

## WEEK 10 LECTURE 4: IMMOBILIZATION OF ENZYME AND ACTIVITY MEASUREMENT

### Aim:

To learn the technique of immobilizing enzyme in alginate beads and determine its enzymatic activity by assay.

### Introduction:

Immobilization is a technique for the combination of enzyme in an insoluble support matrix. The matrix is usually a high molecular weight polymer such as polyacrylamide, starch, cellulose, etc. The advantage of immobilizing enzymes over free enzyme is to increase their stability and efficiency. The immobilized enzymes can also be recovered at the end of the reaction and can be used repeatedly.

### Principle:

An enzyme is usually immobilized onto an inert, insoluble material e.g. calcium alginate. This is produced by the reaction of a mixture of sodium alginate solution with calcium chloride. These beads provide increased resistance to changes in conditions such as pH or temperature. They also allow enzymes to be held in place throughout a reaction, following which they are easily separated from the products and may be used again. The enzyme can be easily immobilized with a number of methods: entrapment, ion exchange adsorption, porous ceramics, and even covalent bonding. There are several methods for the immobilization of enzymes.

**Adsorption:** This is a method that involves electrostatic interaction such as Van der Waals forces, and ionic and hydrogen bonding between the enzymes and the support matrix.

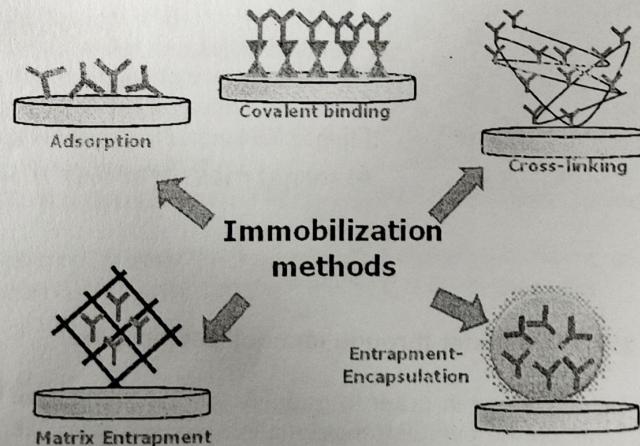


Fig. 1: Different immobilization techniques.

**Covalent Binding:** This method involves the formation of covalent bonds between the enzymes and the support matrix. The bond is normally formed between the functional groups present on the surface of the support and functional groups belonging to amino acid residues on the surface of the enzyme.

**Entrapment:** In this method, the enzyme molecules are mixed with a polyionic polymer material and then crosslinked of the polymer with multivalent cations in an ion exchange reaction to form a lattice structure that traps the enzymes.

**Encapsulation:** This can be achieved by enveloping the enzymes within various forms of semi-permeable membranes.

**Crosslinking:** This involves the joining of enzymes with each other to form large three-dimensional complex structures and can be achieved by physical or chemical methods without any support system.

#### External and internal mass transfer limitations:

Mass transfer is one of the major factors affecting the activity of immobilized enzymes since reactants and products have to be transported through external boundary layers (external mass transfer or film mass transfer) and diffusion through the porous matrix (internal mass transfer). Thus, the effectiveness of immobilized enzyme systems can be reduced by mass transfer limitations. With proper design of an immobilized reactor, external mass transfer resistance can be made negligible, for example, by increasing the velocity of the bulk solution relative to the gel particle. However, internal mass transfer resistance, which is affected by system design parameters such as particle size, enzyme loading, bulk movement, reactant concentration, and reactor type, generally is not negligible. Diffusion through the immobilization matrix ( $D_{eff}$ ) cannot be characterized by the bulk diffusivity ( $D_{aq}$ ) because the pore space is only a fraction of the total volume (exclusion effect) and the matrix increase the path length for a diffusing substrate (obstruction effect). Thus, information about effective diffusivity is essential for describing the kinetics of immobilized enzyme reactions.

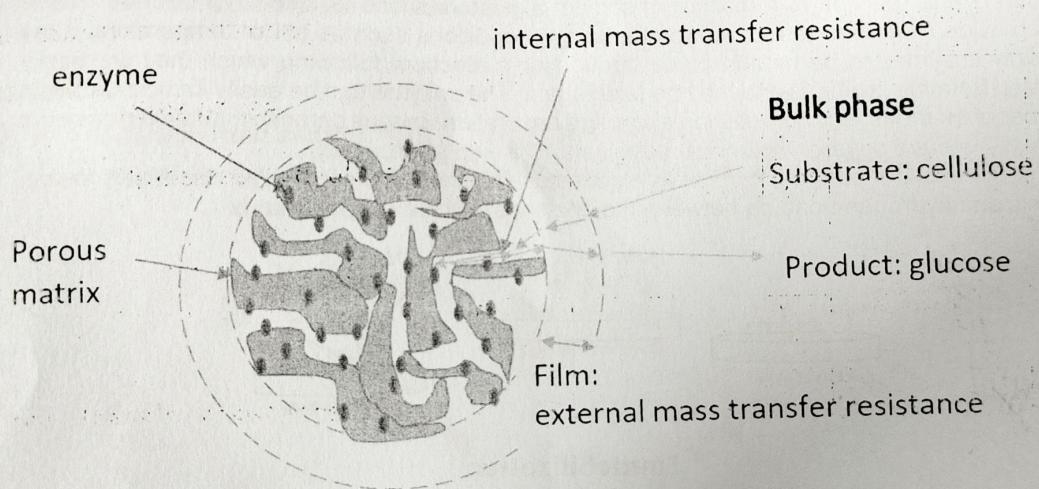


Figure 2: Mass transfer through immobilized enzyme

Some parameters could be calculated in order to quantify the contribution of mass transfer by diffusion into support particles on the overall transformation process rate. Diffusional limitations can be quantitatively expressed by the effectiveness factor,  $\eta$ , defined as the ratio between the average reaction rate and the rate that would be obtained if all enzyme molecules inside the particles were exposed to the same substrate concentration as the bulk liquid, that is, in the absence of diffusional effects.

