other substrates (Jen *et al.* 1996; Willaert and Baron 1996). Both, encapsulation and microporous filter membranes have been used very successfully in industrial settings. Encapsulation has its major application in the medical field where the permeable membrane helps in restricting immunoglobulin and immune cells that are responsible for transplant rejection from coming in contact with the cells (Jen *et al.* 1996). Four basic types of encapsulation methods are coacervation, emulsion/interfacial polymerization, pregel dissolving, and liquid droplet formation (Park and Chang 2000). Higher cell density and a cell free effluent can be achieved through encapsulation methods (Cheong *et al.* 1993). No cell release during the first 60 h of cell encapsulation experiment has been observed (Dembczynski and Jankowski 2002). Major drawbacks in using membrane filters are mass transfer limitation and biofouling (Gryta 2002).

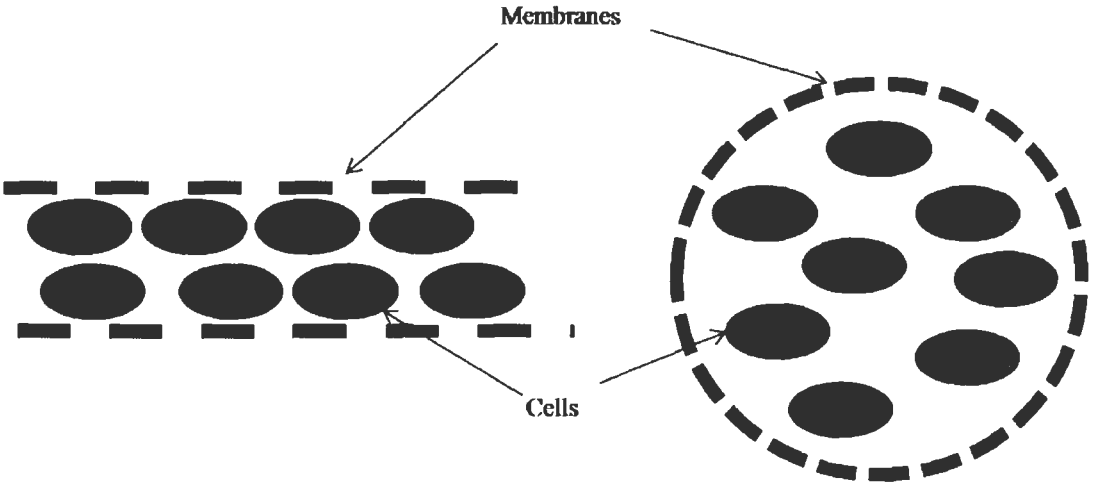

Figure 4: Encapsulation and microporous filter membranes

2.1.3. Matrix entrapment.

Cell entrapment is the uniform confinement of cells in a three dimensional matrix (Figure 5). Hydrogels are the most common matrix for cell entrapment. Hydrogels form a three dimensional mesh structure which keeps cells constrained and the pores of the mesh allows substrate and products to move in and out of the matrix. Some of the matrices are alginates, PVA, k-carrageenen, collagen, pectate, agarose, chitin and chitosan (Jen *et al.* 1996; Willaert and Baron 1996). Entrapped microbial cells have many industrial applications. Few of the industries that carry out bioconversion processes using entrapped cells are alcohol, pharmaceutical, wastewater treatment and biosensor industries (Kourkoutas *et al.* 2004; Quintana and Dalton 1999; Yang and Wang 1990; Nakamura *et al.* 2007). Entrapped cells have also been used for bioremediation and organic degradation purposes, such as carbon and nitrogen removal, atrazine degradation, and azo dye removal (Chen *et al.* 2000; Cunningham *et al.* 2004; Siripattankul *et al.* 2008; Chen *et al.* 2003; Cassidy *et al.* 1996).

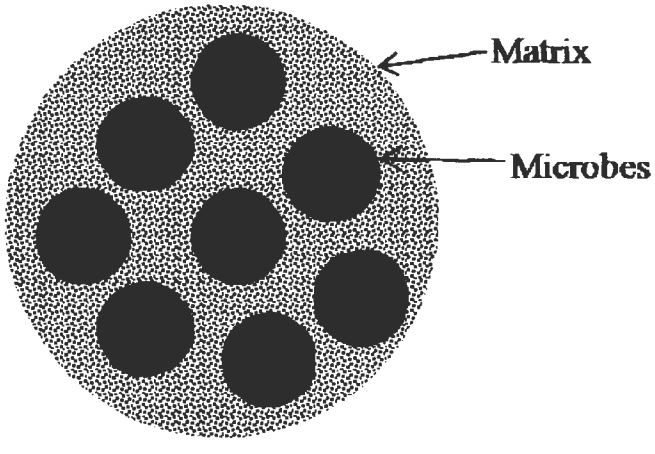

Figure 5: Cell entrapment

Entrapped cells are favored over free cells for their successful use in many of the industries. The advantages of entrapped cells include protection of cells from external

stresses (such as temp and pH), enhancement of biological and physical stabilities of cells and prolonged reuse of the cells (Dervakos and Webb 1991; Kearney *et al.* 1990; Chen *et al.* 2003; Konsoula and Kyriakides 2006; Krasaekoopt *et al.* 2006; Kim *et al.* 2008). Whole cell immobilization helps in increasing the amount of product and decreasing cell concentration in the outlet stream. Removal of a contaminant by sorption onto cells and the hydrogel is another advantage of using entrapped cell systems (Chen *et al.* 2003; Luan *et al.* 2006). Two hydrogels used for cell entrapment in this study are reviewed below, calcium alginate and PPVA.

2.1.3.1. Calcium alginate entrapment.

Calcium alginate is a water-insoluble, gelatinous hydrogel which is formed by interaction between aqueous calcium chloride and sodium alginate (Figure 6). Alginates are non-toxic and are able to form hydrogels under mild conditions. As a hydrogel, alginate absorbs water without readily being dissolved in it. They were first discovered in brown algae by a British chemist E.C.C. Standford in 1881. Afterwards alginate has been isolated from few of the bacterial species such as *Pseudomaonas aeruginosa* and *Azotobacter vinelandii* (Sabra *et al.* 2001).

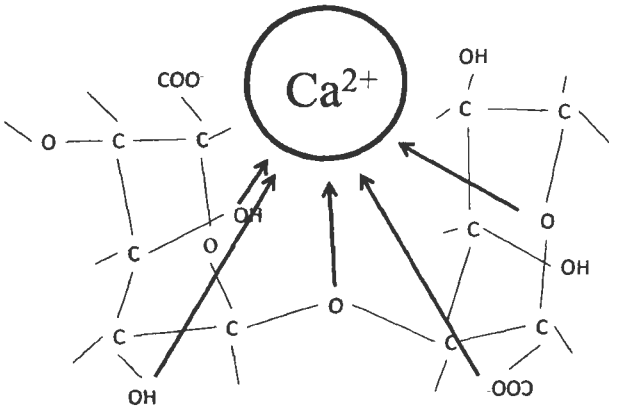

Figure 6: Structure of calcium alginate

Entrapped cells have been successfully applied commercially and industrially (Stormo and Crawford 1992; Chen and Lin 1994; Chen *et al.* 2003). Examples of entrapped cell applications are biodegradation of toxic compounds (Siripattankul *et al.* 2008), bioremediation, biosorption of heavy metals (Cunningham *et al.* 2004; Luan *et al.* 2006), biosensor applications (Fine *et al.* 2006) and metabolite production (Charlet *et al.* 2000).

A large number of cells have been entrapped and studied. A varying array of cells (microbial, animal, human and plant) entrapped in alginate are as follow: *Bacillus subtilis* PE-11 cells (Adinarayana *et al.* 2003; Konsoula and Kyriakides 2006), HepG2; bone marrow derived mesenchymal stromal cells (Bazou *et al.* 2008; Bhakta *et al.* 2009; Khattak *et al.* 2006; Khattak *et al.* 2007), *Lactococcus* ssp. *lactis* (Cachon *et al.* 1995), *Lactobacillus plantarum* (Kearney *et al.* 1990), *Lactobacillus acidophilus* ATCC 43121 (Kim *et al.* 2008), *Bifidobacterium bifidum* ATCC 1994 and *Lactobacillus casei* 01 (Krasaekoopt *et al.* 2006; Sandoval-Castilla *et al.* 2010), heterotrophic bacteria, *Escherichia coli* (*E. coli*), and *Pseudomonas fluorescens* (Toth *et al.* 1988; Pramanik 2009), *Solanum chrysotrichum* (Charlet *et al.* 2000), *Saccharomyces cerevisiae* (Fine *et al.* 2006), *Aspergillus niger, Chlamydomonas reinhardtii* cells (Walsh *et al.* 1996; Vilchez *et al.* 1997; Pashova *et al.* 1999; Santos-Rosa *et al.* 1989), *Nicotiana tabacum* (Gilleta *et al.* 2000), *Agrobacterium radiobacter* (Siripattankul *et al.* 2008), fibroblast (Hunt *et al.* 2009), di-n-butyl phthalate degrading bacteria (Jianlong *et al.* 1999), MC3T3 osteoblasts (Kong *et al.* 2003), *Chlorella vulgaris* (Luan *et al.* 2006), *Phaeodactylum tricornutum* (Moreira *et al.* 2006), *Sphingomonas cloacae* (Pluemsab *et al.* 2007); articular chondrocytes (Schneider *et al.* 2004), and human kidney 293 cells (Sugiura *et al.* 2005).

The molecular formula of alginate is $(AC_6H_7O_6)_n$ where A is a cation. Sodium alginate is a sodium salt of the alginic acid with molecular formula $(NaC_6H_7O_6)_n$. The molecular weight of the alginate depends on the cation attached; for sodium alginate, it is 198.11. Sodium alginate is a white to off white powdered odorless, tasteless and nontoxic (LD_{50} > 5000 mg/kg) solid. The melting, boiling and flash points of sodium alginate are > 300°C (572°F), 495.2°C (at 760 mm Hg) and 211.1°C, respectively. It is soluble in hot water and insoluble in diethyl ether.

Alginates are made up of linear chains of two uronic acids, (1-4)-linked monomers of β-D-mannuronic acid (Figure 7) and α-L-guluronic acid (Figure 8). In the basic structure, these monomers occur in blocks of poly-mannuronic acid, referred as M blocks, and poly-guluronic acid, referred as G blocks, and a combination of both which forms the MG block (Figure 9), which is the alginate polymer (Smidsrød and Skjåk-Braek 1990; Clare 1993). These two monomers come together in varying concentrations or sequence to form many different types of alginate with different properties. For example, diffusion of solutes in the alginate beads depends on the guluronic acid (GS) content of the alginate. The lower the GS content is, the more is the diffusion and vice versa. The GS content also determines the rigidness of the beads (matrices); higher GS alginate beads survive autoclaving conditions (Amsden and Turner 1999).

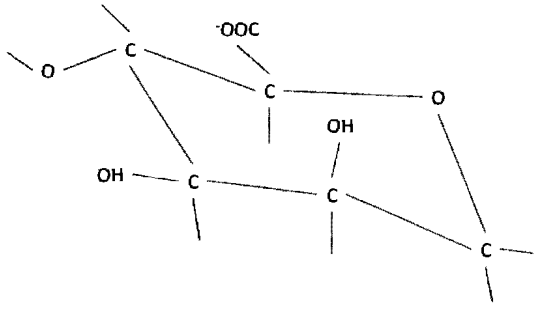

Figure 7: D-mannuronic acid structure

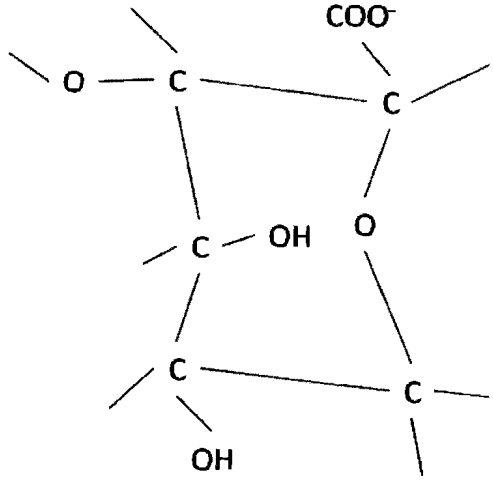

Figure 8: L-guluronic acid structure

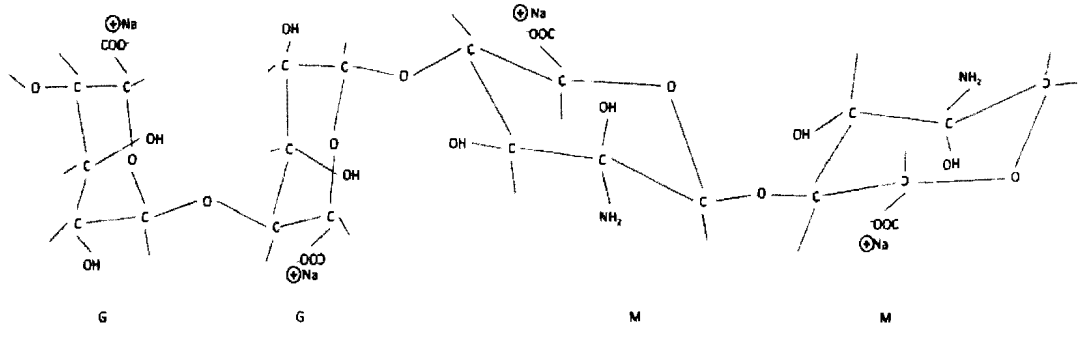

Figure 9: GG, GM and MM blocks in alginate structure

Due to these monomers, alginates can be engineered in a way to get hydrogels with desired properties. For cell entrapment, cells are mixed with the alginate solution which has high viscosity due to the high molecular weight of the alginate. Since the viscosity of this pre-gelled solution is high, the cells have to be mixed in the solution at a high shear force causing their membranes to burst hence affecting the cell viability. The effect on viability of cells in alginate hydrogels depends on the molecular weight of the alginate. Decreasing the molecular weight of the polymer has helped in decreasing the effect on the cell viability which has been shown to increase from 40 to 70% (Kong *et al.* 2003). Hence, monomers in the alginate can be designed to make their hydrogels more compatible with the cells.

Permeability of the alginate towards diffusion of a substrate depends on the physical and chemical characteristics of the diffusing substrate and the alginate gel. These characteristics are as follows: the molecular weight of the diffusing substrate, charge of the substrate, water content of the gel, and gelation time and network densities of alginate matrix (Favre *et al.* 2001; Garbayo *et al.* 2002; Aslani and Kennedy 1996). The properties that define the substrate diffusion into the hydrogel are correlated.

Increases in molecular weight of the diffusing substrate and matrix network density result in less substrate diffusion (Favre *et al.* 2001; Garbayo *et al.* 2002). Charge of substrate can interfere with its diffusion in the matrix. Positively charged ammonium ions were found to have less diffusion than negatively charged nitrate ions while the non-charged glycerol diffused faster than the charged (Garbayo *et al.* 2002).

