# Immobilization of alpha amylase

Literature

A biocatalyst is termed immobilized, if its mobility has been restricted by chemical means. Immobilization of enzymes refers to techniques which represent variety of advantages over free enzyme catalysis including increased stability of enzyme, easy recovery of enzyme, easy separation of reactant and product, repeated or continuous use of a single batch of enzyme1 (Varavinit et al., 2002) which will ultimately save the enzyme, labor and overhead costs (Gerhartz, 1990).

Immobilized enzymes have been widely used for many years in different industrial processes. Usually, immobilization of enzymes is carried out by three principle means, matrix assisted entrapment of enzyme, adsorption on a solid support, ionic or covalent binding (Swaisgood, 1985; Zaborsky, 1973). Entrapment is taken as the most preferable method because it prevents excessive loss of enzyme activity after immobilization, increases enzyme stability in microenvironment of matrix, protects enzyme from microbial contamination (Cabral and Kennedy, 1993).

Physical entrapment of á-amylase in calcium alginate beads has shown to a relatively easy, rapid and safe technique (Dey et al., 2003) in comparison with other immobilization methods. The method method of immobilization should be such that an enzyme faces as little conformational change as possible. The nature of the solid support or matrix plays an important role in retaining the actual confirmation and activity of enzyme in the processes that utilized immobilized biocatalysts.

Thermostable á-amylase is one of the most important and widely used enzymes whose spectrum of application has widened in food, paper and detergent industries (Glazer et al., 1994, Nigam and Singh, 1995). These

industries would find their boosted economy if á-amylase can be re-used which is possible by their immobilization. Therefore, the present study is attempted to immobilize á-amylase produced by Bacillus subtilis KIBGE-HAR by entrapment in calcium alginate beads. We also compared the kinetics of free and immobilized á-amylase in order to explore the benefits of immobilization of enzymes.

Discussions Because enzymes are biological catalysts that promote the rate of reactions but are not themselves consumed in the reactions in which they participate, they may be used repeatedly for as long as they remain active. However, in most of the industrial, analytical, and clinical processes, enzymes are mixed in a solution with substrates and cannot be economically recovered after the exhaustion of the substrates. This single use is obviously quite wasteful when the cost of enzymes is considered.

Thus, there is an incentive to use enzymes in an immobilized or insolubilized form so that they may be retained in a biochemical reactor to catalyze further the subsequent feed. The use of an immobilized enzyme makes it economically feasible to operate an enzymatic process in a continuous mode. Numerous methods exist for enzyme immobilization, sometimes referred to as enzyme insolubilization. The overwhelming majority of the methods can be classified into four main categories: matrix entrapment, microencapsulation, adsorption, and covalent binding. Of these methods, matrix entrapment is the focus of this experiment.

Many entrapment methods are used today, and all are based on the physical occlusion of enzyme molecules within a " caged" gel structure such that the diffusion of enzyme molecules to the surrounding medium is severely

limited, if not rendered totally impossible. What creates the " wires" of the cage is the cross-linking of polymers. A highly cross-linked gel has a fine " wire mesh" structure and can more effectively hold smaller enzymes in its cages. The degree of cross-linking depends on the condition at which polymerization is carried out.

Because there is a statistical variation in the mesh size, some of the enzyme molecules gradually diffuse toward the outer shell of the gel and eventually leak into the surrounding medium. Thus, even in the absence of loss in the intrinsic enzyme activity, there is a need to replenish continually the lost enzymes to compensate for the loss of apparent activity. In addition, because an immobilized enzyme preparation is used for a prolonged period of operation, there is also a gradual, but noticeable, decline in the intrinsic enzyme activity even for the best method. Eventually, the entire immobilized enzyme packing must be replaced.

Besides the leakage of enzymes, another problem associated with the entrapment method of immobilization is the mass transfer resistance to substrates, products, and inhibitors. Because the average diameter of a typical bead of enzyme impregnated gel is much larger compared to the average diffusion length, substrate cannot diffuse deep into the gel matrix, as in any other conventional non-biological immobilized catalysts. At the same time, the diffusional resistance encountered by the product molecules can sometimes cause the product to accumulate near the center of the gel to an undesirable high level, leading to product inhibition for some enzymes. Thus, ideally the network of cross-linking should be coarse enough so that

the passage of substrate and product molecules in and out of a gel bead is as unhindered as possible.

For this reason, entrapment is not suitable for special cases where the substrate has a large molecular weight such that it cannot easily move freely in the gel matrix. Unlike the adsorption and covalent bonding methods, most polymerization reactions that cause cross-linking and gel formation in entrapment methods do not directly involve the formation of bonds between the support material and the enzyme molecules. There are reports that these bonds change the conformation of the enzyme protein and modify the enzyme properties. Since the enzyme molecules do not themselves participate in the polymerization reaction in the entrapment methods, the same entrapment techniques can be successfully applied to a wide range of enzymes with only minor modifications between different enzymes.