$$\eta = \frac{\text{reaction rate with intraparticle diffusion limitations}}{\text{reaction rate without diffusion limitations}}$$

$$= \frac{\text{observed reaction rate with immobilized enzyme}}{\text{reaction rate with free enzyme}}$$

The effectiveness factor is a dimensionless parameter that measures how effectively the catalyst is being used. For near unity, the entire volume of the particle is reacting at the same high rate because the reactant is able to diffuse quickly through the support material. For near zero, the reaction is conducted at the lowest rate. The reactant is unable to penetrate significantly and the reaction rate is limited to a small portion of the particle volume. The diffusional resistance is predominant which lowers the overall reaction rate.

### Reagents and instruments required

Equipment: Flasks, Spectrophotometer, Sample tubes, Micropipette

Chemicals:

1. Tri sodium citrate
2. Citric acid
3. Carboxymethyl cellulose
4. D-Glucose
5. 3,5-Dinitrosalicylic acid (DNS)
6. Sodium potassium tartrate tetrahydrate
7. Sodium hydroxide
8. Hydrochloric acid

Reagents:

Preparation of DNS reagent: 1g of DNS is dissolved in 50ml of distilled water. Add 30g of sodium potassium tartrate tetrahydrate. Then add 20ml of 2N NaOH, which turns the solution to transparent orange yellow colour. The final volume is made to 100 ml with the distilled water.

2. 0.1M Citrate buffer (pH 4.8):

Prepare 80 mL of distilled water in a suitable container.

Add 1.554 g of Tri sodium citrate to the solution.

Add 0.906 g of Citric Acid to the solution.

Adjust solution to final desired pH using HCl or NaOH.

Add distilled water until volume is 100 mL

3. Prepare  $\text{CaCl}_2$  solution (3% or 0.2 M) in distilled water and autoclave it at  $121^\circ\text{C}$  under 15psi

4. Substrate solution (Carboxy Methyl Cellulase): 100 mg in 100 mL of 0.1 M citrate buffer.

5. Enzyme solution (Cellulase): 1 mg in 1 mL of 0.1 M citrate buffer.

Procedure:

Day-1

#### Enzyme Immobilization:

1. In a 2 ml micro-centrifuge tube take 500  $\mu\text{l}$  of the enzyme (Cellulase) and to it add 500  $\mu\text{l}$  of the 5% Sodium alginate solution. Put the cap tightly, and then mix properly.
2. Take 100 ml of Calcium chloride (0.2 M) solution in a conical flask (150 ml size). Crush the ice and keep the flask on ice.
4. Take 200 $\mu\text{l}$  from the alginate and enzyme mix in a micropipette, and add drop wise to the Calcium chloride solution. While adding make sure that the flask is swirled gently (Keep on a magnetic stirrer).
5. Leave the immobilized enzyme beads to harden in the Calcium chloride solution overnight at  $4^\circ\text{C}$  for hardening.
6. The alginate will be ionically cross-linked by the calcium ions.

## Day-2

### Detection of Enzymatic Activity of Immobilized enzymes by Cellulase Assay:

1. Test tubes and microcentrifuge tubes were marked T1, T2, T3, T4 & T5
2. Samples were prepared with the following compositions,

Sl. No.	Samples	CMC	dH <sub>2</sub> O	Enzyme	Beads
1.	T0 (Blank)	0.25 ml	0.75 ml	--	--
2.	T1	0.25 ml	0.74 ml	10 $\mu$ l	--
3.	T2	0.25 ml	0.725 ml	25 $\mu$ l	--
4.	T3	0.25 ml	0.70 ml	50 $\mu$ l	--
5.	T4	0.25 ml	0.675 ml	75 $\mu$ l	--
6.	T5	0.25 ml	0.75 ml	--	10

3. These microcentrifuge tubes were incubated at 50°C for 30 minutes

4. After incubation, T5 tube was spun at 8000 rpm for 2 min.

### DNS Assay:

T0 (Blank)—1.0 ml of the reaction mix.

T1----- 1.0 ml of the reaction mix.

T2----- 1.0 ml of the reaction mix.

T3----- 1.0 ml of the reaction mix.

T4----- 1.0 ml of the reaction mix.

T5----- 1.0 ml of the reaction mix.

Add 1.0 ml of DNS to each tube

Keep in boiling bath for 5 min.



Then cool it down for 5-10 min, and  
Add 0.35 ml of Rochelle salt

Cool it down

Check OD at 540 nm

### Observations:

Sl. No.	Stock Concentration of enzyme (mg/ml)	Sample no.	Vol. of enzyme ( $\mu$ l)	Working concentration of enzyme (mg/ml)	OD <sub>540</sub>
1.	T0 (Blank)	T0 (Blank)			
2.		T1			
3.		T2			
4.		T3			
5.		T4			
6.		T5			

### Calculations and Result:

Concentration of enzyme = 1 mg/ 10 ml  $(1 \text{ mg/ml})$

Enzyme: Alginate = 1:1

OD

Concentration of enzyme ( $\mu\text{g}/\mu\text{l}$ )

Enzyme activity

Concentration of enzyme ( $\mu\text{g}/\mu\text{l}$ )

To determine:

$$\text{Efficiency of immobilized enzyme} = \frac{\text{Activity of the free enzyme} \times 100}{\text{Activity of the immobilized enzyme}}$$