It has been known for more than a century that biological nitrogen fixation is one of the means of introducing the critical element nitrogen into agricultural ecosystems. In the past two decades, there has been a tremendous upsurge in research for the development of new technologies relating to the agronomic, genetic and biochemical aspects of nitrogen fixation. The technique of immobilization was one of the most exciting aspects of biotechnology during the 1970's and it is now maturing into an established technology.

In early 1970's most of the research was centered around immobilization of enzymes. Later on cell immobilization was also developed from the experience gained with enzyme immobilization. Immobilization is a technique which confines a catalytically active enzyme or cell within a reactor system and prevents its entry into the mobile phase which carries the substrate and the product (Rosevear, 1984).

Methods of immobilization

There are four important procedures available for cell and enzyme immobilization, viz., adsorption, entrapment in gels or polymers, covalent coupling and cross linking to insoluble matrices.

Adsorption

Adsorption of an enzyme or cell to a polymer represents the earliest method of immobilization. A range of non-specific or specific bonding forces from simple van der waal's forces to strong ionic bonding are employed. This method has a number of advantages such as the ease and mild ions of preparation of the immobilized material, potential for regeneration of the immobilized catalysts and minimizing leakage of the enzyme during use. However, this technique has its disadvantages such as leaching of enzyme or
cells relatively easily with changes in pH or raised ionic strength (Trevan et al., 1988).

Example of suitable adsorbents are (Chaplin and Bucke, 1990):

1. Ion-exchange matrices
2. Porous carbon
3. Clay
4. Hydrous metal oxides
5. Glasses
6. Polymeric aromatic resins.

Entrapment

Entrapment of biocatalysts within a polymer matrix is in principle easy to perform. The biocatalyst is dissolved in a solution of the polymers precursors and polymerization initiated. Two types of polymers have found wide application viz., polyacrylamide type gels and naturally derived gel materials such as cellulose triacetate, agar, gelatin, carrageenan and alginate. The advantages of this method are the simplicity and mild conditions used in the preparation of the immobilized biocatalyst (Trevan et al., 1988).

Polyacrylamide gels suffer the drawback that the monomers from which they are formed and the free radicals generated during polymerization are toxic (Rosevear, 1984).

Agar gels have been applied in the entrapment of chloroplasts and cyanobacteria (Karube et al., 1981). However, agar has large pore sizes allowing quite sizeable (< 10 µ) cells to escape easily; in addition they are depolymerized by mild heat and are fragile to handle (Trevan et al., 1988).
Alginate is a colourless polymer of mannuronic acid and guluronic acid that is isolated from several species of seaweeds. Its sodium salt is soluble in water and forms a viscous fluid but calcium is water insoluble so that the addition of a solution of sodium alginate to a CaCl₂ solution will precipitate calcium alginate. The three dimensional gel network of calcium alginate is biochemically inert and most of the internal volume of the gel is accessible to diffusion by aqueous reagents. The cytotoxicity is very low (Cheetham et al., 1979). Although calcium is the most commonly used ion, alginate can form strong gels with iron³⁺, aluminium and barium (Rosevear et al., 1984). Calcium alginate gels are elastic and less prone than other gels to fracture due to internal pressure (Rosevear et al., 1984). They have been used for immobilizing a vast number of particulate biocatalysts, including fragile plant cells (Brodelius et al., 1979) and microbial cells (Cheetham et al., 1979).

One of the success stories in immobilization of photosynthetic organisms, particularly with algae and cyanobacteria, is that of polyurethane matrices. Polyurethanes are a class of synthetic polymers used for the manufacture of foams with varied industrial and domestic applications. Urethane is formed by the reaction between an isocyanate and a hydroxyl group

\[
\text{RNCO} + \text{R'OH} \rightarrow \text{RNHCOOR'}
\]

Condensation of the urethanes with other isocyanates, alcohols or amines produces a crosslinked polymer. The isocyanates may be alkyl or aryl—the most commonly used are toluene diisocyanate (TDI) and diphenylmethane diisocyanate (MDI). The hydroxyl compounds usually possess polyether or polyester linkages. During hydrolytic condensations of urethanes to form the polymer, CO₂ gas is evolved and this CO₂ is trapped inside the polymer. By
varying the temperature, prepolymer structure, condensation reagent, etc., polyurethane foams of different porosity, strength and translucency can be produced (Rao and Hall, 1984).

Carrageenan is a marine polysaccharide which has proved useful for cell entrapment. It gels in the presence of alkali metal salts, amines or solvent. Most techniques involve the use of 0.3 M potassium chloride as gelling agent. Unfortunately the gel is too open in structure (Rosevear, 1984).

