

## IMMOBILIZATION OF THERAPEUTICALLY BENEFICIAL ENZYMES

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### ABSTRACT

Immobilization is a process used to restrict the freedom of movement of enzyme or cells. Immobilization is a convenient term covering the methods of generation of heterogenous biocatalysts. The use of immobilized cells as industrial catalysts has many operational advantages associated with the use of free cells together with number of additional merits. Immobilized microorganisms are convenient to handle and permit easy separation of product from the biocatalyst. Adsorption, covalent binding, entrapment, encapsulation, cross linking are the various methods adopted to immobilize the enzymes or whole cells. An support is used to immobilize the cells or enzyme. An ideal support and the choice of matrix for immobilization will depend

on the nature of the bio- catalyst, nature of the reaction and its ultimate application in proper reactor geometry, commercial success has been achieved where the support material has been chosen for its flow characteristic, cost, non-toxicity and immobilization method tailored to give maximum bio- catalytic while retaining the desirable flow characteristic

**Key Words:** Immobilization, catalase, lipase, streptokinase, protease, penicillinase, adsorption.

### INTRODUCTION

Immobilization is a generic term used to describe the retention of biologically active catalyst within the reactor or analytical system <sup>(1)</sup>. The term immobilization is also defined as techniques specifically designed to greatly restrict the freedom of movement of an enzyme/cells <sup>(2)</sup>. In the early period of immobilized enzyme technology, attention was focused primarily on immobilization methodologies. However, as the application of immobilized

enzyme developed, it became apparent that immobilized systems tailored to meet the requirements for each application were needed and more integrated style of development resulted. One of the most important developments that occurred was immobilization of whole cells rather than the cell –free enzymes. Immobilized whole cells can serve as a useful tool for the immobilization of intra cellular enzyme.

### **Rationale for whole cell immobilization <sup>(13)</sup>**

Immobilization is a convenient term covering methods of generation of heterogenous biocatalysts. In the case of enzyme this usually refers to the formulations of insoluble complexes. However, in case of cells which are already insoluble it is mainly concerned with their deployment within a reactor in such a way that they are retained and a cell free product results.

### **Use of immobilized cell <sup>(14)</sup>**

The use of immobilized cells as industrial catalysts has many operational advantages associated with the use of free cells together with number of additional merits. Immobilized microorganisms are convenient to handle and permit easy separation of product from the biocatalyst. They are reusable and suitable for continuous or repeated batch operations at high dilutions rates allowing better process control, which should ultimately improve the yield and product quality.

Immobilized cells in particular trapped ones are less susceptible to phage attack, foam and viscosity owing to the physical protection offered by the polymeric net work. Immobilized cells generally have high operational stability. Immobilized cells have greater potential for multistep processes. Immobilized cells offer greater resistance to environment perturbations. E.g Problem of disposal of spent cell mass is less pronounced with immobilized cells. More effective synthesis of compounds via metabolic pathways requiring more than one enzyme. Decrease or elimination of lag and growth phase for product accumulation associated with non- growth phase fermentation. Reduced end product and substrate inhibition.

### **Rational for enzyme immobilization <sup>(15)</sup>**

Enzymes are nature's catalysts and their ability to catalyze biochemical reactions under mild conditions in a highly specific and efficient manner has led to the interest in their exploitation as industrial catalysts. Enzyme processing traditionally has been accomplished using soluble cell free enzyme preparations. These batch processes are not very economical because bio-

catalysts are used for single operations since conventional recovery methods are wither expensive or cause denaturation and loss of catalytic activity. Also enzymes are relatively unstable. If enzyme is to be reused effectively their stability must be improved and in expensive non- destructive recovery methods must be developed. One of the major activities in the field of biotechnology over the past two decades has been the immobilization of enzymes as a means of achieving both these objectives.

#### Fundamental considerations in selecting a support and method of immobilization <sup>(6)</sup>

Property	Points to be considered
Physical properties	Strength, non- compression of particles available surface area shape/ form (beads/ sheets/fibers), degree of porosity, pore volume permeability, density, space for increased biomass, flow rate and pressure drop.
Chemical properties	Hydrophilicity (water binding by the support) inertness towards enzyme /cell. Available functional groups for modification and regeneration/ reuse of support.
Stability	Storage, residual enzyme active cell, productivity, regenerations of enzyme activity, maintenance of cell viability and mechanical stability of support materials.
Resistance	Bacterial/fungal attack, disruption by chemicals, PH, Temperature, organic solvents, Proteases and cell defense mechanisms( Proteins/ cells)
Safety	Bio compatibility (Invokes an immune response), toxicity of component reagents, health and safety for process workers and end product users, specifications of immobilized preparations for food, pharmaceuticals and medical applications.
Economic	Availability and cost of support, chemicals special equipments, reagents, technical skill required, environmental impact, industrial scale chemical preparations, feasibility for scale up continuous processing, effective working life, reusable support, and size contamination( enzyme/ cell- free products)
Reaction	Flow rate, enzyme/ cell loading and catalytic productivity reaction kinetics, side- reactions, multiple enzyme and or cell systems, batch CSTR ( continuous stirred tank reactor), PBR(packed Bed reactor), ALR ( Air lift reactor) and so on diffusion limitation on mass transfer of cofactors, substrates and products.