The gelation time is the time allowed for sodium alginate to be in contact with calcium chloride to form the alginate gel. The contact time between the two chemicals defines the network density of the alginate gel matrix. During the gelation, the matrix tries to expel any substrate being entrapped (Favre *et al.* 2001). The diffusion of the substrate (nitrate) from the bulk solution into the bead decreases with the increase in the gelation time (10 min to 60 min). The substrate transport through the alginate solution (before gelation) is much easier than through the alginate gel (Garbayo *et al.* 2002).

Alginates are less permeable towards substrate than some other hydrogels such as pectate gels (Toth *et al.* 1988). Permeability of the hydrogel matrix towards a substrate depends on the water content of the hydrogel matrix. This water content further depends on the gelation time, concentration of cations (in alginate matrix), dehydration of the gel and exposure to acids or de-entraping chemicals. The water content decreases with increasing

gelation time, concentration of cations and dehydration of the gel (Aslani and Kennedy 1996).

Dehydration can be stimulated by exposing alginate to heat. Alginate beads decrease in diameter when exposed to high temperature (80°C or more). This dehydration causes the mechanical resistance of the alginate bead to increase and the beads became less sensitive towards dissolution. These changes in alginate properties are due to the rearrangement of polysaccharides under the high temp, leading to expulsion of water from their structure (Serp *et al.* 2002).

In cell entrapment, the three dimensional (3-D) matrix of the hydrogels has certain limitations, which are discussed below with their possible solutions. Depending on the density of the 3-D mesh-like structure of the hydrogel matrix or large size of the bead (diameter > 2 mm) which entraps cells, the oxygen profile can vary inside its structure. This oxygen profile can be different for different regions of the bead and might be as follows: oxygen in the periphery region > middle of the bead > center of the bead. The low oxygen concentration at the center or the middle region (depending on the beads diameter) can affect optimum functioning of the entrapped cells. The amount of oxygen tension inside the bead has been shown to increase by addition of perfluorocarbons, which are known for their ability to dissolve high concentrations of oxygen, to the alginate during entrapment (Khattak *et al.* 2007).

Matrix in the hydrogel structure offers fewer surfaces for cell attachment when compared to the volume of the whole bead. This low surface to volume ratio of the hydrogel restricts having high density of cells entrapped in the matrix. One way to

overcome this is to entrap cell aggregates. A study by Bazou *et al.* (2008) successfully entrapped 3-D Hep G2 cell aggregates which were made using an ultrasonic trap.

Since entrapped cells are more protected than free cells, one of the cell entrapment applications is cell preservation for longer periods. Alginate matrix provides longer shelf life and preservation to the cells, which can be harvested when needed. However, low temperature preservation of these entrapped cells is a problem. This temperature causes the water in the hydrogel and the cells to form ice crystal, resulting in disruption of the bead and the cell membrane. To prevent cell disruption, vitrification can be performed; this process uses antifreeze substances for cryopreservation preventing both cells and beads from being damaged (Bhakta *et al.* 2009).

Different cells might have different compatibility with different concentrations of alginate and calcium chloride. Certain concentrations of either of the entrapping chemicals can affect cell viability. It is advisable to do some preliminary studies by varying concentration of entrapping chemicals and studying effect on cell viability (Charlet *et al.* 2000). These are very crucial issues which impact viability of entrapped cells and should be taken care of for optimum application of entrapped cells.

2.1.3.2. PVA entrapment.

Hermann and Haehnel (1924) first prepared PVA (Figure 10), which is a water soluble, white colored, tasteless, odorless and nontoxic (LD_{50}> 20000 mg/kg [Rat]) granular powder. It is an ethanol homopolymer with a molecular formula [-CH_2CHOH-]-n-(-$CH_2CHOCOCH_3$-)-m and a molecular weight of 30,000-200,000 (Variable based on PVA grade). PVA has a melting point of 200°C (392°F) and a flash point of 79°C (174°F). It is water soluble but insoluble in aliphatic and aromatic hydrocarbons, esters, ketones and oils.

It is a product of hydrolyzing poly vinyl acetate with ethanol and potassium hydroxide. PVA is widely used at industrial and commercial levels. It is used as a sizing, coating and thickening agent (DeMerlis and Schoneker 2003).

Microbial life is prolonged in the PVA matrix (bead like structure) because gel protects cells from harsh environment (such as temp and pH) and allows minimal leakage of bacteria to gel exterior (Chen *et al.* 1998; Kearney *et al.* 1990; Chen *et al.* 2003; Krasaekoopt *et al.* 2006; Kim *et al.* 2008).

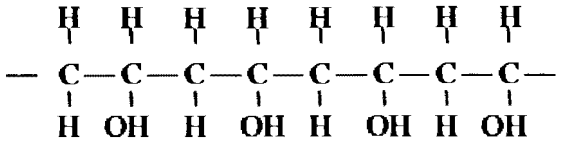

Figure 10: PVA structure

Some of the properties described above make PVA gels appropriate for the purpose of cell entrapment. PVA gels have been mostly used for microbial or enzyme entrapment, some of which are *Alcaligenes eutrophus* (Chang and Tseng 1998), heterotrophic and autotrophic bacteria (Li-seng *et al.* 2007), organic oxidizers, nitrifiers, and denitrifiers (Chen *et al.* 1996; Chen *et al.* 1998; Chen *et al.* 2000), *Aeromonas hydrophila, Comamonas testosteroni* and *Acinetobacter baumannii* (Chen *et al.* 2003; Chen *et al.* 2003); hydrocarbon degrading culture (Cunningham *et al.* 2004), *Pseudomonas fluorescens* (Pramanik 2009), *Agrobacterium radiobacter* (Siripattankul *et al.* 2008), *Candida rugosa* lipase (Dave and Madamwar 2005), *Saccharomyces cerevisiae* (Fine *et al.* 2006), glucoamylase, invertase, and cellulase (Imai *et al.* 1986), *Acidithiobacillus ferrooxidan* (Yujian *et al.* 2006), and *Phanerochaete chrysosporium* (Zhang *et al.* 2008).

PVA gel is prepared by crosslinking PVA molecules. Some of the major PVA gel preparation techniques are chemical based crosslinking (Hashiimoto and Furukawa 1987;

Chang and Tseng 1998; Chen and Lin 1994), freeze and thawing method (Ariga *et al.* 1987; Leenen *et al.* 1996; Cunningham *et al.* 2004) and UV light method (Imai *et al.* 1986). For most of the PVA entrapped cell applications, chemical based crosslinking procedures are used and discussed below. The other two crosslinking methods work as follows. For the freeze and thawing method, a PVA solution is extruded into liquid nitrogen and beads are formed and frozen followed by thawing (4°C) and freezing (-30°C) which are repeated for a few times (Ariga *et al.* 1987; Leenen *et al.* 1996; Cunningham *et al.* 2004). For the UV method, PVA is exposed to UV light for a certain amount of time (Imai *et al.* 1986).

Chemical based cross linking of PVA can be achieved using boric acid (PVA-B) (Hashiimoto and Furukawa 1987), sodium nitrate (PVA-N) (Chang and Tseng 1998), orthophosphate (PVA-P) (Chen and Lin 1994) and sodium dihydrogen phosphate solution (PVA-B-P) (Chang and Tseng 1998). The structures of PVA-B and PVA-P are given in Figures 11 and 12.

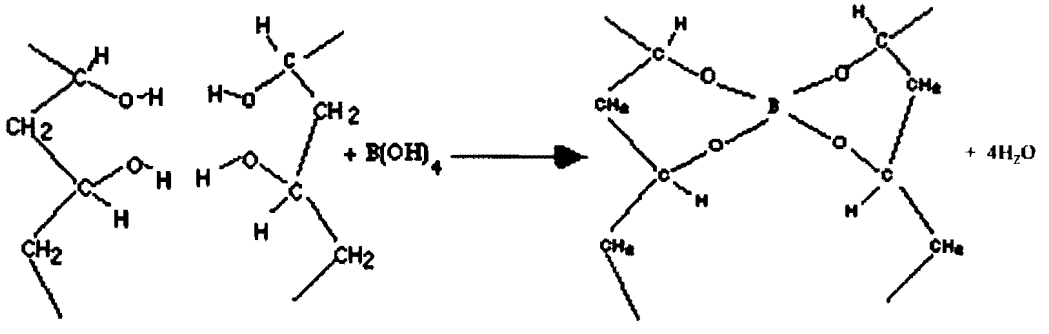

Figure 11: Structure of PVA-boric acid gel

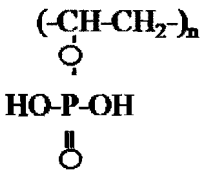

Figure 12: Structure of PVA-orthophosphate gel

The entrapment chemicals provide PVA with different surface groups and different properties. These chemicals define stability, ability to swell, mechanical strength of PVA gel, and activity of cells entrapped. PVA-B and PVA-B-P slightly dissolve and are not very stable when washed with water, while PVA-N and PVA-P beads swell by 1 – 1.5 mm respectively and are very stable. Based on these properties, PVA-B and PVA-B-P are not as good as PVA-N and PVA-P for cell entrapment. The ability of the PVA gel to swell can be reduced by adding glutaraldehyde to the PVA solution (Praptowidodo 2005).

Between PVA-N and PVA-P, some studies suggest one to be better than the other in mechanical strength and bioactivity. Chang and Tseng (1998) observed PVA-N to be more mechanically stronger and better in denitrification activity, while Li-seng *et al.* (2007) observed PVA-P to be better in mechanical strength and oxygen uptake rate. These differences can be due to different concentrations of PVA and cells entrapped.

There are problems associated with PVA entrapment procedure (chemical based procedure): bead agglomeration, less permeability and toxicity of the saturated boric acid (Chang and Tseng 1998; Li-seng *et al.* 2007; Chen *et al.* 1996; Cunningham *et al.* 2004; Dave and Madamwar 2006; Yujian *et al.* 2006). Agglomeration of PVA beads causes difficulty in uniform substrate diffusion in the beads which might affect the viability of entrapped cells. Sodium alginate (used for calcium alginate entrapment) is added to PVA

and entrapment is performed to get rid of the agglomeration problem (Wu and Wisecarver 1992; Chen *et al.* 1996; Dave and Madamwar 2006; Yujian *et al.* 2006).

Similar to alginate, PVA matrix has low substrate permeability. However, this issue has been beneficial in some studies as complete anaerobic conditions can be maintained for microbial degradation. Removal of azo dye was better using PVA entrapped cells as compared to free culture (Chen *et al.* 2003). Chen *et al.* (1996) made PVA more gas permeable by producing alginate and PVA complex and then dissolving alginate in the complex using a phosphate solution. The PVA bead obtained showed permeability increase as much as 62% which made the bead more suitable for denitrification. This was also observed by Li-seng *et al.* (2007) when they used a similar approach using orthophosphate instead of phosphate. Some studies also used PVA and calcium alginate together as an entrapment medium without washing out the alginate. There are several advantages associated with this hybrid medium. The beads or matrices formed are mechanically twice stronger than PVA. The matrix is formed faster without agglomeration and the relative activities of both entrapped microbes and enzymes increase when compared to entrapment by PPVA (Dave and Madamwar 2006; Yujian *et al.* 2006).

Another important issue is the toxicity of the boric acid solution to cells. Decreasing PVA contact time with saturated boric acid is a step towards reducing toxic effect of boric acid on cells being entrapped (Wu and Wisecarver 1992; Chen and Lin 1994). Another method recommended in literature is to use sodium nitrate for making PVA beads rather than boric acid (Chang and Tseng 1998; Li-seng *et al.* 2007). This suggests that the exposure of some entrapment chemicals might be more harmful than the others.

2.1.4. Effects of entrapment on cell growth, physiology, activity and genetic material.

Evaluating the effect of entrapment on cells is very complex. Each of these parameters such as alginate concentration, type of cation, concentration of cation, curing time, bead diameter and nutrient strength can affect cell viability, activity and other physiological characteristics (Adinarayana *et al.* 2004; Amsden and Turner 1999). Both entrapping and de-entrapping chemicals and procedures affect cell viability. Chemicals used for cell entrapment have negative impact on cells. Saturated boric acid is toxic to cells. Calcium chloride (calcium alginate entrapment) has an adverse effect on cell growth, while cells growing in alginate show a cytostatic effect (Charlet *et al.* 2000). De-entrapping procedures such as acid dissolution and heating (70°C) decrease cell viability (discussed in section 3.4.5).

Cells align in the matrix based on oxygen availability and nutrient diffusion. For example, the center of a bead sometimes lacks oxygen and nutrients forcing cells to grow towards the periphery of the bead (Condron *et al.* 1999). However, this is not true for eukaryotic cells, due to their large sizes, they are not able to move once entrapped (Charlet *et al.* 2000). Mixed cultures tend to align in a gradient inside a bead after being entrapped. Cells which require anaerobic environment move towards the center while aerobic cells move towards the periphery. Pure cultures of *Saccharomyces cerevisiae, Aspergillus niger, Chlamydomonas reinhardtii* grow in lens-shape and/or irregular shaped microcolonies in the beads away from the center around the periphery to maintain their activity and growth. (Walsh *et al.* 1996; Vilchez *et al.* 1997; Pashova *et al.* 1999). Three basic regions in the beads, that are evaluated for the presence of cells, are periphery, middle and center (Chen *et al.* 1998; Chen *et al.* 2003). Each region provides a different niche for cells entrapped.

Some of the cells escape from the bead and start to grow in a form of biofilm on the surface of the bead (Chen *et al.* 1998). Physiological changes in a cell occur in immobilized state (biofilm). Microorganisms develop certain surface-sensing responses in the initial stages, although these adhesion steps are not involved in the artificial immobilized systems (Junter *et al.* 2002).

The distribution of cells and their metabolic activity inside a bead can be heterogeneous. A study by Cachon *et al.* (1995) performed controlled linear dissolution along the bead diameter using sodium citrate. An increase in cellular density from the center to the periphery of the matrix was observed. This cellular density was correlated to the oxygen uptake rate of the immobilized cells and a gradient of respiratory activity has been observed. This showed that the activity inside the beads might also depend on the internal transfer of the oxygen and nutrients (Cachon *et al.* 1995; Jianlong *et al.* 1999). Hence, higher cell metabolite production and activity rate can be obtained with smaller beads since they promote maximum internal transfer of both oxygen and nutrients (Toth *et al.* 1989; Cachon *et al.* 1995; Shishido and Toda 1996). A 2.5 fold higher production of enzyme amylase was obtained when *Bacillus subtilis* was entrapped in alginate (Konsoula and Kyriakides 2006).

Although the activity of the entrapped cells increases when compared to the free cultures, the energy content or ATP yield is less than of the free cells. This is observed because there is less space for cells to reproduce hence all the energy generated goes into metabolic activity rather than microbial growth (Chen *et al.* 1998). Both eukaryotic and prokaryotic cells entrapped in alginate have been found to show reduction in the maximum growth rate when compared to their corresponding free cultures. Cells grow slowly in the

entrapped state and slow growing cells produce more metabolites (Charlet *et al.* 2000). The growth kinetics of entrapped cells depends on diffusion properties, bead size and polymer concentration (Chen *et al.* 2003). Their kinetics and number also varies with distance from the center. The periphery has more cells and fast growing bacteria, as compared to the center (Condron *et al.* 1999). The influence of other parameters such as, membrane thickness, initial substrate, and cell concentrations can greatly alter the bulk behaviors of the entrapped cells (Lefebvre and Vincent 1995).