Covalent coupling

The technique of covalent coupling is the creation of permanent chemical bonds between the cells or enzymes and a solid matrix and this technique has been extensively used in the immobilization of enzymes (Rosevear, 1984). The outer surface of cells contain a variety of reactive groups such as hydroxyl, aldehyde, ketone, carboxyl, amino and various substituted aromatic rings. There is a great potential therefore for the creation of covalent bonds with suitable activated carriers. The most commonly used coupling agents are glutaraldehyde, carbodiimide, isocyanate and aminosilane. The great advantage of covalent linkage of enzymes to a support is the permanence of the bond. This ensures that the protein is firmly fixed under all operational conditions, and although highly accessible at the surface of the support, it will not cause contamination of the product (D'Souza, 1989).

Crosslinking

Crosslinking of the enzyme to itself by reaction with a bifunctional reagent, with the possible inclusion of an inert protein is essentially an extension of covalent bonding techniques with all the potential advantages and
problems that this brings. The bifunctional reagent most often used is glutaraldehyde. The technique is cheap and simple but is not often used with pure proteins because it yields very little bulk of immobilized enzyme with a very high intrinsic activity. It is however, widely used in commercial preparations of immobilized enzymes derived from non-viable cells or crude cell extracts, largely because of its low cost (Trevan et al., 1988).

**Effects of immobilization.**

Although there have been relatively few systematic studies, immobilization almost certainly affects the cell physiology and biochemistry which may be either desirable or detrimental depending on the anticipated uses of the catalyst.

**Morphology**

Studies using microscopes tend to show that immobilization has little effect upon the morphology of the algal cells (Brouers et al., 1982; Chevalier and Dela Nove, 1985; Musgrave et al., 1983 and Bailliez et al., 1985). However, immobilized colonies of *Botryococcus* were found to be more regular in shape and to have mean shape diameters 2.5 times those of free cell controls (Bailliez et al., 1986).

Scanning electron microscope studies showed that the foam immobilized cyanobacteria were entrapped inside the pores and adhered closely to the foam matrix. A more detailed examination of *A. azollae* immobilized in polymer foams and in its natural symbiotic niche in *Azolla* using the frozen hydrated SEM technique indicated morphological similarities between the immobilized cells and cells living in symbiotic association (Shi et al., 1987). In both cases a thick mucilage layer covered the cell surface.
Growth

First, it is clear that all of the immobilization techniques described maintained the viability of at least some of the inoculated cells. In studies adopting alginate entrapment, growth of this inoculum has been measured directly by deliberate disruption of the alginate matrix with metal-ion chelators (e.g., phosphate or citrate) and enumeration of released cells using a haemocytometer (Bailliez et al., 1985) disrupted carrageenan gel in 0.9% NaCl at 45°C and directly counted released cells on a Malassez chamber. These direct estimates of cell growth generally suggest that the growth rate of immobilized cells is lower than that of free cells (Bailliez et al., 1985 and Robinson et al., 1985). Distribution of Chlorella cells within calcium alginate beads is limited to the periphery of the beads in many cases (Dainty et al., 1986; Robinson et al., 1985; and Day and Codd, 1985). In working with matrices that cannot easily be disrupted, a variety of indirect estimates of cell growth have been adopted, including chlorophyll content and photosynthetic rate. The chlorophyll content of immobilized cells generally has been found to be higher than that of free cells, possibly as an adaption to self-shading (Robinson et al., 1986). Immobilization of Anabaena azollae and Anabaena variabilis in all the solid matrices such as hollow fibre, polyurethane foam, cotton and silk cotton have resulted in more biomass production under immobilized state than under free-living state (Mahesh and Kannaiyan, 1993). They have suggested that it may be due to the mechanical support provided by the matrices.

Physiology

There have been very few reports on the photosynthetic evolution of oxygen by immobilized algae. The thermophilic cyanobacterium Mastigocladius


*laminosus* for example, has been successfully immobilized on an SnO$_2$ optically transparent electrode, which exhibited both Photosystem I and Photosystem II activities in its intact state, and functioned as a 'living electrode' catalyzing the photodecomposing of water and producing a steady electrical current for 20 days (Ochiai *et al.*, 1980).

Brouers *et al.*, (1982) have showed unchanged photosynthetic oxygen evolution for 25 days in *Chlorella* and *Scenedesmus obliquus* after immobilization in urethane prepolymer. It has also been reported recently that undiminished capacities for photosynthetic oxygen evolution was obtained over a six month period after the immobilization of *Chlorella emersonii* by entrapment in calcium alginate gel (Day and Codd, 1985). An alginate matrix conserved the photosynthetic activity and fluorescence characteristics of the green alga *Scenedesmus obliquus* (Jeanfils and Collard, 1983); chloroplasts (Jeanfils *et al.*, 1986) and the cyanobacterium *Phormidium laminosum* (Brouers and Hall, 1985).