#### Techniques for whole cell/ enzyme immobilization

A large number of techniques are now available for immobilization of cells on different supports. Most of these techniques used for immobilization of soluble enzymes have also been found to be suitable for cells and organelles. Selection of a technique for immobilization will depend on the nature of the cell, nature of the chemical conversion and its ultimate application in a proper reactor geometry.<sup>(7)</sup> There are five principle methods

for immobilization of enzymes/ cells. Adsorption, covalent binding, entrapment, encapsulation and cross linking <sup>(8)</sup>. A combination of two or more of these methods has also been employed. No single system can be applicable to all the enzymes or the cells in view of the differences in their composition and over all charges distribution. The substrate characteristic may also influence the choice of the method of immobilization.

### **Adsorption**

Immobilization of adsorption is the simplest method and involves reversible surface interaction between enzyme / cell and support material. The forces involved are mostly electrostatic, such as Vanderwaals forces, ionic and hydrogen bonding interactions, although hydrophobic bonding can be significant. These forces are very weak but sufficiently large in number to enable reasonable binding. For example it is known that yeast cells have a surface chemistry that is substantially negatively charged. So that use of a positively charged support will enable immobilization. Existing surface chemistry between the enzyme/ cells and support is utilized so no chemical activation/ modification of immobilization. The procedure consists of mixing together the biological components and a support with adsorption properties, under suitable conditions of pH, ionic strength and so on for a period of incubation, followed by collection of the immobilized materials and extensive washing to remove non- bound biological components. Immobilization of yeast *saccharomyces ceravisiae* cells on glass can be improved by the adsorption of aluminium ions on the cell surface <sup>(9)</sup>. The adsorption of aluminium ensures a neutralization of the cell surface and allows the adhesion of the cells to negatively charged surfaces, glass and poly carbons.

### **Advantage**

Little or no damage to enzyme/ cells. Simple, cheap and quick to obtain immobilization. No chemical changes to support or enzyme/ cell. Reversible to allow regeneration with fresh enzymes/ cells

### **Disadvantages**

Leakage of enzyme/ cells from the support / contamination of product. Non- specific binding. Over loading on the support. Steric hindrance by the support.

### **Covalent binding**

This method of immobilization involves the formation of a covalent bond between the enzyme/ cell and support material. The bond is normally formed between functional groups

present on the surface of the support and functional groups belonging to amino acids residues on the surface of the enzyme. A number of amino acid functional groups are suitable for participation in covalent bond formation. Those that are most often involved are the main amino group ( $\text{NH}_2$ ) of lysine or arginine, the carboxyl groups ( $\text{CO}_2\text{H}$ ) of aspartic acid or glutamic acid, the hydroxyl group ( $\text{OH}$ ) of serine or threonine and the sulfhydryl group ( $\text{SH}$ ) of cysteine. Many varied support materials are available for covalent binding and the extensive range of supports available reflects the fact that no ideal support exists.<sup>(10)</sup> Therefore, the advantages and disadvantages of support must be taken into account when considering possible procedures for a given enzyme immobilization. Many factors may influence the selection of a particular support and hydrophilicity is the most important factor for maintaining enzyme activity in a support environment. Consequently, polysaccharide polymers, which are very hydrophilic are popular support materials for enzyme immobilization.<sup>(11)</sup>

For example cellulose, dextran (Sephadex), starch and agarose (Sephacrose) are used for enzyme immobilization. The sugar residues in these polymers contain hydroxyl groups, which are ideal functional groups for chemical activation to provide covalent bond formation. The polysaccharide supports are susceptible to microbial / fungal disintegration and organic solvents can shrinkage of the gels. The supports are usually used in bead form. There are many reaction procedures for coupling an enzyme and support in a covalent bond, however most reactions fall into the following categories 1. Formation of an isourea linkage 2. Formation of an diazo linkage 3. Formation of a peptide bond. 4. An alkylation reaction. Basically two steps are involved in covalent binding of enzymes to support materials.<sup>(12)</sup> First functional groups on the support materials has to be activated by a specific reagents, and second the enzyme is added in a coupling reactions to form covalent bond with the support materials. Normally the activation reaction is designated to make the functional groups on the support strongly electrophilic (electron deficient). In the coupling reactions, these groups will react with strong nucleophiles (electron donating) such as amino ( $\text{NH}_2$ ) functional groups of certain amino acids on the surface of the enzyme, to form a covalent bond. Cyanogen bromide ( $\text{CNBr}$ ) is often used to activate the hydroxyl functional groups in polysaccharide support materials. In this method, the enzyme and support are joined via isourea linkage. In the case of carbodiimide activation, the support material should have carboxyl ( $\text{COOH}$ ) functional group, and the enzyme and support are joined via a peptide bond. If the support material contains an aromatic amino functional group, it can be diazotized using nitrous acid.