Under entrapped conditions, cell viability can be maintained for a longer period of time (Fine *et al.* 2006). In addition, the entrapment prolongs the activity of cells and enzymes. Some experiments show that a good level of enzymatic activity was maintained for a period of about 9 days (Adinarayana *et al.* 2004). Thermal stability of *Candida rugosa* lipase was increased up to 10 times. The enzyme had a shelf life of 10 months and had higher esterification ability as compared to its free form (Dave and Madamwar 2005).

Free bacterial cells have a half life of 2.5 – 5.1 days and entrapped cells have half life of 14 days (Toth *et al.* 1989). Cell activity and respiration are higher in the entrapped state for *E. coli*, *Pseudomonas fluorescens* and mixed bacteria (Chen *et al.* 1998; Chen *et al.* 2000). However, some studies have reported more reduction in viability by entrapped cells when compared to free cells. This is when the end product generated by the cells is an acid which due to mass transfer limitation accumulates inside the bead causing growth cession (Dembczynski and Jankowski 2002).

Cells not only change their niche and colony shape inside the bead but also might alter the shape of their cell membrane. Due to partial shrinkage of plasma membrane, transformation of rod-like bacteria to oval ones is observed (Toth *et al.* 1989; Pramanik

2009). Plasmolysis is also observed in plant cells. Due to hypertonic calcium chloride solution, entrapped plant cells become spherical, while bacteria in free cultures are usually elongated (Charlet *et al.* 2000). *Nicotiana tabacum* cells, which are elongated when they are in a free form, exhibited spherical morphology when entrapped. This was because the cells inside the beads formed dense spherical aggregates (Gilleta *et al.* 2000).

Exposure of entrapped cells to nutrient deficient or stressful conditions results in alteration of their physiology. Under these deficient conditions, nucleic acids and enzymes are regulated first and then respiration is adjusted (Toth *et al.* 1989). The amounts of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are higher in entrapped cells as compared to free cells. RNA profiling and proteomic studies of calcium alginate entrapped cells have been performed (Kruckeberg *et al.* 2009). The respiration of entrapped cells takes time to regain while the time for entrapped cells to regain its regular growth depends on the time of exposure to deficient conditions. The entrapment itself creates a stressful condition for cells and results in change in their metabolism, certain stress response genes also gets activated (Pramanik 2009). Charlet *et al.* (2000) observed accumulation of starch containing organelles in entrapped cells.

2.2. Microbial viability assays

A viability assay is a procedure where a property or concentration of viable cells (analyte) is measured. Defining microbial viability is a very complex task and requires a good understanding of microbial physiology. To understand viability it is important to know all the parameters that contribute towards a viable cell. Certain cellular parameters such as metabolic activity, responsiveness, cellular integrity and nucleic acid are well established cell viability indicators (Keer and Birch 2003). Different assessment techniques

used for estimating calcium alginate and PPVA entrapped biomass and viable cells are described in Tables 1 and 2.

Almost all the assays described in Tables 1 and 2 require dissolution or slicing of the bead for biomass or cell viability assessments. For the assays which do not require the dissolution or slicing of the bead, the size of the bead is few hundred micrometers and entrapped cells can be easily stained and enumerated microscopically. However, most of the practical applications involve cells entrapped in beads of size $\geq$ 1 mm. Described below is the literature review of selected assays in Table 2 that are relevant to this study.

Table 1: Calcium alginate and PVA entrapped biomass quantifying techniques

Method	**Cells**	**De-entrapment**	**References**
Protein based BCA (Bicinchoninic acid) assay	Denitrifires, *Clostridium pasteurianum*	Slicing or dissolution	Chen *et al.* 1998, Chen *et al.* 2000, Chen *et al.* 2003, Chen *et al.* 2003, Lovitt *et al.* 1986
Dry weight	*Nicotiana tabacu, B. subtilis* PE-11, *Bacillus subtilis,* mixed culture, *Alcaligenes faecalis, Comamonas testosterone, Pseudomonas putida, Pseudomonas putida UV4 cells, E. Coli JM 109.*	Dissolution	Gillet *et al.* 2000, Adinarayana *et al.* 2004, Chen *et al.* 2003, Konsoula *et al.* 2006, Pramanik and Khan 2008, Pramanik and Khan 2009, Quintana and Dalton 1999
Miscellaneous 1. Conductive 2. Image analysis (Pixels) 3. Carbon^{14} labeled valine	1. *Clostridium pasteurianum* 2. *Lactobacillus plantarum* 3. Heterotrophic bacteria, *E. coli*, and *Pseudomonas fluorescens*	Slicing or dissolution	1. Lovitt *et al.* 1986 2. Condron *et al.* 1999 3. Toth *et al.* 1988

Table 2: Calcium alginate and PVA entrapped cell viability quantifying techniques

Method	Cells	De-entrapment	References
Activity based 1. MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) 2. WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) 3. INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride) 4. Alamar blue 5. FDA (Fluorescein diacetate) 6. LDH (Lactate dehydrogenase)	1. Fibroblast t3t cells, HepG2 liver cells 2. HepG2 3. Mixed culture 4. Bone marrow derived mesenchymal stromal cells 5. *Nicotiana tabacum, Solanum chrysotrichum* 6. HepG2 liver cells	Dissolution for all except for Khattak et al. 2006 and 2007 (bead size few 100 µm)	1. Rokstad *et al.* 2006, Hunt *et al.* 2009, Khattak *et al.* 2006, Khattak *et al.* 2007 2. Bazou *et al.* 2008 3. Cunningham *et al.* 2004 4. Bhakta *et al.* 2009 5. Gillet *et al.* 2000, Charlet *et al.* 2000 6. Bazou *et al.* 2008, Khattak *et al.* 2006, Khattak *et al.* 2007
Colony forming units	*Phanerochaete chrysosporium, Phaeodactylum tricornutum, Lactobacillus casei, Lactobacillus acidophilus 547, Bifidobacterium bifidum, Lactobacillus casei, Sphingomonas cloacae; Lactobacillus acidophilus, Lactobacillus rhamnosus,* Agrobacterium radiobacter J14a (J14a) and a mixed culture (MC), *Lactobacillus plantarum*	Dissolution	Zhang *et al.* 2008, Moreira *et al.* 2006, Sandoval-Castilla *et al.* 2010, Krasaekoopt *et al.* 2006, Pluemsab *et al.* 2007, Kim *et al.* 2008, Dembczynski *et al.* 2002, Siripattankul *et al.* 2008, Kearney *et al.* 1990
Membrane integrity 1. LIVE/DEAD assay 2. Trypan Blue 3. Not specified	1. Bone marrow derived mesenchymal stromal cells; *Solanum chrysotrichum; Fibroblast t3t cells;* Mixed culture; HepG2 liver cells; *Agrobacterium radiobacter* J14a (J14a) and a mixed culture (MC) 2. HepG2 liver cells; Chondrocytes; MC3T3 osteoblasts 3. *Chlorella vulgaris*	Dissolution for all except for Khattak et al. 2006 and 2007 (bead size few 100 µm)	1. Bhakta *et al.* 2009; Hunt *et al.* 2009; Cunningham *et al.* 2004; Khattak *et al.* 2007; Charlet *et al.* 2000; Siripattankul *et al.* 2008 2. Khattak *et al.* 2006; Khattak *et al.* 2007; Schneider *et al.* 2004; Kong *et al.* 2003; 3. Luan *et al.* 2006
Nucleic acid PicoGreen	Bone marrow derived mesenchymal stromal cells	Dissolution	Bhakta *et al.* 2009
ATP based	Chondrocytes, yeast	Dissolution	Schneider *et al.* 2004, Navrátil *et al.* 2000
Miscellaneous 1. GFP gene encoded 2. Luc reporter gene encoded	1. Human kidney 293 cells 2. Yeast cells	Slicing or dissolution	1. Sugiura *et al.* 2005 2. Fine *et al.* 2006

2.2.1. XTT assay.

XTT was first synthesized in 1988 by Paull *et al.* to evaluate 10 million microculture samples for a new anticancer drug screening program at the National Cancer Institute, Bethesda, MD (Scudiero *et al.* 1988). XTT is a bioreducible water soluble tetrazolium salt. The reduced form of XTT is formazan, an intense orange colored substance, which unlike other tetrazolium salts does not require organic solvents to dissolve (Goodwin *et al.* 1995). Formazan is a chromogenic product of the reduction of tetrazolium salts by dehydrogenase (DH). It has a variety of colors from dark blue to deep red to orange, depending on the original tetrazolium salt used as the substrate for the reaction. The formazan dyes are commonly used in cell proliferation and toxicity assays.

Tetrazolium salts have been previously used to correlate formazan production to the electron transport system (ETS) activity. Reduction of XTT and other tetrazolium salts by DH of the bacterial ETS, has also been well established (Roslev and King 1993; Hatzinger *et al.* 2003; McCluskey *et al.* 2005; Mosmann 1983; Zimmermann *et al.* 1978). DH takes hydrogen from the substrate and transfer to XTT, an artificial electron acceptor, at the end of the ETS chain.

Tetrazolium ring bears a positive charge and two sulfonic acid group negative charges in the XTT salt, the net charge being -1, which makes the salt water soluble. As the tetrazolium ring is bioreduced, formazan is formed, the positive charge is lost and resulting a net charge of -2 making it highly water soluble (Figure 13) (Paull *et al.* 1988). These characteristics make XTT a widely used assay, which is quicker and easier to perform than other insoluble formazan production tetrazolium assays (Stevens and Olsen 2002).

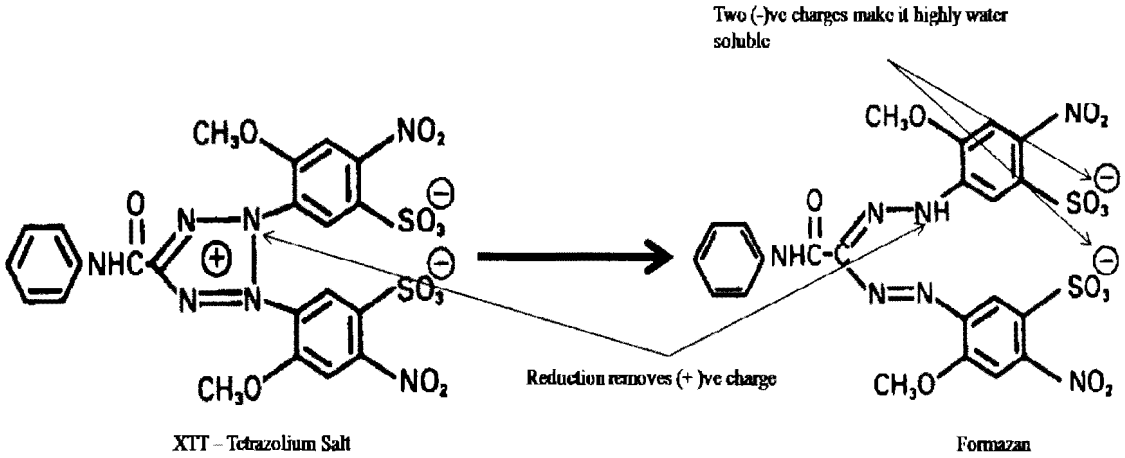

Figure 13: Structure of XTT and its reduced form (formazan)

Few of the tetrazolium salts including XTT require the addition of an intermediate electron acceptor, such as phenazine methylsulfate (PMS), to accelerate their bioreduction and the production of their formazan (Bartlett *et al.* 1979). PMS when used with XTT and MTT, cells generated higher formazan absorbance values with XTT than with MTT (Roehm *et al.* 1991) .

XTT has been successfully used to estimate the viability of a broad range of bacteria, *Methylosinus trichosporium OB3b*, *Pseudomonas putida*, *E. coli*, *Bacilus subtilis*, *Listeria monocytogenes*, *Brucella abortus*, *Staphylococcus aureus*, coagulase-negative staphylococci (CoNS) *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* and multidrug-resistant strains of *Mycobacterium tuberculosis* (Roslev and King 1993; Stevens and Olsen 2002; Cerca *et al.* 2005; De Logu *et al.* 2003). After successfully studying pure planktonic cultures, XTT has also been used to evaluate microbial aggregates, of mixed and pure cultures. For immobilized cell systems such as self aggregated mixed cultures, XTT bioreduction (formazan production) has been well correlated with oxygen uptake, substrate removal and extracellular redox activity (Bensaid *et al.* 2000; Wuertz *et al.* 1998;

McCluskey *et al.* 2005). For pure culture biofilm immobilized systems, the effect of drugs and antibiotics on microbial viability has been studied using the XTT assay (Cerca *et al.* 2005).

MTT has been used to evaluate in situ viability of eukaryotic cells encapsulated in calcium alginate (Khattak *et al.* 2006). MTT forms a water insoluble formazan and for quantitative estimation, organic solvents are used for the dissolution of formazan, adding an extra step in the process (Kim *et al.* 1994). The insolubility of formazan produced by MTT and other tetrazolium salts including, INT and CTC, can lead to an underestimation of viable cells. This problem is not encountered when using XTT dye (Roslev and King 1993).

The XTT colorimetric assay can quantify fungal spores and fungal growth by measuring its metabolism (Stentelaire *et al.* 2000; Antachopoulos *et al.* 2005). Many clinical trials using XTT have been conducted to estimate the end point viability of fungi *Rhizopus oryzae*, *Cunninghamella* spp., *Mucor* spp., *Absidia corymbifera*, *Candida albicans* and *Aspergillus* spp. in antifungal agent susceptibility testing (Hawser *et al.* 1998; Hawser *et al.* 2001; Meshulam *et al.* 1995; Antachopoulos *et al.* 2005). Both planktonic and plastic adherent *Candida albicans* have been evaluated for their viability using XTT (Hawser 1996a; Hawser 1996b).

T cells, several cytokine-dependent cell lines, melanoma cells, cryo-preserved cardiac valve tissue and *Leishmania promastigotes* have been evaluated using the XTT assay (Jost *et al.* 1992; Lu *et al.* 1997; Williams *et al.* 2003). Free radical production in living tissues, such as superoxide radical anions generated by xanthine–xanthine oxidase (XO) and XO activity, are linearly related to the reduction of XTT. Superoxide dismutase

(SOD), the antioxidative enzyme that carries out dismutation of superoxide into oxygen and hydrogen peroxide, inhibits XTT reduction. The inhibition activity of SOD can also be estimated using XTT (Sutherland and Learmonth 1997; Ukeda *et al.* 2002). To reduce the number of animal experiments, the XTT assay was used *in vitro* to study the effects of surfactants and metal salts for their cytotoxic potency in human keratinocyte cells (Brosin *et al.* 1997).

Bioreduced XTT, formazan, has been found to inhibit the replication of Human Immunodeficiency Virus-1 type clinical isolates (Zhao *et al.* 2004). Aminoreductone, 1-(butylamino)-1,2-dehydro-1,4-dideoxy-3-hexulose in the Maillard reaction, an intermediate between milk protein and lactose, has been found to reduce XTT (Ukeda *et al.* 1995; Shimamura *et al.* 2000). The phenolic compounds, catechins and catechols, also reduce XTT (Vyas *et al.* 2002). The XTT is a simple, inexpensive, safe and sensitive assay to perform (Jost *et al.* 1992; Meshulam *et al.* 1995; De Logu *et al.* 2003). The assay has been used to evaluate a broad range of cells, from eukaryotic to prokaryotic cells and some of the chemical substances as reviewed above.