Oxygen evolution was greater in the immobilized state suggesting a fundamental change of metabolism. Immobilization enhances the stability of protein-chlorophyll complexes in *Botryococcus* (Bailliez *et al.*, 1986) and *Euglena* (Tamponnet *et al.*, 1985).

The induction kinetics of variable fluorescence of immobilized *Porphyridium cruentum* cells in a urethane prepolymer showed the same characteristics as observed with free-living cells and photosynthetic oxygen evolution was the same for both free-living and immobilized cells (Thepenier *et al.*, 1985). Robinson *et al.*, (1985) found the mean respiratory rate of entrapped *Chlorella* to be lower than that of free cells.
Enzymes of nitrogen metabolism

Nitrogenase

Cyanobacteria are unique organisms which possess peculiar differentiated cells called heterocysts and there is evidence that under aerobic conditions, these heterocysts are the major sites of $N_2$ fixation with light as the sole source of energy and water as the ultimate reductant (Fogg et al., 1973). Heterocysts do not fix $CO_2$ or carry out photolysis of $H_2O$, but they house photosystem I of photosynthesis and the nitrogenase enzyme complex (Tel-Or and Stewart, 1977).

The conversion of $N_2$ into ammonia is catalyzed by the enzyme complex nitrogenase. It consists of two oxygen sensitive subunits - a larger Fe protein having a molecular weight of 2,20,000 daltons and a smaller Mo-Fe protein having a molecular weight of 50,000 - 60,000 daltons. The enzyme catalyses the reduction of various substrates including $N_2$, protons, cyanide, nitrous oxide, acetylene and amide. All cyanobacterial nitrogenases require ATP, reductant and $Mg^{2+}$ (Stewart, 1980).

Nitrogenase activity of *Anabaena azollae* assayed as acetylene reduction was always higher in immobilized than in free-living cells; after 50 hrs continuous illumination the total amount of $C_2H_2$ reduced by freshly immobilized cells was six fold greater than the amount reduced by free-living *A. azollae*. Even after 40 days of immobilization in alginate beads or in polyvinyl foam the nitrogenase activity was still high whereas at that time no activity could be detected in the free-living controls (Shi et al., 1987). The heterocyst frequency increased relative to free-living cells, reaching a level after 10-14 days of 14-17% and thus approaching that of cells in the symbiotic association (Shi et al., 1987) Heterocyst frequency and nitrogenase activity were examined in a
cyanobiont *Nostoc* ANTH and its L-methionine -DL-sulphoximine resistant mutant (MSXr) immobilized in polyvinyl foam, polyurethane foam or calcium alginate and compared to control. Immobilization of the cells in all matrices increased heterocyst frequency and nitrogenase activity in both strains, with maximum being in polyvinyl foam (Singh, 1993). Mahesh and Kannaiyan (1993) have reported increased heterocyst frequency in *Anabaena azollae* and *Anabaena variabilis* of 5 week old culture under immobilized state and they have suggested that this may be due to the accumulation and congregation of the algal cells inside the pores. They have also suggested high nitrogenase activity under immobilized state which may be due to higher heterocyst frequency under immobilized state.

**Glutamine synthetase**

It has been well established that the most important route of ammonia assimilation in N₂ fixing cyanobacteria involves glutamine synthetase and glutamine oxoglutarate amino transferase (GS-GOGAT) pathway (Stewart *et al.*, 1975; Meeks *et al.*, 1978). Alternate ammonia assimilatory pathways employing either glutamate dehydrogenase or alanine dehydrogenase have been shown to be absent or of minor physiological significance in cyanobacteria (Meeks *et al.*, 1977). The conversion of glutamate to glutamine, catalyzed by glutamine synthetase, is a highly significant step in nitrogen metabolism, since glutamine can serve as nitrogen donor in the biosynthesis of many metabolites (Stacey *et al.*, 1979). This enzyme has been studied using crude extracts and partially purified preparations from *Anabaena cylindrica* and a divalent cation requirement satisfied by Mg²⁺, Mn²⁺, Ca²⁺, Co²⁺ and Zn²⁺ but not Cu²⁺ or Ba²⁺ with a pH optimum of 7.2 and temperature optimum near 37°C. (Stewart *et al.*, 1975)
Stacey et al. (1977) have purified glutamine synthetase from the cyanobacterium *Anabaena* sp. CA. The highest specific activities were obtained from cells grown in the presence of atmospheric N$_2$ or KNO$_3$; when ammonium chloride was used as the nitrogen source, the specific activity was reduced by approximately 40%. Stacey et al., (1979) have further examined the catalytic and regulatory properties of the enzyme purified from *Anabaena* sp. CA. The enzyme showed a marked specificity for Mg$^{2+}$ in the biosynthetic assay and Mn$^{2+}$ in the transferase assay. They have also suggested a variety of regulatory mechanisms that control glutamine synthetase activity in cyanobacteria.