Subsequent addition of enzyme leads to the formations of a diazo linkage between the reactive diazo group on the support and the ring structure of an aromatic amino acid such as tyrosine. <sup>(13)</sup>

### **Entrapment**

Immobilization by entrapment differs from adsorption and covalent bonding in that enzyme molecules are free in solution, but restricted in movement by the lattice structure of a gel. The porosity of the gel lattice is controlled to ensure that the structure is tight enough to prevent leakage of enzyme or cells yet at the same time allow free movement of substrate and product. Inevitably, the support will act as a barrier to mass transfer and although this can have serious complications for reaction kinetics, it can have useful advantage since harmful cell proteins and enzymes are prevented from interactions with the immobilized bio-catalyst. There are several major methods of entrapment 1. Ionotropic gelation of macromolecules with multivalent cations (e.g alginate) 2. Temperature- induced gelation (e.g agar, agarose, gelatin) 3. Organic polymerization by chemical/ photochemical reaction (e.g. Polyacrylamide) 4. Precipitation from an immiscible solvent (e.g Polystyrene)

Entrapment can be achieved by mixing an enzyme with polyionic polymer material and then cross linking the polymer with multivalent cations in an ion exchange reactions to form a lattice structure that traps the enzymes/ cells (ionotropic gelation). Temperature change is a simple method of gelation by phase transition using 1-4 solutions of agar, agarose or gelatin. However, the gel, formed are soft and unstable. A significant development in this area has been the introduction of K- carrageen in polymers that can form gels by ionotropic gelation and by temperature- induced phase transition which has introduced a greater degree of flexibility in gelation systems for immobilization. Alternatively, it is possible to mix the enzyme in the interstitial spaces of the lattice. The latter method is more widely used and a number of acrylic monomers are available for the formation of hydrophilic copolymers. For example, acrylamide monomers is polymerized to form polyacrylamide and methylacrylamide and methylacrylate is polymerized to form polymethacrylate, in addition to the monomer, a cross linking agent is added during polymerization to form cross linkages between the polymers chains and help to create a three dimensional net work lattice. The pore size of the gel and its mechanical properties are determined by relative amounts of monomers and cross linking agents. It is therefore possible to vary these concentration to influence the lattice structure. The formed polymer may be broken up into particles of a

desired size, or polymerization can be arranged to form beads of defined size. This system has been extensively investigated for the immobilization of animal cells and ethanol fermentation using yeast cells.<sup>(14)</sup>

### **Encapsulation**

Encapsulation of enzyme and or cells can be achieved by enveloping the biological components within various forms of semi permeable membrane. It is similar to entrapment in that the enzyme/ cells are free in solution, but restricted in space. Large proteins or enzyme cannot pass out of or into the capsule, but small substrate, but small substrate and products can pass freely across the semi permeable membrane. Many materials have been used to construct microcapsules varying from 10-100  $\mu\text{m}$  in diameter, for example, nylon and cellulose nitrate have proven popular. The problems associated with diffusion are more acute and may result in rupture of the membrane if products from a reaction accumulate rapidly. A further problem is that the immobilized cell or enzyme particles may have a density fairly similar to that of the bulk solution with consequent problems in reactor configuration, flow dynamics and so on, it is also possible to use biological cells as capsule, and a notable example of this is the use of erythrocytes ( red blood cells). The membrane of the erythrocytes is normally only permeable to small molecules. However, when erythrocytes are placed in hypotonic solutions, they swell stretching the cell membrane and substantially increasing the permeability. In these conditions, erythrocytes proteins diffuse out of the cell and enzymes can diffuse into cell. Returning the swollen erythrocytes to an isotonic solution enables the cell membrane to return to its normal state and the enzyme trapped inside the cell does not leak out. A distinct advantage of this method is co immobilization. Cells and /or enzyme may be immobilized in any desired combination to suit particular application.<sup>(15)</sup>

### **Cross linking**

This type of immobilization is support free and involves joining the cells( or the enzyme) to each other to form a large, three dimensional complex structure, and can be achieved by chemical or physical method. Chemical methods of cross linking normally involve covalent bond formation between the cells by means of a bi- or multifunctional reagent such as Glutaraldehyde and toluene diisocyanate. However, the toxicity of such reagents is a limiting factor in applying this method to living cells and many enzymes; both albumin and gelatin have been used to provide additional protein molecules as spacers to minimize the close proximity problems that can be caused by cross linking a single enzyme.

Physical cross linking of cells by flocculation is well known in the biotechnology industry and does lead to high cell densities. Flocculating agents, such as polyamines, polyethyleneimine, polystyrene sulfonates and various phosphates have been used extensively and are well characterized. Cross linking is rarely used as the only means of immobilization because the absence of mechanical properties and poor stability are severe limitations. Cross linking is most often used to enhance other methods of immobilization, normally by reducing cell leakage in other systems.<sup>(16)</sup>

### **Choice of support for immobilization <sup>(17)</sup>**

An ideal support and the choice of matrix for immobilization will depend on the nature of the bio-catalyst, nature of the reaction and its ultimate application in proper reactor geometry, commercial success has been achieved where the support material has been chosen for its flow characteristic, cost, non-toxicity and immobilization method tailored to give maximum bio-catalytic while retaining the desirable flow characteristic.