Although XTT is a widely used assay with many advantages, some disadvantages have also been reported. XTT is valuable for quantification but it cannot be assumed that in mixed cultures there is necessarily a linear relationship between organism number and colorimetric signal, since different strains will have different metabolic activity. Some strains have been shown to retain a significant amount of intracellular product, which can become soluble after dimethyl sulfoxide. Some studies have reported instability in formazan production due to XTT/PMS complexes; a 5 min delay before initial mixing of

cells decreases formazan production. The acidity or alkalinity of the solution (pH) also increases or decreases formazan production (Kuhn *et al.* 2003).

2.2.2. ATP assay.

ATP is a principal energy carrier molecule found in every living microbial cell. It regulates many biochemical pathways inside the cell and was discovered in 1929 by Karl Lohmann. It is also one of the monomers used in the synthesis of RNA and DNA. ATP is found in every viable microbial cell and is an established indicator of viability. These characteristics make ATP an essential cellular viability parameter.

An ATP assay has been widely used in estimating bacteria in drinking water (Berney *et al.* 2008), ground water (Eydal and Pedersen 2007), and aerosol (Seshadri *et al.* 2009). Both mixed and pure cultures of prokaryotic cells (such as *Staphylococcus epidermidis* and *E. coli*) and eukaryotic cells (such as 3T3 fibroblasts) have been assessed for viability using the ATP assay both in suspension and biofilm forms (Dexter *et al.* 2003; Sule *et al.* 2009). The ATP assay has been successfully used for rapidly assessing active biomass immobilized in granular activated carbon used for filtration of drinking water (Velten *et al.* 2007).

The BacTiter-Glo™ Microbial Cell Viability ATP Assay is a widely used commercial kit. It determines the number of viable bacterial cells based on the quantification of ATP present. In the presence of a combination of a substrate and an enzyme (luciferin and luciferase, respectively), ATP is converted to a luminescent signal. This reaction is called the luciferase reaction in which the mono-oxygenation of luciferin is catalyzed by luciferase in the presence of Mg^{2+}, ATP, and molecular oxygen (Figure 14).

The amount of luminescent signal produced is proportional to the amount of ATP present which further corresponds to the number of viable cells present (Steinberg 1995).

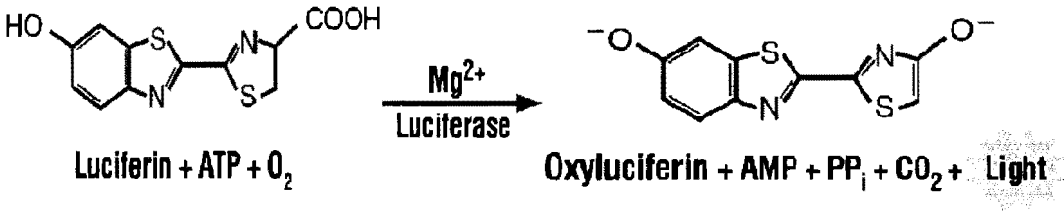

Figure 14: The luciferase reaction

2.2.3. Live/Dead assay.

The live/dead assay is a novel two-color fluorescence based microscopy assay for determining bacterial viability by assessing bacterial membrane integrity. The two nucleic acid stains are SYTO® 9 for green-fluorescence and propidium iodide (PI; phenanthridium, 3,8-diamino-5-[3-(diethylmethylammonio)propyl]-6-phenyl-diiodide) for red-fluorescence. Both of these stains have special characteristics in their ability to penetrate bacterial membranes and strongly intercalate with the DNA. The SYTO 9 stain labels all the bacteria with both, intact and damaged membranes and propidium iodide only penetrates bacteria with damaged membranes. Since PI has more affinity towards the DNA, it replaces SYTO 9 stain fluorescence when both dead and live bacteria are assessed simultaneously.

The SYTO and PI dyes are cell-permeant nucleic acid stains that can be used to stain RNA and DNA in both live and dead bacterial cells. These dyes show a large fluorescence enhancement upon intercalating with nucleic acids. For PI, one molecule of dye is known to bind every 4–5 base pairs of DNA and the fluorescence increases 20- to 30-fold when the dye binds to nucleic acid as the fluorescence excitation maximum is shifted. The dye has been used for fluorescence microscopy, confocal laser scanning

microscopy, flow cytometry and fluorometry. The live/dead assay has been successfully used for bone marrow-derived mesenchymal stromal cells, *Solanum chrysotrichum, Fibroblast t3t cells,* bacterial mixed cultures, HepG2 liver cells and *Agrobacterium radiobacter* J14a (Bhakta *et al.* 2009; Hunt *et al.* 2009; Cunningham *et al.* 2004; Khattak *et al.* 2007; Boulos *et al.* 1999; Charlet *et al.* 2000; Siripattankul *et al.* 2008).

CHAPTER 3

ASSESSING TETRAZOLIUM AND ATP ASSAYS FOR RAPID IN SITU VIABILITY QUANTIFICATION OF BACTERIAL CELLS ENTRAPPED IN HYDROGEL BEADS

3.1. Introduction

Bacterial cell entrapment is defined as a process that restricts free movement and physically isolates cells from their external environment. Entrapped cells are favored over free cells in several industries that carry out bioconversion processes such as food and beverage, pharmaceutical, wastewater treatment and biosensor (Kourkoutas *et al.* 2004; Quintana and Dalton 1999; Yang and Wang 1990; Nakamura *et al.* 2007). Entrapped cells have also been used for bioremediation including denitrification, atrazine degradation, and azo dye removal (Chen *et al.* 2000; Siripattanakul *et al.* 2008; Chen *et al.* 2003; Cassidy *et al.* 1996).

The advantages of entrapped cells include protection of cells from external stresses, enhancement of biological and physical stabilities of cells, and prolonged reuse of cells (Dervakos and Webb 1991; Konsoula and Kyriakides 2006). Different hydrogel matrices such as calcium alginate, PPVA and k-carrageenan have been used to entrap bacteria (Jen *et al.* 1996). A few studies have reported loss in cell viability due to the cell entrapment by these matrices (Yang *et al.* 1994; Chen and Lin 1994). The main cause of cell viability loss during the entrapment process is due to high viscosity of the pre-gelled solution, which requires the mixing of cells at a very high shear force resulting in cellular membrane disruption (Kong *et al.* 2003).

The amount of live biomass is one of the most critical parameters in most cell entrapment applications. Thus, quantifying the live biomass entrapped in hydrogel matrices is critical. Estimations of bacterial viability will help in understanding and controlling the biotechnological processes associated with the applications of entrapped cells. It is therefore important to identify fast, reliable, and cost efficient viability assays currently being used for free cells that can be adapted for viability estimation of entrapped cells.

To date, there is no reliable procedure for indicating the viability of entrapped bacteria without de-entrapping them. The de-entrapment requires additions of acids, chelating agents and heat which may affect the cell viability. For example, the de-entrapment of calcium alginate requires citric acid or ethylenediaminetetraacetic acid (EDTA) while the dissolution of PPVA involves heating it at 70°C for 15 min. This limitation is one of the challenges to understanding the viability, growth and metabolism of entrapped cells (Kong *et al.* 2003; Pramanik and Khan 2008; Pramanik and Khan 2009).

Certain cellular parameters such as metabolic activity, responsiveness, cellular integrity, and nucleic acid (specific messenger ribonucleic acid transcripts), are well established cell viability indicators (Keer and Birch 2003). XTT, a tetrazolium salt, can be reduced by metabolically active cells to form water-soluble formazan, which gives a good estimate of live biomass. Reduction of XTT leaves formazan with a net charge of -2, therefore making it highly water soluble (Paull *et al.* 1988). This approach has been widely studied and used for free and naturally immobilized (biofilm) bacterial viability assessment (Roslev and King 1993; Stevens and Olsen 1993; Cerca *et al.* 2005; De Logu *et al.* 2003). It was applied on activated sludge and the results (formazan production) correlated well with oxygen uptake and substrate removal (Bensaid *et al.* 2000).

MTT, another tetrazolium salt has been used to evaluate in situ viability of eukaryotic cells encapsulated in calcium alginate and on a variety of naturally occurring fungal spores (Khattak *et al.* 2006; Stentelaire *et al.* 2001). Bioreduced MTT forms water insoluble formazan. For viable cell quantification, organic solvents are used for the dissolution of formazan, adding an extra step to the process (Kim *et al.* 1994). The incomplete dissolution of MTT and other tetrazolium salts that produce insoluble formazan, such as INT, and CTC, can lead to an underestimation of viable cells. This problem is not encountered with XTT (Roslev and King 1993).

The BacTiter-Glo™ Microbial Cell Viability Assay is a method for determining the number of viable bacterial cells in a culture based on the quantification of ATP present. ATP is a principal energy carrier molecule found in every viable microbial cell and is an established indicator of viability (Schneider and Gourse 2004). In the presence of a combination of a substrate and an enzyme (luciferin and luciferase, respectively), ATP is converted to a luminescent signal (light). This reaction is called the luciferase reaction in which the mono-oxygenation of luciferin is catalyzed by luciferase in the presence of Mg^{2+}, ATP, and molecular oxygen. Oxyluciferin, adenosine monophosphate (AMP), inorganic phosphate (PPi), and CO_2 are the other products (Equation 1).

$$\text{Luciferin} + \text{ATP} + O_2 \xrightarrow{\text{Luciferase} + Mg^{2+}} \text{Oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{Light} \quad (1)$$

The amount of luminescent signal produced is proportional to the amount of ATP present which further corresponds to the number of viable cells present (Steinberg 1995). The ATP assay has been extensively used and well established for its use in studying naturally occurring immobilized microbial systems such as biofilm (Sule *et al.* 2009).

A need for understanding the biotechnology of entrapped cells has led to the investigation of alternative methods for viability quantification. Measurements of the cellular ATP and the bioreduced XTT correspond to the metabolic activity and the responsiveness of live bacteria (Keer and Birch 2003). Here, these two viability assays are investigated for in situ quantification of hydrogel entrapped bacterial cells using *E. coli* as a model. Calcium alginate and PPVA were used as the entrapment media because they are extensively studied and commercially applied hydrogel matrices (Kourkoutas *et al.* 2004; Quintana and Dalton 1999; Yang and Wang 1990; Nakamura *et al.* 2007; Chen *et al.* 2000; Siripattanakul *et al.* 2008; Chen *et al.* 2003; Cassidy *et al.* 1996; Dervakos and Webb 1991; Konsoula and Kyriakides 2006; Jen *et al.* 1996; Yang *et al.* 1994; Chen and Lin 1994; Kong *et al.* 2003; Pramanik and Khan 2008; Pramanik and Khan 2009; Khattak *et al.* 2006).

3.2. Materials and Methods

3.2.1. Chemicals and test kits.

Sodium alginate, PVA, calcium chloride, boric acid, sodium orthophosphate, sodium chloride, sodium citrate, XTT and PMS were purchased from VWR International, Inc. (West Chester, PA, USA). These chemicals were used in cell immobilization and de-immobilization procedures except XTT and PMS which were for the viability assay. The ATP-based BacTiter-Glo™ assay and ATP disodium salt were from Promega, Inc. (Madison, WI, USA).

3.2.2. Bacterial strain and culture.

E. coli K-12 JM109 strain was from Promega, Inc. (Catalog number P9751, Madison, WI, USA). The strain was plated onto Luria–Bertani plates (LB; 1% tryptone,

0.5% yeast extract, 0.5% NaCl, 1.5% agar) and incubated overnight at 37°C. A single colony from the overnight incubation was used to prepare a liquid culture of LB, which was also incubated overnight at 37°C with constant shaking to aerate the bacteria.

3.2.3. Cell entrapment procedures.

3.2.3.1. Calcium alginate entrapment.

Sodium alginate was dissolved at 80°C in deionized water (DW) until it formed a homogeneous solution. A 10 ml aliquot of the overnight liquid culture was transferred to 40 ml of the aqueous sodium alginate solution (2.0%, w/v) and homogeneously mixed to achieve uniform distribution of bacterial cells. Spherical beads were produced by dropping the mixture into a 3.5% (w/v) of calcium chloride solution using a 1 ml syringe. The beads were continuously stirred and soaked in calcium chloride for 1 hr to complete the gelation process (Konsoula and Kyriakides 2006). The diameter of the beads was 2 mm (measured using an optical microscope, BX61^{TM}, Olympus, PA, USA).

3.2.3.2. PPVA entrapment.

A 10% (w/v) aqueous PVA solution and the mixture between the culture and the solution were prepared in the same manner as described above for the sodium alginate entrapment. Spherical beads were produced by dropping the mixture of the PVA solution and culture into a saturated solution of boric acid using a 1 ml syringe. The beads were soaked in boric acid for 30 min and later transferred to a saturated sodium orthophosphate solution in which they were soaked for 1 hr (Chen and Lin 1994). The diameter of the beads was 2 mm (measured using an optical microscope, BX61^{TM}, Olympus, PA, USA).

3.2.4. XTT assay.

3.2.4.1. XTT preparation.

XTT and PMS which is an electron-coupling agent necessary for accelerated bioreduction of XTT (Bartlett *et al.* 1976) were separately dissolved in sterile DW (autoclaved at 121°C for 20 min) at a final concentration of 1 mg/ml. Both solutions were heated at 60°C for 15 min and allowed to cool to room temperature to attain complete dissolution. The solutions were filter sterilized using a 0.22 μm pore size membrane (Millipore, USA) and stored at 4°C in the dark until used. A XTT/PMS reagent was made by combining both solutions at 10:1 (XTT:PMS) volumetric ratio. The reagent was made before each experiment.

3.2.4.2. Effect of XTT concentration on formazan production.

To study the effect of the XTT concentration on the formazan production, different concentrations of the XTT reagent were evaluated using free bacterial cells. *E. coli* K-12 JM109 was cultured as described above. One hundred microliters of the overnight culture was subjected to the XTT/PMS reagent with different final concentrations of XTT (100 to 500 μM) and PMS (20 to 100 μM). The samples were incubated at 37°C in the dark for 5 hr, before they were measured for absorbance at 490 nm (A_{490nm}) in an EL808 ultra microplate reader (Biotek instrument Inc., Winooski, VT, USA).

3.2.4.3. The XTT assay.

Preliminary experiments were conducted to determine optimal assay parameters including time of XTT reduction, volume of DW, and maximum absorption wavelength of the formazan produced. The optimized assay is as follows: In 24-well microtiter plates, the beads with entrapped bacteria, 500 μl of DW, and 200 μl of XTT/PMS reagent were added.

The mixture was incubated for 5 hr at 37°C in the dark. The formazan produced was transferred to a 96 well-plate and A_{490nm} was measured.