They include

a. repression and derepression of enzyme synthesis by alterations in the nitrogen supply to the cell,

b. modulation of enzyme activity in response to divalent cations,

c. feed back inhibition by end products of glutamine metabolism,

d. covalent modification by adenylation and

e. energy charge.

**Nitrate reductase**

Reduction of nitrate to ammonium in cyanobacteria is catalyzed by nitrate assimilating enzymes. It takes place in two successive steps. First, nitrate is reduced to nitrite catalyzed by the enzyme nitrate reductase. The resulting nitrite is then reduced to ammonium catalyzed by nitrite reductase.

Nitrate reductase from the unicellular cyanobacterium *Anacystis nidulans* has been purified to homogeneity and characterized as a protein with a molecular weight of 75,000 daltons, having only one polypeptide chain and exhibiting a Km value for nitrate of 0.7 mM (Candau, 1979). Molybdenum plays an essential role in the catalytic activity (Candau, 1979; Peschek, 1979).
Nitrate reductase from all cyanobacteria studied to date depend on reduced ferredoxin as the immediate physiological electron donor (Hattori and Myers, 1966; Hattori and Myers, 1967; Manzano et al., 1976; Ortega et al., 1976; Mendez et al., 1981).

Nitrate reductase synthesis in cyanobacteria is markedly influenced by the nitrogen source available in the external medium (Herrero et al., 1981; 1985). It has been shown that the activity of nitrate reductase is reduced in the absence of a source of combined nitrogen (Herrero et al., 1984).

Nitrite reductase

Nitrite reductase of cyanobacteria contains sirohaem and an iron-sulphur center, uses reduced ferredoxin as the electron donor and it is partially membrane associated (Hattori and Uesugi, 1968; Peschek, 1979; Mendez et al., 1981). It is a single polypeptide with a molecular weight of 68,000 daltons (Hattori and Uesugi, 1968) or 50,000 daltons (Flores et al., 1983) and occurs in both heterocysts and vegetative cells (Rai and Bergman, 1986).

Production of metabolites

Few studies have been made comparing the immobilized and free systems on extracellular production of metabolites.

Photoproduction of ammonia by N₂-fixing cyanobacteria immobilized in a calcium alginate matrix was demonstrated by Musgrave et al., (1982) and Kerby et al., (1986) using either inactivation of glutamine synthetase (GS) by L-methionine-D, L-sulphoximine (MSX) or using GS-deficient selected mutant strains. Continuous photoproduction of aminoacids was also demonstrated for a period of 10 weeks in air-lift reactors from selected mutant cyanobacterial strains immobilized in calcium-alginate beads ((Kerby et al., 1987). Hall and
Brouers et al. (1986) have also reported photoproduction of ammonia up to 400 μmol and 100 μmol mg⁻¹ Chl d⁻¹, in batch reactors from polyvinyl foam immobilized Anabaena azollae and Mastigocladus laminosus respectively, whereas control free-living cultures produced less than 10 μmol ammonia under the same conditions in both cases.

Jeanfils and Loudeche (1986) studied the effect of nitrite anaerobiosis production of ammonia by Anabaena cylindrica immobilized in alginate, in a batch reactor. Addition of nitrite to the medium increased the ammonia production as cells used the nitrite reductase pathway to form ammonia. When reactors were placed in anaerobiosis by N₂ bubbling, ammonia production was sustained for several days and the total ammonia formed was about two fold higher than in aerobiosis.

Park et al., (1990) have observed continuous production of ammonia for one week in "trickling-medium" photobioreactors using polyvinyl immobilized Anabaena azollae.

Wang et al., (1991) reported sustained photoproduction of ammonia for a period of 16 days in photobioreactors with and without the inhibitor MSX (L-methionine-D, L-sulfoximine) by Anabaena azollae immobilized in hollow fibres made from polysulphone and polypropylene. The ammonia concentration in the effluent reached 600-700 μg without MSX in the medium.

In addition to the metabolites listed above, it must be emphasized that other compounds synthesized by algae and cyanobacteria such as antibiotics, vitamins, plant growth regulators, pigments, etc., with particular interest in pharmaceutical, agricultural or agro-food industry are envisaged as potential products from photobioreactors by immobilized photosynthetic cell systems.