### **Supports used immobilization**

Both organic and inorganic supports have been used for immobilizing cells and enzyme. The inorganic supports are generally rapid and give the greatest protection against turbulent environment. They less prone to microbial attack and are quite stable over a wide pH range and in a variety of solvents. They also have good hydraulic properties and can be regenerated. On the other hand, organic polymers are flexible and elastic contain more reactive groups which an enzyme can be attached. They can also be cast into any geometry including membrane, beads etc.

### **Example for inorganic supports**

Alumina, bentonite, colloidal silica, controller pore glass, ceramics, charcoal, glass particles, hydroxylapatite, iron oxide, horn blends. Kaolinite, magnetite nickel oxide, sand, steel zirconia. Examples for organic supports- agar, agarose, cellulose chitin, chitosan, calcium alginate, Carrageenin, colloidion, dextran, nylon, ocium basilicum seeds, polyacrylamide, polystyrene, poly acrylic acid, poly vinyl alcohol, poly urethane pre polymers.

### **Agar**

Agar is the dried, hydrophilic, colloidal concentrate from a decoction of various red algae, particularly species of “ Gelidium” ( class- Rhodophyceae <sup>(18)</sup>) Agar is made chiefly from *Gelidium elegans*, *G. amansil*, *G. polycladum* belonging to the family Gelidiaceae. Small

amounts of other genera may be included such as *Gracilaria lichenoides* and *Gracilaria confervoides*. Agar is sometimes referred to as “Japanese isinglass”. These algae grow along the eastern coast of Asia and the coasts of North America and Europe. Most of the commercial supply comes from Japan, Spain, Portugal and Morocco. Mexico, New Zealand, South Africa and United States are also significant producers (<sup>[19]</sup>)

Agar usually occurs as bundles consisting of these membranous agglutinated strips or in cut, flaked or granulated forms. It may be dull yellowish orange, yellowish gray to pale yellow or colorless. It is tough when damp, brittle when dry, odorless or slightly odorous and has a mucilaginous taste. Agar is insoluble in cold water but if one part of agar is boiled for 10 minutes with 65 times its weight of water, it yields a firm gel when cooled.

### Constituents

Agar is predominantly the calcium salt of a sulphuric ester of a carbohydrate complex R (O, SO<sub>2</sub>, O<sub>2</sub>, Ca). It is now known to be a heterogeneous polysaccharide, the two principal constituents of which are agarose and agaropectin (<sup>[20]</sup>). Agarose is a neutral galactose polymer which is principally responsible for the gel strength of agar. It consists of alternate residues of 3,6-anhydro-L-galactose and D-galactose (the disaccharide known as agarobiose). The structure of agaropectin, responsible for the viscosity of agar solutions, the structure of these constituents have not been fully established.

### Uses

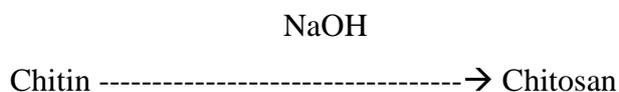
Agar is extensively used as a gel in bacteriological, culture media and as an aid in food processing and other industrial processes.

### Role of agar on immobilized bacterial and algae systems.

- The bacteria *Pseudomonas putida* was immobilized on agar for the application of degradation of caffeine.
- Methogenic bacteria was immobilized on agar for phenol degradation.
- *Rhodospirillum rubrum* and *Clostridium butyricum* were immobilized on agar for the purpose of H<sub>2</sub> production.
- The algae *Mastigocladus laminosus* was immobilized on agar for the study of hydrogen production.
- *Anacystis nidulans* was immobilized on agar for the evolution of oxygen.

**Chitosan** <sup>[21]</sup>

Chitosan a polymer of 2- amino- glucose is a commercially available biopolymer, usually derived from chitin via alkaline hydrolysis.

**Resources of chitin/ chitosan**

The major source of chitin are shell fish, krill, clams, oysters, squid, fungi and insects. Through the main source of chitin is animals it is frequently present as a cell wall materials in plants, replacing cellulose or sometimes occurring together with cellulose. This polymer in the deacetylated form, 1,2 chitosin is present in various fungi e.g Zygomycetes contain both chitin and chitosan.

Most commercial polysaccharides e.g cellulose, dextran, pectin, alginic acid, agar, agarose, carrageenins and heparin are neutral or acidic. Chitin and chitosan are the only abundant basic polysaccharides. Their unique properties include solubility behavior in various media, solution, viscosity, polyelectrolytic behavior, polyoxysalt formation, ability to form films, chelate metal ions and optical and structural characteristics.

Chitin is highly hydrophobic and is insoluble in water and most organic solvents. It is soluble in hexafluron, isopropanol , hexa fluoro acetone, chloroalcohols in conjunction with aqueous solutions of mineral acids and dimethyl acetamide containing 5% lithium chloride. On deacetylation with strong yields the free base chitosan. Chitosan is insoluble in water but insoluble in dilute acids. Chitin and chitosan are now produced commercially in Japan, U.S.A, India, Poland, Norway and Australia.

**Application of chitin and Chitosan** <sup>[22]</sup>

Chitosan is a versatile polymer and the interest in chitosan is due to the large variety of useful forms that are commercially available or can be made available.