3.2.4.4. Formazan retention in beads.

Retention of formazan in the beads could lead to an underestimation of viable cells. An experiment to address this concern was conducted. Calcium alginate and PPVA beads were produced as described above but without bacteria. Fifty beads of each material were separately exposed to formazan. To obtain formazan used in the experiment, XTT was bioreduced by incubating 500 µl of *E. coli* K-12 JM109 (cultured as described above) with 200 µl of XTT/PMS reagent at 37°C in the dark for 5 hr. The formazan produced from the incubation was collected by centrifugation (16,000 *g* for 1 min). Seven hundred microliters of formazan were added to the beads in 24-well microtiter plates and incubated at 37°C in the dark for 5 hr. The beads were also incubated with DW as a control. A color change of the beads was monitored and A_{490nm} of formazan before and after its exposure to the beads was measured.

3.2.5. ATP (BacTiter-Glo™) assay.

3.2.5.1. Reagent preparation and optimization.

The BacTiter-Glo buffer was mixed with the lyophilized BacTiter-Glo substrate and equilibrated at room temperature to form the ATP reagent. To determine the amount of reagent to be used, different volumes of the ATP reagent were evaluated against a range of number of calcium alginate beads with entrapped bacteria in a 24-well plate. The ATP assay was performed as described below.

3.2.5.2. The assay procedure.

The bioluminescence reaction was started by the addition of 400 µl of the BacTiter-Glo reagent to each well of a 24-well plate. An additional 600 µl of DW was added to the PPVA beads to completely submerge them. The incubation time was 5 min at room temperature. Bioluminescence was determined using a TN20/20 luminometer (Turner Designs, Sunnyvale, CA, USA). ATP-per-well was calculated by converting the bioluminescence signal (relative luminescence units, RLUs) to the ATP concentration (nM) using a linear regression equation obtained from the ATP standard curve (described below). ATP-per-bead was calculated as follows: ATP-per-bead = Total ATP concentration per well/number of beads per well.

3.2.5.3. ATP standard curve.

The luminescence intensity (RLU) of a known amount of ATP in serial dilutions was plotted to verify the linearity of the response. A 10 mM ATP stock solution was prepared using ATP disodium salt and sterile DW. Serial decimal dilutions to produce standard ATP solutions were performed by diluting the ATP stock solution with sterile DW. From each dilution, 1 µL and 5 µL aliquots were further diluted in a 1:100 ratio. One hundred microliters of the aliquot were placed in a 1.5 ml plastic centrifuge tube, an equal volume of the BacTiter-Glo reagent was added, and the sample tube was placed in a luminometer and the RLU was recorded.

3.2.6. Dissolution of beads and plate count of de-entrapped bacteria.

The procedures for dissolving the calcium alginate and PPVA beads are as follows: To each well of the 24-well plate containing calcium alginate beads, 1000 µl of 0.30 M sodium citrate prepared in a 0.15 M sodium chloride solution was added. The 24-well plate

was incubated for 45 min at 37°C and vigorously vortexed at regular intervals. One milliliter DW was added to each well of the 24-well plate containing PPVA beads and the plate was incubated at 70°C for 15 min. One hundred microliters of the dissolved bead solutions was used to perform a 10-fold serial dilution and plate count. The dilutions were spread onto LB-agar plates and counted after an incubation of 15-20 hrs at 37°C.

3.2.7. Viability experimental design and procedure.

Each viability assay experiment was conducted in triplicate. A single factorial randomized complete block design (RCBD) was adopted, where each triplicate represented a block. Each well of the 24-well plate contained a range of entrapped bacterial beads (0 to 50) which were subjected to all three viability assays. For further validation of the assays, the beads containing dead bacteria were then mixed with the beads containing viable bacteria in various percentage ratios (100/0, 75/25, 50/50, 25/75, and 0/100). RLUs, formazan measurements, and colony forming units per milliliter (cfu/ml) enumeration were performed for each mixture. For dead cell entrapment, bacteria were killed by autoclaving at 120°C for 20 min.

3.2.8. Effect of de-entrapment procedures on viability of free bacteria.

One milliliter of the overnight culture was transferred to 1.5 ml microcentrifuge tubes and centrifuged at 16,000X*g* for 2 min. The supernatant was discarded and the pellet was re-suspended in 1 ml of 0.30 M sodium citrate or 50 mM EDTA or DW. Samples with sodium citrate and EDTA were incubated for 45 min at 37°C (Calcium alginate bead de-entrapment procedure), while samples with DW were incubated at 70°C for 15 min (PPVA bead de-entrapment procedure). In addition, samples with DW were incubated at 37°C for 45 min as a control for the alginate de-entrapment. Note that the PPVA de-entrapment did

not need a control because there was only one treatment (temperature). After the incubation, all samples were centrifuged at 16,000 *g* for 2 min. The supernatant was discarded and the pellet was re-suspended in 1 ml of DW. Each sample was evaluated using the ATP assay, XTT assay and plate count method.

3.2.9. Statistical analysis.

Results from the triplicate experiments were statistically analyzed using the SAS program (Version 9.1, SAS Institute Inc., Cary, NC). RLUs, formazan production and colony forming units per milliliter (cfu/ml) served as the dependent variables. Entrapment media, de-entrapment procedures and number of beads constituted the independent variables. The data from the RCBD setup were analyzed by the analysis of variance (ANOVA) and Bonferroni *t*-test (Kuehl 2000) at 5% significance level. The use of the Bonferroni *t*-test over the regular *t*-test was to control the experiment wise error rate by dividing the significance level by the number of data points per viability experiment. ANOVA was used to analyze separately, different number of beads for each individual viability assay, concentration of XTT, amount of reagent used and the effect of de-entrapment procedures on the viability of the free bacterial culture. A follow up with multiple pair wise comparison for each of the experiment was performed.

3.3. Results

3.3.1. XTT assay.

3.3.1.1. Relationship between XTT concentration and formazan production.

Production of formazan showed a sigmoidal relationship with the XTT concentration used in the assay (Figure 15). The XTT concentration of 380 μM was chosen as the concentration prescribed in the procedure instead of 475 μM, because there was no

significant difference in formazan production ($p > 0.8352$) between the two. It should be noted that to prepare XTT and PMS, DW was used instead of the commonly used phosphate buffered saline (PBS) (Paull *et al.* 1988; Roslev and King 1993; Bensaid *et al.* 2000; Bartlett *et al.* 1976; Hatzinger *et al.* 2003), as precipitation was observed when PBS was in contact with the calcium alginate beads.

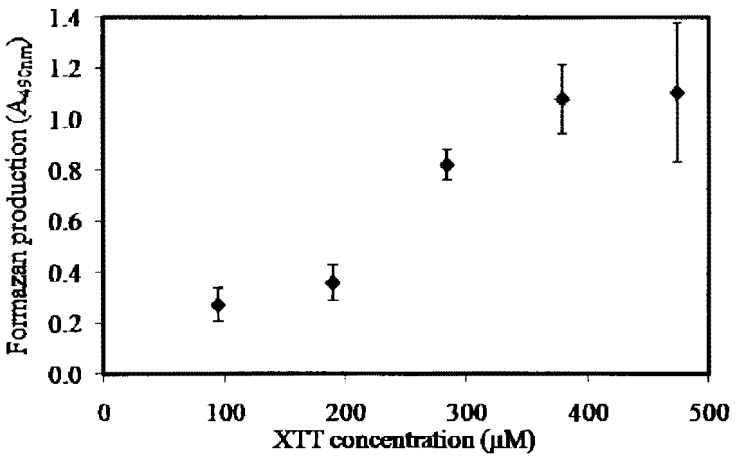

Figure 15: Effect of XTT concentration on production of formazan by *E. coli* K-12 JM109 (mean ± standard deviation)

3.3.1.2. XTT assay response to various numbers of entrapped cells.

Production of the formazan showed a good correlation with the number of calcium alginate beads containing *E. coli* (Figure 16). The number of PPVA beads did not correlate with the formazan production. Increasing the XTT concentration or the number of bacteria did not help in establishing the XTT procedure as a viability assay for the PPVA entrapped cells. The formazan detection in the bulk solution decreased as the number of PPVA beads increased (Data not shown). The incubation period for the PPVA beads with the XTT was increased to 12 h, but formazan remained undetectable in the bulk solution.

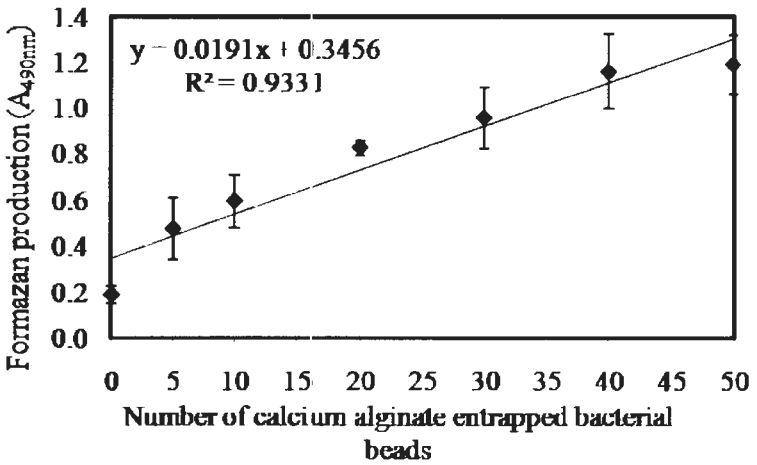

Figure 16: Formazan production (mean ± standard deviation) as a function of number of calcium alginate entrapped bacterial beads

3.3.1.3. Formazan retention in beads.

Figure 17A and C shows no changes in the color of the beads and A_{490nm} of the bulk solution when exposed to DW (control). A slight change in the color of the calcium alginate beads was observed when exposed to formazan (Figure 17B) and the A_{490nm} of the bulk solution decreased by 8%. The color of the PPVA beads (with no bacteria) changed from white to orange when exposed to formazan (Figure 17D). The A_{490nm} of the bulk solution decreased 40% after the PPVA beads were exposed to formazan.

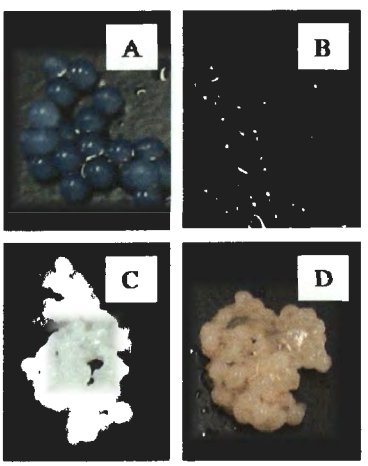

Figure 17: Calcium alginate and PPVA beads (no bacteria) exposed to distilled water (control) (A and C) and formazan (B and D)

3.3.2. ATP assay.

3.3.2.1. ATP standard curve and reagent optimization.

Figure 18A shows a strong log-log relationship between ATP concentration and RLUs. *E. coli* K-12 JM109 entrapped in calcium alginate beads were used to determine the amount of the BacTiter-Glo reagent required (Figure 18B). The beads were completely submerged when the reagent volume was 400 µl, allowing reagent diffusion into the entire bead. The reagent volume of 400 µl was found to provide a linear relationship between the number of bacterial beads and RLUs. However, reagent volumes of 100 µl and 200 µl did not result in a good correlation between RLUs and number of beads. RLUs initially increased (0 to 10 beads) before reaching comparable values from 10 to 50 beads (Figure 18B).

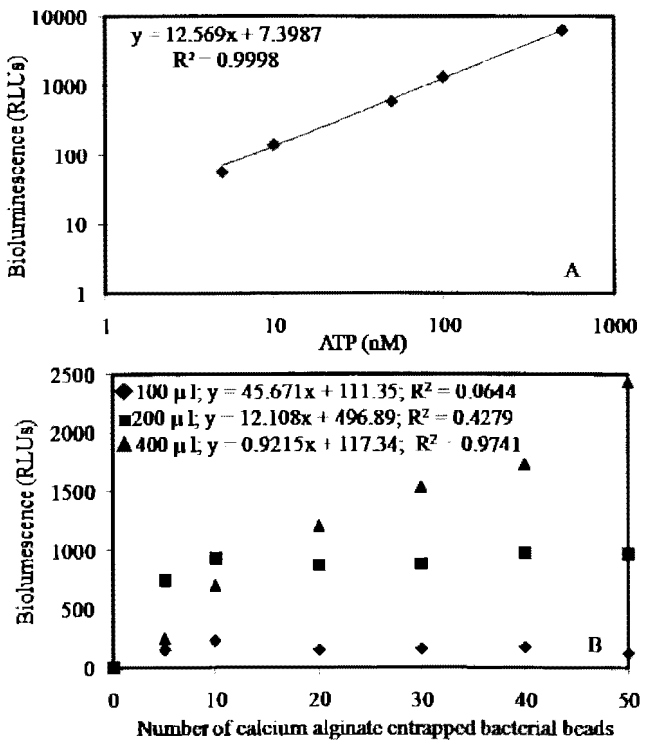

Figure 18: ATP standard curve (A), RLU values shown are mean ± standard deviation. The ATP reagent volumes of 100 µl (♦), 200 µl (■) and 400 µl (▲) were assessed to determine the optimum value for use in the assay (B)

3.3.2.2. ATP assay response to various numbers of entrapped cells.

In the ATP assay, RLUs were correlated with the number of beads (Figure 19A and B) at r^2 = 0.991 (CA) and r^2 = 0.962 (PPVA). The RLUs obtained from the assay were converted to the ATP concentration (nM), using the following regression equation from Fig. 18A: y = 12.569x + 7.3987. The PPVA beads contained 0.202±0.0038 nM of ATP per bead, while the calcium alginate beads had 3.76±0.3026 nM of ATP per bead.

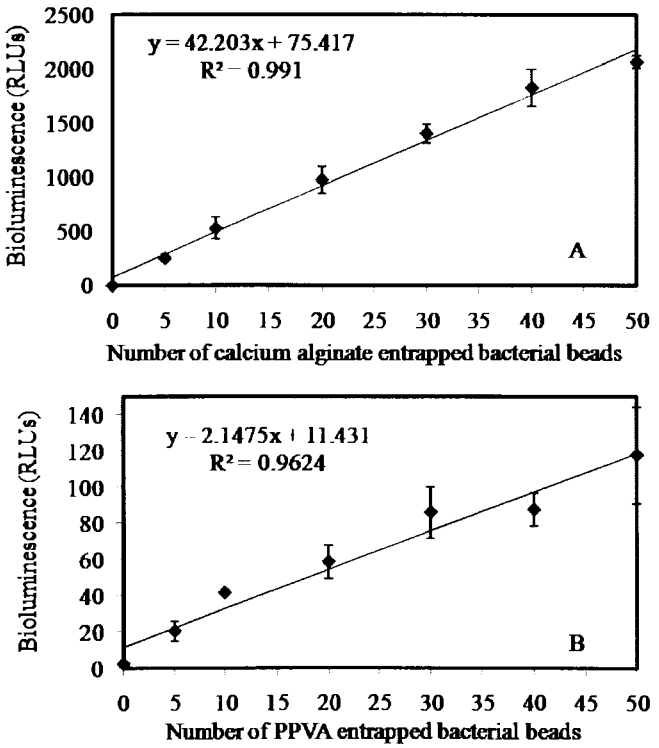

Figure 19: RLUs (mean ± standard deviation) as a function of number of calcium alginate (A) and PPVA (B) entrapped bacterial beads

3.3.3. Plate count enumeration of bacteria in entrapment.

A linear correlation was observed between cfu/ml and the number of entrapped bacterial beads using the plate count (Figure 20A and B). Both of the entrapment media did not completely dissolve while de-entrapping the cells for estimating the cfu/ml. The

calcium alginate beads reduced in size and became slimier, while the PPVA beads went from being white, opaque and rigid to being transparent, aqueous and slimy.