Review of literature

Three different commonly used entrapment media will be introduced in this experiment: polyacrylamide, calcium alginate, and gelatin. All these gels can be formed with a simple set of equipment and share similar procedures. In all the protocols, enzymes are well mixed with monomers/polymers and cross-linking agents in a solution. The solution is then exposed to polymerization promoters to start the process of gel formation. The solution is poured into a mold to achieve the desired shapes. A gel block may be cut into smaller cubes to increase the surface area. Commercially, it is common to force the unpolymerized solution through a set of nozzles to form spherical beads, whose size can be controlled by adjusting the back pressure.

The resulting beads may be further hardened to enhance structural integrity. Of the three gels, polyacrylamide is the most widely used matrix for entrapping enzymes. It has the advantage that it is non-ionic. The consequence is that the properties of the enzymes are only minimally modified in the presence of the gel matrix. At the same time, the diffusion of the charged substrate and products is not affected, neither. However, dimethylaminopropionitrile, the polymerization initiator, is highly toxic and must be handled with great care. The requirement to purge the monomer solution with nitrogen is also troublesome, although not totally crippling. Calcium alginate is just as widely used as polyacrylamide.

Unlike polyacrylamide gels, gelation of calcium alginate does not depend on the formation of more permanent covalent bonds between polymer chains. Rather, polymer molecules are cross-linked by calcium ions. Because of this, calcium alginate beads can be formed in extremely mild conditions, which ensure that enzyme activity yields of over 80% can be routinely achieved. However, just as easily as calcium ions can be exchanged for sodium ions, they can also be displaced by other ions. This property can both be advantageous and disadvantageous. If needed, enzymes or microbial cells can be easily recovered by dissolving the gel in a sodium solution. On the other hand, proper caution must be exercised to ensure that the substrate solution does not contain high concentrations of those ions that can disintegrate the gel.

The main attraction of using gelatin as the immobilization media is that the gel formation process requires only simple equipment and that the reagents are relatively inexpensive and nontoxic. The retention of enzymatic activities

for immobilization with a gelatin gel is typically 25-50% of the original free enzyme. Gelatin gel has the advantage that the mass transfer resistance is relatively low compared to other entrapment methods, but the rate of enzyme loss due to leakage is high. In summary, the efficiency of an immobilization process can be measured by the following criteria. Most important of all, a high percentage of the enzymes must be initially retained in gel matrices. Secondly, the enzyme activity must be preserved. And thirdly, the enzymes must be physically restrained from diffusing back into the substrate solution at a later time. It is quite difficult to create a fine and uniform mesh so as to prevent the entrapped enzymes from leaking out of the matrices. On the other hand, highly cross-linked matrices can result in higher mass transfer resistances for both the substrate and the product. Bacterial alpha-amylase will be used in this experiment to demonstrate and compare the effectiveness of various entrapment techniques. The class will be divided into groups of three. Before attempting the experiment, identify the major steps and devise a work plan among the group members.

Each member should be explicitly assigned an equal portion of the responsibilities. For example, one member may be responsible for making all three types of gels, and other members may be assigned the tasks of measuring the enzyme leakage and immobilized enzyme activities. Alternatively, each member may be responsible for remain responsible throughout all phases of the study associated only one type of gel. It is the responsibility of the entire group to make sure that each member is carrying out the work according to the plan, whatever the plan is. Work closely

together and help each other if needed to coordinate the smooth execution of the plan. Share the data at the end.

Procedures 1. Prepare immobilized enzyme beads: Immobilize alpha-amylase by entrapping it inside gel matrices according to the immobilization protocols accompanying this write-up. See Note 1. 2. Immobilized enzyme activities: Follow a similar procedure as in the previous amylase experiment to measure the activities of the immobilized enzymes. Instead of the enzyme solution, immerse about 1 g of the gel beads prepared in the above step in 10 ml of the buffered starch solution at pH= 7. 0. Constantly shake the mixture to make sure that the solution is not stagnant. As before, add 0. 5 ml of the reacted starch solution to 5ml of the 0. 1N HCl stopping solution. Mix with the iodine solution to detect the presence of residual starch. Rinse the gel beads with water and reuse the same beads twice more, each time measuring the enzyme activities. 3. Shift in the optimal enzymatic condition -- pH effect:

Study the effect of pH on the activities of the immobilized enzymes. Refer to the previous experiment on alpha-amylase. Because of the time limitation, perform this part for only one of the gels of your choice. 4. Recovery from adverse pH condition: Immerse about 1g of the gel bead in 5 ml of the 1N HCl solution. After shaking for 15 minutes, discard the HCl solution and thoroughly wash the gel beads with water. Measure the enzyme activity with buffered (pH= 7. 0) starch solution. Repeat for a 1N KOH solution. 5. Recovery of enzymes: Dissolve the gels and measure the enzyme activities afterward. 6. Enzyme leakage and inactivation: Immerse 5 ml of the fresh gel

which has not been exposed to adverse pH or temperature conditions in 5 ml of water in a test tube for over 24 hours.

Record the duration allowed for enzyme leakage. Measure and report the amylase activity in the surrounding water by following the same procedure as in the previous experiments. In addition measure the activities of the immobilized enzyme gel beads. 7. For Curious Students: Follow the same procedure as in the previous amylase experiment to study the effect of temperature on the activities of the immobilized enzymes. Is there any shift in the optimal temperature?