**Clarification and purification**

- 1.It can remove transition metals, vegetables matter, proteins, suspended solids from vegetables, process wastes and activated sludge and clarify beverages.
- 2.The largest commercial use of chitosan, at present, is in the clarification of waste water.

3. The important food related applications are in purifying drinking water, recovering protein for animal feeds, clarifying wastes from vegetables, poultry, egg and fish processing, clarifying, fruit juices and recovery of micro algae.

### **Chromatography** <sup>[23]</sup>

1. Because of the presence of free amino and hydroxyl groups Chitosan is a good chromatographic support.
2. The resistance of Chitosan towards  $\gamma$ -radiations and ability to absorb metal ions make it suitable for the removal of radio active solutes.

### **Paper and textiles industries**

1. Chitosan coating improved the bursting strength, punctures resistance, water proofness, tensile strength, water vapor transmission rate and grease resistance of craft paper and could be used as a good substitute for glassine paper.
2. The chelating ability, adhesive property and ionic bond forming characteristic of chitosan find potential application in textiles.

### **Medical and pharmaceutical uses**

1. Chitin and its derivatives in solution have found use as bacteriostatic agent, drug delivery vehicle, spermicide, surgical glove powder, haemostatic agents, enzyme immobilization, contact lens, artificial skin and wound dressings.
2. Other miscellaneous pharmaceutical applications such as anticholesteremic materials, antigastritis agents, antisore compositions, antibilirubinemia agent and anticoagulants.

### **Role of Chitin/ Chitosan in immobilization**

1. The following cell were immobilized on chitin for the purpose of synthesis of L-Tryptophan and nitrification of waste water respectively E.coli cells and Nitrosomonas-Europaea cells.
2. The following enzyme were immobilized on chitin and its derivatives
  - $\alpha$ - Chymotrypsin for plastein synthesis.
  - $\beta$ -D-galactosidase for hydrolysis of lactose.
  - D-Glucose isomerase for the preparation of D- gluconic acid and for the conversion of D-Glucose to D- Fructose.
  - $\beta$ -D-galactosidase for hydrolysis of cellobiose
  - Lysozyme for the preparation of pharmaceuticals.

## **Role of fermentation technology along with the immobilization techniques**

### **Historical perspective <sup>(24)</sup>**

Historically the technology evolved to the production of increasingly more value products such as antibiotics. More recently the advent of recombinant technology has dictated the development of novel process for the production of totally synthetic products such as monoclonal antibodies, as well as scaling up of the process, to such an extent that the incorporation of modern engineering concepts and methods in the development, design and control of fermentation operations is becoming universal.

Fermentation can be defined as follows: chemical reactions catalyzed by enzyme systems, which in turn are produced during the growth of the microorganism <sup>(25)</sup>. The growth of a microorganism is a complex process and the transformation of a nutrient into a metabolic end product usually involves a large number of individual chemical reactions.

### **Applications <sup>(26)</sup>**

The following applications are become commercially established

1. Production of alcohols ( Ethanol, butanol)
2. Production of organic acids ( acetic acid, citric acid)
3. Production of fine chemicals ( vitamin C precursor, Riboflavin, Cobalamine).
4. Production of antibiotics( secondary metabolites)

### **Fermenters <sup>(27)</sup>**

The industrial usage of microorganisms often requires that they be grown in large vessels containing considerable quantities of nutritive media. These vessels are commonly called fermenters and they can be quite complicated in design, since frequently they must provide for the control and observations of many facets of microbial growth and bio- synthesis.

### **Bio-reactors <sup>(28)</sup>**

It can be defined as a system or multiple vessels in which microbial or biochemical reactions are carried out employing free cells, immobilized cells, free enzymes or immobilized enzymes.

### **Biosensors <sup>(29)</sup>**

It is the term used for a whole class of sensors that utilize biochemical reactions to determine a specific compound. An immobilized enzyme or cell is combined with a transducer to

monitor a specific change in the micro environment.

### **Types of bio- reactors**

Especially the following two different types of bio- reactors can be used for immobilized cell/ enzyme system.

#### **1. Packed bed reactor**

In these reactors enzyme/ cells are either attached by adsorption, chemical bonding (cross linked or covalently bound) entrapment on suitable carrier or encapsulated and placed / packed in different types of vessel configuration to serve as flow reactors. Reactors with physical adsorption of cells or enzymes encounter practical difficulties because the adsorbed enzyme / cell is weakly bound and is lost easily during the operations. Covalently or cross linked immobilized reactor systems require mild processing conditions. Large changes in pH and temperature are not permitted. Micro encapsulated reactor system is subjected to the requirement of substrate diffusivity across the same permeable membrane which contains the enzyme or cells. Despite these demerits the greatest advantage of high productivity is offered by the system.

#### **2. Membrane reactor**

This reactor consists of semi permeable membrane of cellulose acetate or other polymeric materials having tailored molecular cut off characteristic placed at the bottom of the vessel. The membrane permits the separations of the product freely across the film while the substrate and the enzyme are rejected in to the reactor. One of the major advantages of this reactor is the possibility of rapid removal of inhibitory or toxic metabolic products of lower molecular weight. High productivity also has been reported in such a system.