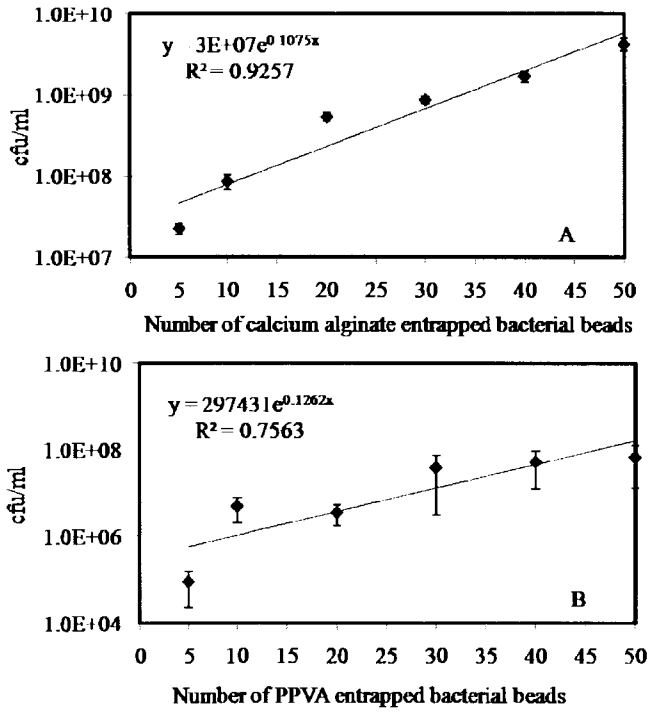

Figure 20: The cfu/ml (mean ± standard deviation) as a function of number of calcium alginate (A) and PPVA (B) entrapped bacterial beads

3.3.4. Validation of XTT and ATP assays with known ratios of live and dead entrapped cells.

For further validation of all the assays, both live and heat killed bacteria were entrapped separately, this was done for both of the entrapment media. Different ratios of the live and dead bacterial beads were evaluated using the three viability assays (Figure 21A-E). Each well of the 24-well plate contained a total of 50 beads, out of which 0%, 25%, 50%, 75% and 100% contained entrapped viable bacteria and the remainder contained entrapped heat killed bacteria. Good linear correlations were observed between the key assay indicator and the percentage of live bacterial beads ($r^2 > 0.88$) (Figure 21A-E). Table 3 shows statistical results for different assays and entrapment media. Based on

the statistical grouping, the ATP assay was the most sensitive and the plate count method was the least sensitive in quantifying viable cells entrapped in hydrogel.

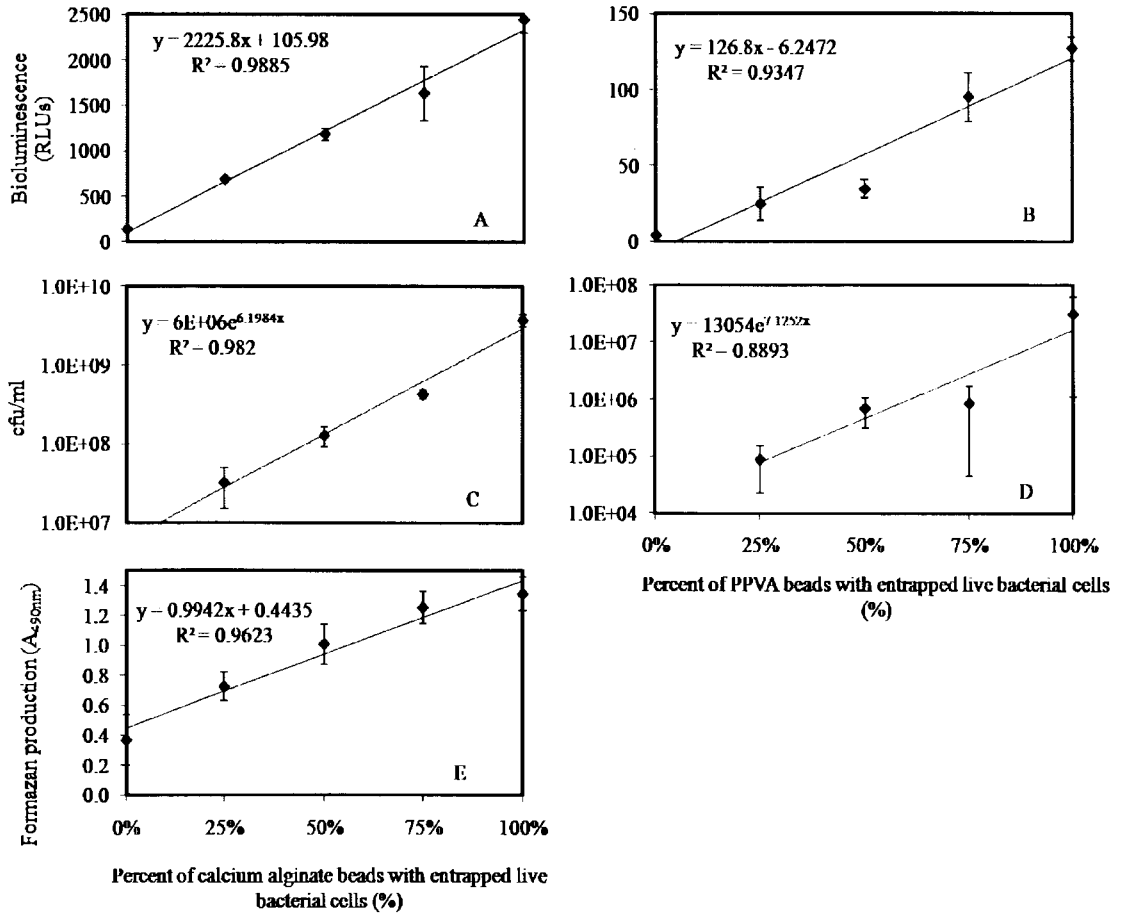

Figure 21: Bioluminescence (A and B), cfu/ml (C and D) and formazan production (E) (mean ± standard deviation) as a function of percentage of calcium alginate and PPVA beads with entrapped live bacterial cells

Table 3: Statistical grouping (*t*-test with Bonferroni adjustment) of number and percentage of live bacteria entrapped beads.

	PPVA beads				Calcium alginate beads					
No. of beads	**Plate count**		**ATP assay**		**Plate count**		**XTT assay**		**ATP assay**	
Bonferroni grouping[a]										
50	A		A		A		A		A	
40	A		A	B	B		A	B	A	B
30	A		A	B	B	C	A	B	C	B
20	A		C	B	B	C	C	B	C	D
10	A		C	B		C	C	D	E	D
5	A		C			C	E	D	E	F
0			C				E			F
Percent of live beads										
100%			A		A		A		A	
75%	A		B		B		A	B	B	
50%	A		C		B		A	B	B	C
25%	A		C	D	B		C	B	D	C
0%	A			D	B		C		D	

[a]To interpret the results in the table, choose the assay and entrapment media of interest and read the letters in the column(s) alphabetically. Different alphabets represent differences between the means of two or more values of the variables which in this case are the number or percentage of beads with a particular assay and entrapment media. Sharing the same alphabet indicates no significant difference (p-value > 0.05) in mean values of the variables.

3.3.5. Effect of de-entrapment procedure.

Sodium citrate, EDTA and heat (70°C) reduced the viability as determined by each assay. As shown in Figure 22A-C, heating at 70°C resulted in comparable or more reduction in bacterial viability when compared to the other two de-entrapment procedures. For the ATP assay (Figure 22A), the RLU values are less than half of the control (note logarithmic scale of y-axis) suggesting that all three de-entrapment processes have negative

effects on the RLUs and biomass. Formazan production decreased by 20% to 40% compared to the control (Figure 22B). The plate counts also showed a decrease of approximately 1 log cfu/ml (Figure 22C) in all treated samples but this was not significantly different from the control ($p = 0.2137$).

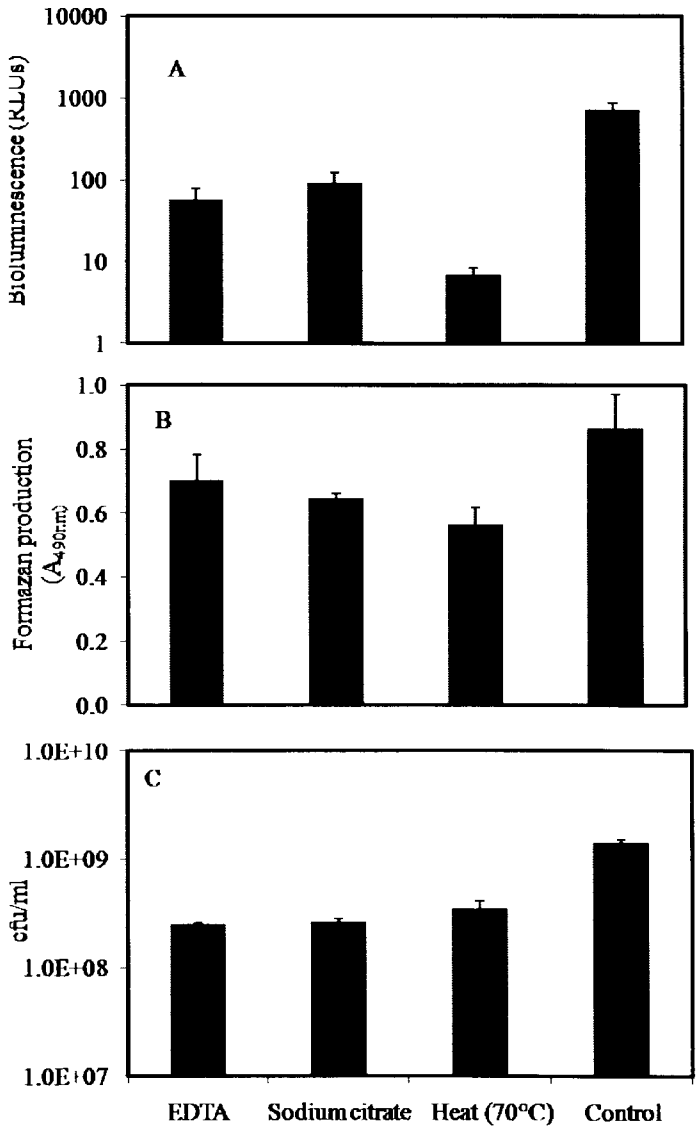

Figure 22: Effects of de-entrapment processes on bacterial viability based on XTT assay (A), ATP assay (B) and plate count method (C). All the values reported are mean ± standard deviation

3.4. Discussion

Several studies (Roslev and King 1993; Bensaid *et al.* 2000; McCluskey *et al.* 2005) have evaluated a higher concentration range (0.5 to 8 mM) of XTT to determine the optimum concentration used in their experiments. A lower concentration range of XTT (Figure 15) was evaluated in this research as higher concentrations have shown to be toxic towards certain bacterial populations (Hatzinger *et al.* 2003). The sigmoidal relationship between XTT concentration and formazan production in the present study (Figure 15) concurs with the results of Roslev and King 1993 and Bensaid *et al.* 2000.

XTT was preferred over other tetrazolium salts because incomplete solubility of MTT, INT and CTC can lead to an underestimation of viable cells, while XTT has shown good correlations with viable bacterial cells (Roslev and King 1993; Hatzinger *et al.* 2003; McCluskey *et al.* 2005; Mosmann 1983; Zimmermann *et al.* 1978). The data (Figure 16) show a strong relationship between the number of alginate beads containing entrapped bacteria and formazan production. However, the correlation between XTT and the number of PPVA beads containing entrapped bacteria was poor. Possible explanations for an underestimation of the bacterial viability in this study, particularly the poor performance of the assay for PPVA entrapped bacteria, include XTT diffusion limitation by the bead structure and/or retention of formazan in the beads.

Shishido and Toda 1996, simulated microbial substrate degradation and oxygen profiling in calcium alginate beads and concluded that gel matrix and mass transfer resistance do not affect substrate degradation. They found that smaller size beads were more effective in degrading substrate and there were no problems in substrate diffusion or degradation with a bead size of 2 mm. For PVA beads, a lower polymer concentration of

7% (w/w) and a smaller diameter (2.7 mm to 3.7 mm) have been recommended to avoid diffusion problems (Chen *et al.* 2003). A slight change in color of the calcium alginate beads (Figure 17B) and reduction in A_{490nm} of the bulk solution were observed when the beads were exposed to formazan. This was due to the high solubility of formazan and hydrogel characteristic of alginate. Exposing formazan to the PPVA beads changed the color of the beads (Figure 17D) and a drastic decrease in A_{490nm} of the bulk solution was observed. Hydroxides on PPVA make it a prolific water sorbent (Liu *et al.* 2009) while the negative charges on formazan make them water soluble. These two characteristics keep the produced formazan from coming into the bulk solution. The degree of formazan retention in the PPVA beads depends on surface charge, polymer concentration and bead size.

The optimal amount of ATP reagent to be used was determined to be 400 µl (Figure 18B). At this volume, the cellular ATP represented by RLUs relates strongly to the amount of live biomass entrapped in calcium alginate and PPVA (Figure 19A and B). The other two volumes, 100 µl and 200 µl, did not relate RLUs to the number of beads. In addition, the reagent volumes were a limiting factor in the luciferase reaction. The PPVA beads resulted in lower RLUs when compared to the alginate beads even though both were inoculated with the same number of bacteria. There were two possible reasons for this observation. Firstly, 400 µl of the reagent was insufficient for complete submergence of PPVA beads requiring it to be diluted with 600 µl of DW. This leads to dilution of the bioluminescence signal. Secondly, there might have been greater retention of the reagent in the PPVA beads than the calcium alginate beads.

Figures 17, 20A and B, and 21A-E demonstrate that plate counts, formazan production and ATP can all be related to entrapped bacterial populations. All the assays

show a linear relationship with increasing number of beads containing entrapped bacteria. The statistical analysis results (Table 3) help in determining whether the assays studied would be able to differentiate the signals produced by different numbers of beads. For example, the ATP assay showed the greatest sensitivity and resolution when quantifying the viability of entrapped bacteria. With the ATP assay, it was possible to differentiate intra groupings of 10, 30, 50 and 5, 20, 40 calcium alginate beads, while the plate count method was only able to distinguish among 5, 40 and 50 calcium alginate beads.

A negative effect of the de-entrapment procedures on microbial viability was observed (Fig. 22A-E). RLUs, formazan production and plate counts were reduced because of the de-entrapment processes. The chelating agents and high temperatures ($\geq$ 60°C) have been shown to affect the viability of bacteria by disrupting the cellular membrane (Haque and Russell 1974; Groh *et al.* 1996). While using the ATP assay, it was found that the incomplete removal of EDTA or sodium citrate resulted in lower RLU readings. Mg^{2+}, which is essential for catalysis by luciferase in the ATP assay, is chelated by EDTA, lowering the bioluminescence signal.

The linear regression equations for the ATP assay show high R^2 and the steep slopes of the regression lines suggest high sensitivity. The ATP assay showed excellent resolution with small changes in the number of beads or percentage of beads with viable bacteria resulting in a detectable change in RLUs. This was not observed for the XTT assay. Table 4 compares the key features of all three viability assays. The total detection time for the ATP assay is only 5 min, while the XTT and the plate count methods take a few and several hours respectively. It should be noted that 5 min was determined to be sufficient for a substrate to diffuse into the bead (Aslani and Kennedy 1996; Favre *et al.*

2001; Garbayo *et al.* 2002). The incubation time and sensitivity of the ATP assay make it a suitable tool for tracking the viability of entrapped bacteria without having to dissolve the matrix.

Table 4: Comparison of key features of the three viability assays studied.

Viability assay	Entrapment Media	Cellular response Measurement	Total assay time[a]	Units
XTT assay	CA	Detection of respiration	5-6 hrs	O.D.(A_{490nm})
ATP assay	CA, PPVA	Bioluminescence or ATP	5-15 min	RLU or nM
Plate count method	CA, PPVA	Bacterial colonies	12-15 hrs	cfu/ml

[a]Total assay time including incubation, chemical preparation and measurement time.

3.5. Summary

This study investigates the XTT and ATP assays for rapid in situ viability enumeration of hydrogel entrapped bacteria. There are good linear correlations between the indicators (ATP content and XTT reduction/formazan production) of the two assays explored and the plate count for entrapped bacteria. The ATP assay works for both types of entrapment media explored, while the XTT assay is applicable to only calcium alginate. The PPVA beads retain XTT and formazan resulting in the inapplicability of the XTT assay for viability assessment of the PPVA entrapped cells. RLUs and formazan production are established as in situ viability indicators for entrapped bacteria. Using the ATP assay, it is possible to calculate the amount of ATP per bead.

CHAPTER 4

A METHOD TO DETERMINE THE EFECT OF ENTRAPMENT PROCEDURES ON BACTERIAL CELL VIABILITY USING FLUORESCENT NUCLEIC ACID STAINING

4.1. Introduction

The microbial entrapment has applications in commercial and industrial wastewater treatment (Chen and Lin 1994; Fiol *et al.* 2006; Hameed 2007; Chen *et al.* 2003), biodegradation of toxic compounds (Siripattanakul *et al.* 2008), bioremediation, biosorption of heavy metals (Arica *et al.* 2001; Luan *et al.* 2006), microbial fuel cells, biohydrogen production (Wang *et al.* 2010a), fermentation (Najafpour *et al.* 2004) and metabolite production (Anisha and Prema 2008). Advantages of entrapped cells include protection from environmental stresses (pH and temperature) and enhancement of biological and physical stabilities of the microorganisms (Dervakos and Webb 1991; Charlet *et al.* 2000). In addition, relative to free cells, higher biomass concentrations and higher activity can also be achieved through cell entrapment (Dervakos and Webb 1991; Anisha and Prema 2008).

It has been reported that biotechnological processes by entrapped cells are better than free cells, due to the added protection (Yang *et al.* 1994). However, other studies suggest that cell viability may be reduced during the cell entrapment procedure (Yang *et al.* 1994; Chen and Lin 1994; Chang and Tseng 1998; Charlet *et al.* 2000; Li-seng *et al.* 2007). The effect of entrapment procedure on cell viability likely differs depending on techniques, procedures, and the matrix used for entrapment. Hydrogels are the most commonly used entrapment matrices; this includes carrageenan, PVA, and alginate.

There are several existing assays for the determination of cell viability. One of the most commonly used methods is the heterotrophic plate count. The standard plate count assay is a classical technique which depends on the capability of specific microorganisms to proliferate and form colonies on solid media. The assay is relatively simple but takes a significant amount of time (1-5 days). The standard plate count assay also has limitations for non-culturable, dormant, and inactive cells (Lahtinen *et al.* 2005). The plate count technique cannot be used to accurately determine the viability of entrapped cells because the cells must be de-entrapped before the application of the method. In all cases, the de-entrapment requires the addition of heat or chemicals which could affect the viability of the cells. For example, the de-entrapment of PPVA entrapped cells requires the bead to be heated to more than 70°C and calcium alginate bead de-entrapment requires an addition of acid or chelating agents. Using these approaches to study the effects of entrapment procedure on cell viability will result in inaccurate results as the de-entrapment will further reduce the viability. To be able to achieve a desirable cell number inside the hydrogel matrix it is important to develop a method which determines the effect of entrapment procedure on cell viability without further harming the cell.

A number of fluorescence-based assays have been developed to determine free and immobilized (biofilm) cell viability, including the commercially available LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Molecular Probes, OR, USA) (Kakimoto *et al.* 2007; Sankaran *et al.* 2008; Chae *et al.* 2009; Lee *et al.* 2009; Wang *et al.* 2010b; Sarkar *et al.* 2010). The principle of this approach is to use fluorescent dyes to differentially label live and dead cells. Viable and non-viable cells are then enumerated using a microscope with an epifluorescence attachment. The assay is sensitive and takes much less time (less than one

hour) compared to classical assays. The LIVE/DEAD® assay has been successfully used to differentially label and quantify live and dead bacterial cells for environmental applications (Quéric *et al.* 2004; Biggerstaff *et al.* 2006). Few studies have used this assay for entrapped cells by dissolving or dissecting the hydrogel (Charlet *et al.* 2000; Cunningham *et al.* 2004). This disruption could affect the viability of cells and could give incorrect results.

The primary objective of research described in this chapter was to develop a method to determine the effect of entrapment procedures on cell viability (without matrix dissolution) using the LIVE/DEAD® assay. The method development focused on the ability to quantify the viability of cells after the entrapment procedures, which are known to affect the survivability of cells (Chang and Tseng 1998; Charlet *et al.* 2000; Li-seng *et al.* 2007). PPVA and calcium alginate were chosen as entrapment matrices in this study since they are widely used in environmental applications. Both pure and mixed cultures were used in this study to examine the broader applicability of the developed method.

4.2. Materials and Methods

4.2.1. Chemicals.

PVA (99.0-99.8% fully hydrolyzed, molecular weight 77,000-79,000, J. T. Baker), sodium alginate (unspecified grade, Pfaltz and Bauer), and chemicals used for cell entrapment were obtained from VWR International Co., PA, USA. The bacterial viability kit used was the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit L7012 or L13152 (Molecular Probes, OR, USA).

4.2.2. Bacterial cultures and growth conditions.

E. coli K-12 JM109 (JM109), *Agrobacterium radiobacter* J14a (J14a), an atrazine-degrading mixed culture (ADMC), and a denitrifying mixed bacterial culture (DNMC)

were used in this study. These cultures were selected because of their use in other research activities in the Environmental Engineering Laboratory, North Dakota State University and represented a broad range of activities and origins (Hill and Khan 2008; Siripattanakul *et al.* 2008).

JM109 was from Promega, Inc. (Catalog number P9751, Madison, WI, USA). The strain was plated onto LB plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar) and incubated overnight at 37°C. A single colony from the overnight incubation was used to prepare a liquid culture of LB, which was also incubated overnight at 37°C but with constant shaking to aerate the bacteria. Ten milliliter of the overnight culture was centrifuged, the supernatant was discarded and the pellet was used for entrapment (described below).

J14a was obtained from the National Soil Tilth Laboratory, Ames, IA, USA. ADMC was enriched from atrazine contaminated soil collected from a field site in Oakes, ND, USA, following the procedure of Siripattanakul *et al.* (2008). J14a and ADMC were cultivated following Siripattanakul *et al.* (2008).

DNMC was acclimated from mixed liquor suspended solids obtained from the Moorhead Wastewater Treatment Facility, Moorhead, MN, USA. The MLSS was acclimated under anaerobic conditions with a hydraulic retention time of 1 day for 4 months. DNMC was acclimated and cultivated based on the procedure described by Hill and Khan (2008). The concentration of cells in each culture used in this study was approximately 4-5 log CFU/ml.

4.2.3. Experimental approach.

Typically, the final forms of PPVA and calcium alginate entrapped cell matrices are spherical beads. However, fluorescence microscopy based assays require samples which permit penetration of dye and light. A previously used method used to achieve this is by dissolving or thinly slicing the matrix (Charlet *et al.* 2000; Cunningham *et al.* 2004). However, both dissolving and slicing the bead could affect cell viability and/or can cause cell leaching, leading to a false result. Therefore, a modified entrapment and sample preparation method was developed for determining the viability and studying the effect of entrapment procedures on cells using the LIVE/DEAD® assay. This new method is compared to the traditional method. Here the traditional method is denoted as Method 1 which involves dissolution of the entrapment matrix while entrapping bacterial cells directly onto the slide is Method 2 (Figure 23).

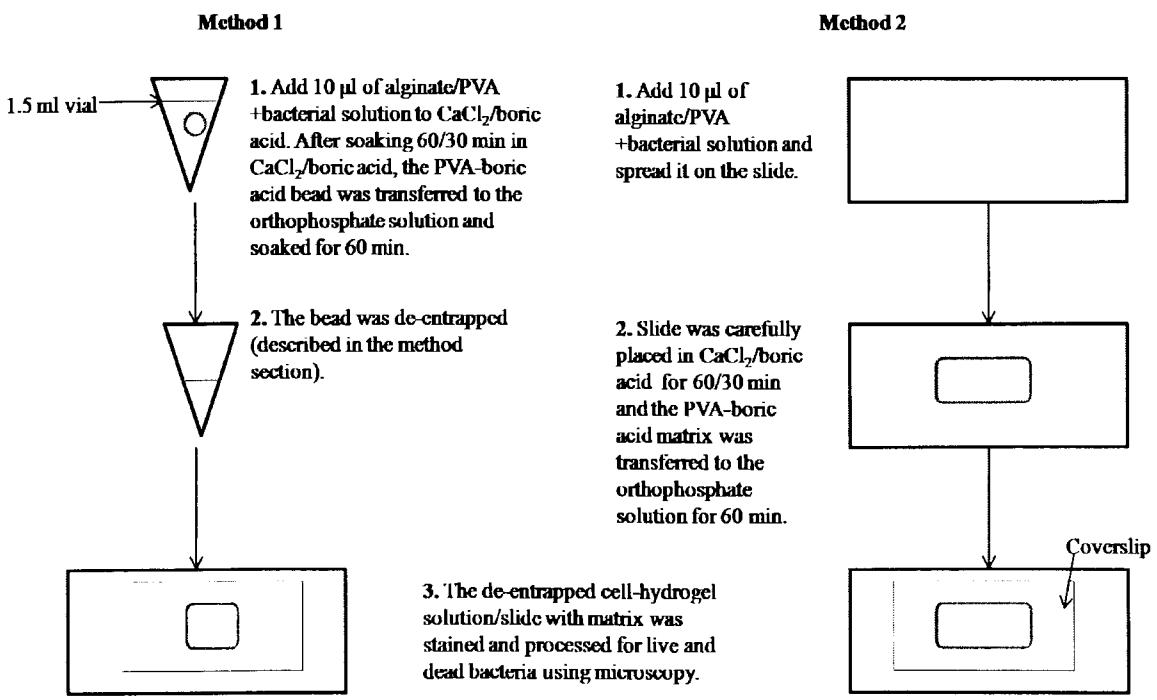

Figure 23: Flow diagram briefly explaining methods 1 and 2 used to determine viability of entrapped bacterial cells.

4.2.4. Cell entrapment and de-entrapment.

4.2.4.1. Method 1(Bead entrapment).

4.2.4.1.1. Calcium alginate entrapment.

The procedure of entrapping bacteria in calcium alginate was modified from the procedure by van Ginkel *et al.* (1983). Sodium alginate was dissolved at 80°C in DW until it formed a homogeneous 2 % (w/v) alginate solution. Bacterial pellets were prepared as described above and were homogeneously mixed in the alginate solution. Spherical beads were produced by dropping a mixture of a 10 µl of sodium alginate solution and the culture into a 3.5% (w/v) of calcium chloride solution using a 1 ml syringe. The beads were submerged for 60 min.

4.2.4.1.2. PPVA entrapment.

The procedure of entrapping bacteria in PPVA was based on the procedure by Siripattanakul *et al.* (2008). A 10% (w/v) aqueous PVA solution and the mixture of the culture and the solution were prepared in the same manner as described above for the calcium alginate entrapment. A spherical bead was produced by dropping10 µl of the PVA solution and culture into a saturated solution of boric acid using a 1 ml syringe. Finally, the bead was allowed to soak in a phosphate solution (1 M at pH 7) for 60 min for PVA phosphorylation.

4.2.4.1.3. De-entrapment procedures.

For dissolving the calcium alginate bead, 100 µl of 0.30 M sodium citrate prepared in a 0.15 M sodium chloride solution was added to bead and kept at 37°C for 20 min. For dissolving the PPVA bead, 100 µl of DW was added to the bead and incubated at 70°C for

15 min. Both, calcium alginate and PPVA bead were vigorously vortexed from time to time.

4.2.4.2. Method 2 (Slide entrapment).

4.2.4.2.1. Calcium alginate entrapment on slide.

One drop (~10 µl) of the sodium alginate and bacterial solution (prepared as described above in section *4.2.4.1.1*) was placed on a microscope slide and spread into a thin layer, approximately 500 µm in thickness using a micropipette tip. The microscope slide was submerged in a 3.5% (w/v) $CaCl_2$ solution for 60 min.

4.2.4.2.2. PPVA entrapment on slide.

One drop (~10 µl) of the PVA and bacterial solution (prepared as described above in section *4.2.4.1.1*) was placed on a microscope slide and spread into a thin layer, approximately 500 µm in thickness. The microscope slide was submerged in saturated boric acid for 30 min for PVA-boron cross-linking. Finally, the slide was allowed to soak in a phosphate solution (1 M at pH 7) for 60 min for PVA phosphorylation.

4.2.5. LIVE/DEAD® staining and viability determination.

The staining method was based on the manufacturer's instructions for the *Bac*Light™ LIVE/DEAD® kit (L7012 or L13152, Molecular Probe, OR, USA). The kit employs two nucleic acid stains, which can be used to differentiate between live and dead cells. SYTO®9 is a green fluorescent dye which freely enters both live and dead cells. PI only enters dead cells as it requires a damaged cell membrane for entry. PI replaces SYTO®9 in dead cells with the result that live cells fluoresce green and dead cells fluoresce red. The viability of cells was determined by enumerating the live and dead cells using a

microscope (BX61™, Olympus, PA, USA) with an epifluorescence attachment. In our study, live and dead cells were enumerated in 20 microscopic fields at 60× or 1000× magnification. SYTO®9 and PI excitation wavelengths were 480 nm and 535 nm respectively. Emission wavelengths were 535 (green) and 610 (red) nm, respectively.

Using the assay described above, the extent to which spectral overlap occurred when using each filter set with SYTO®9 and PI was determined. Then the optimal dye ratio to use in the assay was determined. The LIVE/DEAD® assay was used to study the effect of method 1 and the results were compared to method 2. This was done only for JM109 with three different ratios of live and dead bacterial cultures (1:0; 1:1; 0:1). Finally, the usefulness of method 2 with the LIVE/DEAD® assay when applied to other entrapped cultures was examined.