### **Over view of ethanol production<sup>(30)</sup>**

Ethanol production used to be considered the answer too many developing countries that faced a depressed sugar cane market, soaring oil prices and an increasing foreign debt. Ethanol is a high quality fuel and a substitute for gasoline. With the growing interest in ethanol, more biotechnology research has to be performed.

Diverting sugarcane into ethanol production was deemed to provide a solution for energy as well as agricultural and foreign exchange problems. Many countries such as Costa Rica, Malawi, Kenya, Zimbabwe and the USA have started operating ethanol programmes over the past decade.

**Reduced air pollution** <sup>(31)</sup>

Apart from being a gasoline extender, ethanol gains attraction because it is a relatively clean burning fuel. According to the American environmental protection Agency (AEPA), emission of cars is responsible for 40 % of air pollution in US metropolis. The AEPA attributes 66% of all carbon monoxide emissions to imperfect combustion in motor vehicle engines. For this reason ethanol production also merits attention blending ethanol I gasoline will reduce lead and carbon dioxide emissions.

**Lipase** <sup>[ 32,33,34,35, 36]</sup>

Lipase are of considerably commercial and industrial potential. The yeast *Candida rugosa* is regarded as a good producer of lipase. Its growth on olive oil or oleic acid appears to be a good procedure for lipase production. The application of immobilized biocatalyst for the production of extra cellular enzyme is well documented for the production of useful low molecular mass components.

Immobilization of *Candida rugosa* cells on a solid support for industrial production of lipase could offer Several advantages. These are improvement of microbial cell stability, higher dilution rates without culture washed out and facilitation of continuous operation are significantly simplified when compared with conventional batch processing. To alleviate mass transfer resistance of organic substrate, immobilization of microbial cells on a support with optimum hydrophobic / hydrophilic balance. The immobilization techniques has to be carried out by entrapping yeast cells in calcium alginate gels A fluidized bed reactor provides good contact and interactions between solids and fluids because of its large surface area, the particulate solid is freely exposed to the fluids. A fluidized bed reactor has high heat and mass transfer coefficients and it has good medium and particle mixing. Yeast cells of an inoculation culture were suspended in sterile sodium alginate by stirring in order to obtain 2 % alginate solution.

Preparation of the mixed matrices of PH foam and Ca- alginate gel was done by adding a prefixed amount of treated dry PU form particles to the alginate solution before its sterilizations in order to improve the affinity of Ca- alginate beads towards the organic substrate. Mixed matrix beads were prepared consisting of different PU/Ca alginate proportions ( 5 %, 20 %, 40 %, w/w). The mixtures were pumbed through a thin hypodermic needle into a 1.5 % CaCl<sub>2</sub>. 2 H<sub>2</sub>O solution and then left to harden over night. The gel beads had a diameter of about 1 mm. After immobilization gel beads were incubated in feed

medium with 40 g/l of glycerol as a carbon source for 2 days in order to increase the cell concentration to  $10^9$  cells/ gel.

### **Release of immobilized cells**

A 1.5 % solution of sodium citrate was used to free yeast from calcium alginate without any loss of viability. The beads were previously washed with saline to eliminate free  $\text{Ca}^{2+}$ . A cell suspension from mixed PU/ $\text{Ca}^{2+}$ - alginate beads was prepared by the same method. Dissolution of mixed beads was followed by continuous gently shaking for 15 minutes to facilitate the washing free of the PU foam particles from biomass and  $\text{Ca}^{2+}$ - alginate aggregates. The resultant cell suspension were used to estimate the immobilized biomass. Biomass determination and cell viability cells were counted by direct microscopy enumeration. The methylene blue staining method was used to determine yeast viability.

### **Lipase activity in aqueous phase assay**

5 ml of filtered sample was mixed with 5 ml of 0.03 M calcium chloride 10 ml of 0.2 M TRIS- maleate 0.2 M sodium hydroxide buffer Ph 8.2 and 5 ml of olive oil. After 50 minutes of incubation at  $37^{\circ}\text{C}$ , two phases were separated in a boiling water bath. The amount of liberated fatty acids was titrated with 0.05 M organic KOH (ethanol trichloroethylene 1:1). Activity was expressed as units/ ml of broth.

One unit (U) of lipase activity was defined as the amount necessary to hydroxylase  $1\ \mu\text{mol}$  ester bond/minute under assay condition. PU foam preparation pieces of foam ( $8\ \text{mm}^3$ ) were washed twice in distilled water. The pieces were then comminuted to get particles of  $54.74\ \mu$  and added to 1-1-Erlenmeyer flask containing 600 ml distilled water and autoclaved for 20 minutes at 1.2 atmosphere to ensure thorough leading of all impurities. The water was then poured off and fresh distilled water was added against to repeat the operations. Finally treated PU foam was dried at  $40\text{-}60^{\circ}\text{C}$ .