4.2.5.1. Assessment of spectral overlap.

The extent of spectral overlap was determined using a heat killed suspension of JM109 or J14a. The suspension was killed by heating to 121°C for 15 min. Heat killed cells were labeled with SYTO®9 alone, PI alone, and a 1:1 ratio of SYTO®9:PI (this last step was in accordance with the manufacturer's instructions). Each sample of labeled cells was viewed using SYTO®9 and PI specific filter sets.

4.2.5.2. Effect of dye ratios on labeling.

The kit manufacturer recommends a 1:1 ratio of dyes; however, they suggest that a different dye ratio may be more effective. The ratio of dyes used in the assay may potentially impact the assessment of cell viability. For example, all live cells may not fluoresce with a 1:1 ratio of SYTO®9 to PI leading to underestimation of viability. In this

case, the optimal dye ratio may be 2:1. Three different ratios of dye combinations, SYTO®9: PI of 1:1, 1:2, and 2:1, were tested using JM109 and J14a suspensions.

4.2.5.3. Application of the LIVE/DEAD® assay to entrapped cells.

The viabilities of four bacterial cultures (JM109, J14a, ADMC, and DNMC) were tested. The viability of JM109 was evaluated using both methods (1 and 2) and both entrapment media. J14a and ADMC were entrapped in PPVA, and DNMC was entrapped in calcium alginate. They were evaluated using method 2. Free cells of all three cultures were also tested for viability comparisons before and after entrapment. Live and dead cells of all samples were counted using the protocol described earlier. The viabilities of JM109, J14a and ADMC were tested twice to ensure the reproducibility of the procedure.

4.3. Results and Discussion

4.3.1. Assessment of spectral overlap.

The heat killed J14a or JM109 culture was stained with each dye individually and with the combined dyes to determine the extent of fluorescence overlap between filters. Figure 24A shows the result of a sample stained only with SYTO®9 and viewed under the green filter. As expected, cells appeared fluorescent green under the green filter, but did not appear under the red filter (data not shown). Similarly, Figure 24B shows the result of heat killed cells stained only with PI and viewed under the red filter. The heat killed cells stained with PI fluoresced under the red filter but not the green filter (data not shown). When both SYTO®9 and PI were used at a 1:1 ratio, the heat killed J14a cells appeared red under the red filter and were not visible under the green filter (Figure 24C and Figure 24D). This demonstrates that no spectral overlap occurs between filters.

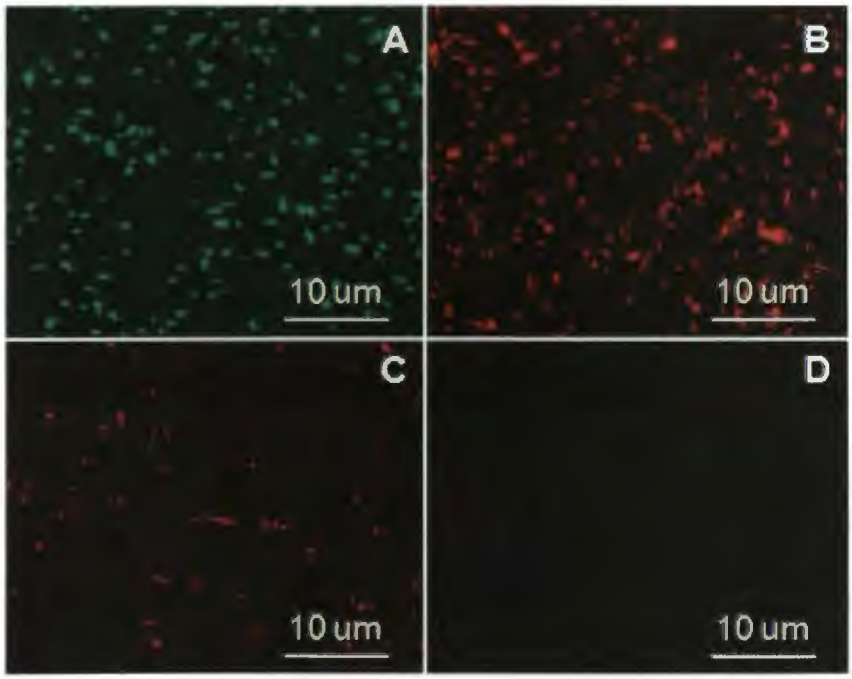

Figure 24: Images of dead J14a suspension stained with different dyes: A) only SYTO®9 using the green filter; B) only PI using red filter; C) both SYTO®9 and PI using the red filter; D) both SYTO®9 and PI using the green filter (C and D are from the same frame).

4.3.2. Effect of dye ratios on labeling.

The results of the 2:1 and 1:2 ratios of SYTO®9 to PI did not differ from that of the 1:1 ratio (data not shown). Therefore, a 1:1 ratio was used for the rest of this study (free DNMC and ADMC, entrapped J14a, DNMC, and ADMC) and provided satisfactory results without a need to conduct the dye adjustment experiments.

4.3.3. Application of the LIVE/DEAD® assay to entrapped cells.

4.3.3.1. Cell staining.

Prior to the testing of methods 1 and 2, there was a concern that the entrapment matrices in method 2 would interfere with the assay, either by preventing fluorescent dyes and light from penetrating the target cells or by contributing to background autofluorescence. Figure 25 shows that while some autofluorescence did occur, the bacteria

could be easily differentiated from the entrapment matrices, enabling viability determination. For Method 1, no background fluorescence was observed as matrix was dissolved (data not shown). For Method 2, the staining results of the entrapped bacteria are shown in Figure 25. Figure 25A and Figure 25B (from the same frame) show the results of the DNMC entrapped in calcium alginate under the green (FITC) and red (TRITC) filters, respectively. Figure 25C and Figure 25D (from the same frame) show the ADMC entrapped in PPVA under the green and red filters, respectively. Background fluorescence was present in both matrices; however, target bacteria were easily differentiated from this background noise. Images similar to those of Figure 25C and Figure 25D were obtained for J14a entrapped in PPVA and JM109 entrapped in calcium alginate and PPVA (data not shown).

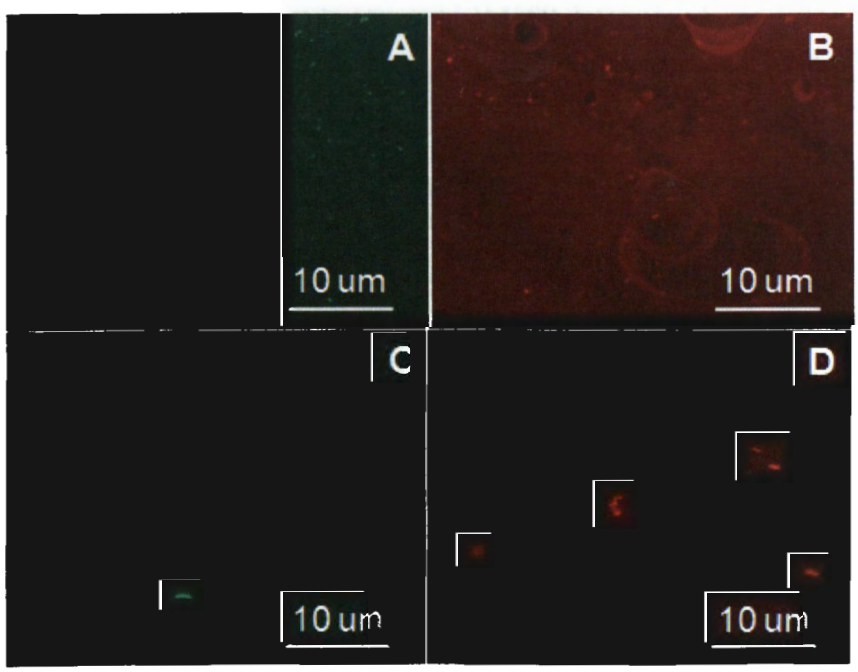

Figure 25: Images of bacterial cells entrapped in calcium alginate and PPVA. A) Cells in calcium alginate matrix using FITC filter; B) Cells in calcium alginate matrix using TRITC filter; C) Cells in PPVA matrix using FITC filter; D) Cells in PPVA matrix using TRITC filter (A and B, and C and D are from the same frames, respectively).

4.3.3.2. Cell quantification.

Different ratios of live and dead cells of JM109 were entrapped and the cell viability was estimated. Only live JM109 cells were used to evaluate the two methods. Table 5 shows percentage of live and dead cells obtained from estimating the viability of JM109 cells (only live cells in the matrices) using both methods and both entrapment media. The use of method 1 showed reduction in the viability of cells when compared to the use of method 2. To further evaluate and confirm the results, a different ratio of live to dead JM109 cells was used. Figure 26 also shows reduction in the percentage of live JM109 cells (ratio of live:dead cells entrapped was 1:1) for both methods and both entrapment media when compared to the free culture. This result was similar to the data reported in Table 5.

Table 5: Ratios of live and dead entrapped JM109 determined using two different methods.

Matrix	Cell viability	Method 1	Method 2
Calcium alginate	Live cells	43±4	51±5
	Dead cells	56±4	48±5
PPVA	Live cells	39±4	50±4
	Dead cells	60±4	49±4

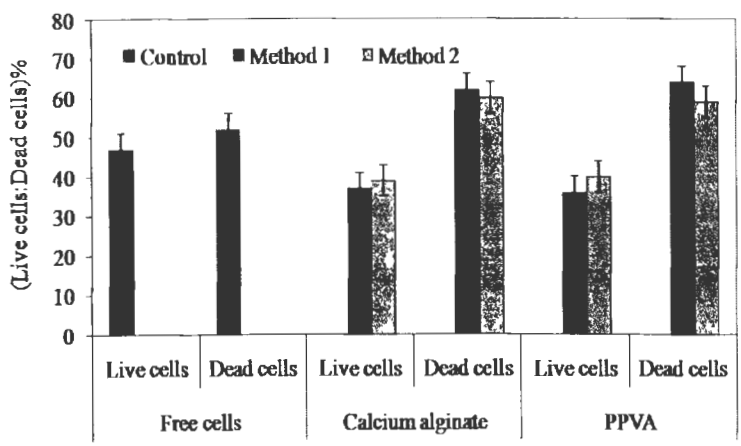

Figure 26: Effects of methods 1 and 2 and entrapment procedure on the percentage of live and dead JM109 is evaluated. Ratio of live to dead cells entrapped was 1:1.

After confirming the results, the effects of entrapment on the viability of the remaining bacterial cultures (J14a, ADMC, and DNMC) were also evaluated using only method 2. The results of cell quantification before and after the entrapment in PPVA and calcium alginate matrices for these bacterial cultures are detailed in Table 6. The ratio of live bacterial cells before the entrapment ranged from 54 to 74%, while the ratio of live cells after the entrapment was 39 to 62%. As seen in Tables 5 and 6, for viability determination, approximately half of the bacterial population before the entrapment was identified as dead. This was expected since the samples were collected at the late exponential growth phase. As stated earlier, the entrapped cell samples had less live cell portions compared to the cells before entrapment.

Table 6: Ratio of live and dead cells using Method 2.

Culture	**Sample description**	**Ratio (%)**	
		Live cells	**Dead cells**
JM109	Before entrapment	65±6	35±6
	After entrapping in calcium alginate	51±5	48±5
	After entrapping in PPVA	50±4	49±4
J14a	Before entrapping in PPVA	54±5	46±5
	After entrapping in PPVA	39±4	61±4
ADMC	Before entrapping in PPVA	63±5	37±5
	After entrapping in PPVA	41±3	59±3
DNMC	Before entrapping in calcium alginate	74	26
	After entrapping in calcium alginate	62	38

All the results indicate that both methods reduced bacterial viability when compared to free culture. This effect was larger for Method 1. This suggests that Method 1 led to an underestimation of the percent of live entrapped bacteria. Reduction in viability can be due to the entrapment and de-entrapment processes. The live cell loss due to the entrapment could be for several reasons such as entrapment chemical exposure or physical stress from entrapment conditions. The saturated boric acid used in the PPVA entrapment

procedure is toxic to the cells and calcium chloride and alginate also reduces their viability (Chang and Tseng 1998; Charlet *et al.* 2000; Li-seng *et al.* 2007). There is more reduction in cell viability when using Method 1 (Table 5 and Figure 26), which could be due to the reduction in cell viability due to the de-entrapment procedures. Both sodium citrate and heat (70°C) used for calcium alginate and PPVA dissolution, respectively, are known to reduce cell viability (Haque and Russell 1974; Groh *et al.* 1996).

4.4. Summary

Currently used methods for quantifying the effect of cell entrapment procedures on cell viability involve de-entrapment of the cells by dissolving the hydrogel (for estimating cell viability). Using these methods may underestimate live bacteria as the de-entrapment can further reduce the viability. Achieving a desirable cell number inside the hydrogel matrix is important for entrapped cell applications. To be able to do so a method which accurately determines the effect of entrapment procedure on cell viability without further harming the cell is needed. A method for applying the commercially available LIVE/DEAD® assay to determine the effect of two entrapment procedures on bacterial cell viability was successfully developed. This new method did not require the de-entrapment of the cells from the matrix. The method was applied to pure and mixed cultures entrapped in calcium alginate and PPVA. The percent of live bacterial cells before the entrapment ranged from 54 to 74%, while the percent of live cells following the entrapment based on the new method was 39 to 62%. This new method can be used for optimizing entrapment procedures.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

This thesis research work was divided into two parts. The first part dealt with an investigation of the XTT and the ATP assays for rapid in situ viability enumeration of hydrogel entrapped bacteria. Results from the XTT and ATP assays were compared to the traditional plate count method and showed that the XTT and ATP assays are suitable for estimating the number of entrapped cells without having to dissolve the hydrogel. The ATP assay is more rapid and less laborious than the XTT and plate count techniques. All three indicating parameters corresponding to the methods assessed (ATP content, microbial activity and colony forming ability) are essential, and their information is complementary and will help in further understanding bacterial growth and metabolism in entrapped media.

The XTT and ATP assays should be further tested for their applicability under different conditions such as entrapped mixed cultures. A future investigation of the applicability of the XTT and the ATP assays on different sizes of beads, matrix type and types of cells entrapped is recommended. The ATP and XTT assays should be used to evaluate the effects of entrapment procedure on cell viability. An adsorption study of substrate (Luciferin) and enzyme (Luciferase), from the ATP assay, onto PPVA and alginate beads should be carried out to determine the degree of cell number underestimation (due to the adsorption). More specific viability estimation indicators such as messenger ribonucleic acids should be investigated for their applicability to entrapped cells.

The second part of this work involved the development of a method to study the effect of hydrogel entrapment procedures on cell viability. The method developed involved

entrapping cells onto a slide followed by staining with the LIVE/DEAD® assay. This method was compared to a traditional method which requires dissolution of the entrapping matrix and then staining with the LIVE/DEAD® assay. The advantages of the new method developed to study the effect of entrapment are that it is a simple and rapid with no need for de-entrapment. The method could potentially be used for other entrapment matrices not tested in our study. A slight background due to the staining of the hydrogel matrix is observed while using the LIVE/DEAD® assay. Washing the stained hydrogel with DW should be experimented to overcome this problem.

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