### **Catalase** (<sup>[37,38,39,40,41]</sup>)

Catalase is an enzyme normally found in peroxisomes of nearly an aerobic cells and serve to protect the cells from toxic effects of  $\text{H}_2\text{O}_2$  by catalyzing its decomposition into molecular oxygen and water without production of free radical. The reaction of catalase in the decomposition of hydrogen peroxide or glucose biosensors system. There are many limitation for the free enzyme. Enzymes are unstable, having a short life span in the circulation and causing toxic reactions.

These drawbacks can be partially eliminated by immobilizing enzyme onto solid carriers. Hence immobilization is an indispensable step in the preparation of the biocatalyst for industrial application

#### Use

- The reaction of catalase in the decomposition of hydrogen peroxide is  $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$ .
- Here has useful application in various industrial fields such as food industry textile industry and in the analytical field as a components of  $\text{H}_2\text{O}_2$  or glucose biosensors system
- Chitosan is produced commercially by deacetylation of chitin which is a poly N- acetyl glucosamine and a transformed oligosaccharides obtained by the structural element in the exoskeleton of crustaceans and cell wall of fungi.
- It has a number of commercial and possible biomedical application.
- Gel membrane and beads forms of chitosan have been used in the enzyme immobilization as supporting materials.

#### Methods of immobilization

Three grams of chitosan flasks were added into 100 ml of distilled water and stirred for 10 minutes. 1 ml of glacial acetic acid was then added and stirred continuously for 2 hours at room temperature. To prepare the cross linking agent glyoxal (30.0 ml 40%) was added to 240 ml distilled water and heated at  $80^\circ\text{C}$  for 10 minutes and cooled to room temperature. Then the solution was mixed with 270 ml (3 % w/v) tetra sodium pyrophosphate solution. The fresh catalase solution ( 100  $\mu\text{g}/\text{ml}$ ) prepared in 50 mM phosphate buffer with 7.0 was added in chitosan solution. After 2 hours adsorption at  $4^\circ\text{C}$ , this suspension was injected into the cross linking agent. The beads were cured for 45 minutes and washed 5 times with 50 mM cold phosphate buffer ( PH 7.0). The immobilized catalase was freeze dried for 3 hours under vacuum and stored at  $4^\circ\text{C}$  for later use

#### Penicillinase immobilization ( <sup>[42,43,44,45]</sup> )

Penicillinase is an enzyme used to find our the concentration of penicillin in standard solution and in fermentation broth in the antibiotic manufacturing industry. In this process immobilization of penicillinase play an important role. Penicillinase is immobilized by cross linking with a very fine film of gluteraldehyde which is sprayed on to the sensitive ends of the two different pH glass electrode. Penicillinase immobilization on open tubular reactors was accomplished by adding about 5 mg of enzyme to 3 ml of phosphate buffer pH 7.0. This

solution was pumped into the reactor which is immediately refrigerated and stored over night at 4 °C. for immobilization of penicillinase on finalized controlled pore glass (CPG). About 10 mg of enzyme was added to 10 ml of buffer and 0.10 g of CPG in a vial. The mixture was shaken and immediately refrigerated and stored over night. The specific activity of the enzyme solution used was about 2000 U/mg.

### **Protease** ( [46,47,48,49,50] )

Proteases are important because of their physiological role in dietary protein digestion and cellular protein processing.  $\alpha$ -chymotrypsin, trypsin – serine protease, Papain – thiol protease. Most familiar proteases of animal origin are pancreatic  $\alpha$ -chymotrypsin, trypsin and rennin.

### **Uses**

- $\alpha$ -chymotrypsin is specific for the hydrolysis of peptide bonds, in which carboxyl groups are provided by one of the three aromatic amino acids, “Phenyl alanine, tyrosine or Tryptophan”. It is used extensively in the deallergenizing of milk protein hydrolysates.
- Trypsin- hydrolyze peptide bond in which carboxyl groups are contributed by the lysine and arginine residues and this is the main intestinal enzyme responsible for hydrolysis of protein.
- Papain- used as a preservative agent during beer finishing operations. Because of its ability to hydrolyze the peptide bond of the connective tissue protein collagen, elastin and actinomyosin. It is used as a meat tenderizer in the meat industry. It acts as a biocatalyst for amino acid ester and peptide synthesis.

### **Immobilization of enzyme**

- The enzyme was immobilized by carrier binding method in which enzymes are made to bind to the support materials by covalently.
- For immobilization, 30 mg of tri (4- formyl phenoxy) cyanurate and 90 mg of  $\alpha$ -chymotrypsin and papain enzyme were taken separately in 10 ml of 10 mM acetate buffer pH 5 and stirred continuously for 20 minutes at room temperature.
- The amino group of enzyme was made to bind reversibly by the aromatic aldehyde group of tri (4- formyl phenoxy) cyanurate to form Schiff base.

### **Streptokinase** ( [51,52,53,54,55] )

Immobilization of streptokinase is an FDA approved drug that has been used in the treatment of thrombosis, pulmonary embolism and other clotting induced maladies. The enzyme was originally discovered by Tillett in 1933 in hemolytic streptococci and was subsequently

known to be an activator of human plasminogen. The enzyme is capable of promoting lysis of experimentally induced thrombi as well as of spontaneously occurring venous and arterial thrombi. Repeated injections of streptokinase rapidly leads to an immune response. Streptokinase is a potent antigen and problems relating to its antigenicity caused a major shift from interest in streptokinase to urokinase. Streptokinase has a relatively short half life in vivo and frequent injections are necessary in order to promote a continuous fibrinolytic action. (100,000 units per hour were administered for a period of 24 hours). The circulating half-life of streptokinase is about 30 hours. The major problem that is encountered with the use of urokinase or streptokinase is an increased tendency for bleeding, especially when high levels of enzyme are used. This effect considerably limits the usefulness of enzymes, especially in the post-surgical stage where the need for an effective thrombolytic agent may be quite great. Streptokinase would be preferable to urokinase as a thrombolytic agent, not for its antigenic effect.

The advantage may be

- Streptokinase may be readily obtained in larger quantities where as source of urokinase is limited.
- Circulating half-life of streptokinase is much longer than that of urokinase even smaller amounts would be sufficient to achieve the same thrombolytic agents.
- Urokinase does not induce an immune response. The enzyme has an extremely short circulating half-life (8-10) minutes.
- It has found wide application in the treatment of embolism. The greater benefit was found in patients with acute emboli (less than 10 days old) where as only marginal benefit were observed in patients with sub-acute or chronic emboli.
- Urokinase as well as streptokinase have successfully been used in the treatment of myocardial infarctions, cerebral infarctions, deep vein thrombosis and pulmonary emboli.
- Immobilized streptokinase has been prepared by using a diazotized co-polymer of p- amino phenylalanine and leucine stability of urokinase was found to increase as a result of immobilization with nylon.
- Preparations were found to be stable at 4 °C for about 6 months.

## Steps for immobilization

### Partial hydrolysis

Nylon 66 is partially hydrolyzed by heating the nylon for 1 hour at 50 ° C in sufficient 4 N HCl to completely covering the nylon. After partial hydrolysis the nylon is thoroughly washed with water to remove all acids and is subsequently dried in air overnight. The nylon is finally put in an oven at 90 ° C for 1 and half hours to ensure complete dryness.

### Preparation of PNB nylon

The dry nylon is put into a round bottom flask and covered with a solution of 10 % triethanolamine in chloroform. Fifty mg of P- nitrobenzoyl chloride per gram of nylon is then added and mixture is refluxed for atleast 12 hours. At the end of this period the pNA-nylon is thoroughly washed with dry chloroform and dried in air.

### Reduction

The PNB- nylon is put into a round bottom flask and covered with a solution of 10 % dithionite in water. The mixture is refluxed for 1 hour. The nylon is then thoroughly rinsed with acidified water until no more dilutions is detectable.

### Diazotization and coupling

The wet PNB nylon is transferred to a beaker which is placed on ice. The nylon is covered with 200 ml of 1 N HCl and 20 ml of 2 N sodium nitrite and the mixture is kept in ice for 1 hour.

The nylon is then thoroughly washed with ice cold water and then diazotized nylon is spread out in a flat dish that is placed on ice. 5 ml of sterile water containing 20,000 units of streptokinase are added to the nylon and the coupling is allowed to proceed for 1 hour in ice. All the nylon should be kept in constant touch with streptokinase solution during the period in order to ensure uniform coupling. After coupling is completed the streptokinase- nylon is thoroughly washed with sterile water until soluble streptokinase has been removed. The streptokinase – nylon may be stored moist at 0 ° C – 5 ° C. The immobilization of streptokinase greatly increases its stability *in vivo*. Immobilized streptokinase appears to exert its action for at least half a year without any apparent loss in activity.

Such a stability represents a tremendous increase over the circulatory half- life of soluble streptokinase which is about 30 hours and suggest that immobilized streptokinase may be

useful in cases where prolong therapy is required. Immobilized streptokinase could successfully be used in the treatment of thromboembolix disease that require prolonged fibrinolytic therapy. In addition, immobilized streptokinase could be valuable in preventive therapy in case of anticipated thromboembolic problems.

## CONCLUSION

Lipases was produced from the yeast *Candida rugosa* Yeast cells of an inoculation culture were suspended in sterile sodium alginate by stirring in order to obtain 2 % alginate solution. This immobilized enzyme could be used to hydrolysis the lipids that are present in our food. Catalase is an enzyme normally found in peroxisomes of nearly an aerobic cells and serve to protect the cells from toxic effects of H<sub>2</sub>O<sub>2</sub> by catalyzing its decomposition into molecular oxygen and water without production of free radical, this immobilized enzyme could be used to act as an antioxidant to reduced the severity of the diseases which occurs due to the accumulation of H<sub>2</sub>O<sub>2</sub>. Penicillinase is an enzyme used to find our the concentration of penicillin in standard solution and in fermentation broth in the antibiotic manufacturing industry. Protease are important because of their physiological role in dietary protein digestion and cellular protein processing. Streptokinase is capable of promoting lysis of experimentally induced thrombi as well as of spontaneously occurring venous and arterial thrombi. Being a biocatalyst enzymes are very much useful to carry out various biochemical reactions. It is not only useful in industrial purpose but also in medicinal purpose. It would be very much useful if we could immobilize various enzymes in a proper mode which could retains the stability of the enzyme.

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