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16/MHS01/175

BCH 404

QUESTIONS

1. Enumerate and discuss the principal techniques for immobilization of enzymes
2. What is the generation time of a bacteria population that increases from 15,000 to 15,000,000 cells in ten minutes?

ANSWERS

1. Most enzymes are relatively unstable, and have high production and separation costs, displaying a disadvantage in that the recovery of active enzymes in the reaction mixture after use is technically very difficult. Immobilized enzymes have received great attention from those who wish to use the enzyme immobilization technology for specific purposes in the medical and industrial sectors. The term “Immobilized enzymes” is defined as “Enzymes that is physically attached to specific solid supports and thus confined, and which can be used repeatedly and continuously while maintaining their catalytic activities”. In recent years, enzymatic productivity has been rapidly growing through the improvement of genetic engineering technology, microbial cultivation technology and wild type strain screening technology in parallel with the understanding of enzymatic biosynthesis mechanism.

Immobilization is defined as the imprisonment of enzymes in a district support or matrix. The support or matrix on which the enzymes are immobilized allows the exchange of medium containing substrate or effector or inhibitor molecules. The practice of immobilization of cells is very old and the first immobilized enzyme was amino acylase of *Aspergillus oryzae* for the production of L-amino acids in Japan.

TECHNIQUES OF ENZYME IMMOBILIZATION

In an enzymatic reaction, an enzyme acts as a biological catalyst that promotes the reaction rate and is not worn out during the reactions. Thus, it allows for the repeatedly use of enzyme as long as the enzyme remain active. So far, a variety of immobilization procedures have been developed for immobilizing enzyme on a solid surface. The different enzyme immobilization methods are grouped as follows: 1. Adsorption; 2. Covalent bonding; 3. Entrapment; 4. Cross-linking.

ADSORPTION

Enzyme immobilization onto biosensor transducer solid surface by adsorption is one of the most straightforward methods in immobilization. The adsorption mechanisms are based on weak bonds such as Van der Waal’s forces, electrostatic and hydrophobic interactions. Enzyme is dissolved in solution and the solid support is placed in contact with the enzyme solution for a fixed period of time under suitable conditions which sustain enzyme activity. The unadsorbed enzyme molecules are then removed from the surface by washing with buffer. Immobilization by adsorption is a simple and economical process which is reagent-free, low cost and is generally non-destructive toward enzyme activity because it does not involve any functionalization of the support. Nevertheless, this technique presents drawbacks: enzymes are loosely bound to the support by weak physical bonding so that changes in temperature, pH or ionic strength may result in enzyme desorption/leaching. In addition, biosensors based on adsorbed enzyme suffer from poor operational and storage stability because apart from enzyme leaching, non-specific adsorption of other proteins or substances on the transducer surface may cause contamination and interference to signal. Immobilization by adsorption is commonly divided into 3 sub categories as follows: 1. Physical adsorption; 2. Electrostatic binding; 3. Hydrophobic adsorption.

COVALENT BONDING

Enzyme immobilization by covalent binding is one of the most widely used methods, in which stable complexes between functional groups on enzyme molecules and a support matrix are formed through covalent bondings. The functional group present on enzyme, through which a covalent bond with support could be established, should be non-essential for enzymatic activity which usually involves binding via the side chains of lysine (ε-amino group), cysteine (thiol group) and aspartic and glutamic acids (carboxylic group). The enzyme functional groups that could be utilized in covalent coupling include: Amino group, carboxylic group, phenolic group, sulfhydryl group, thiol group, imidazole group, indole group and hydroxyl group. The binding procedure of enzyme to the solid support generally goes through two stages: (1) activation of the surface using linker molecules such as glutaraldehyde or carbodiimide and (2) enzyme covalent coupling to the activated support. Linker molecules are multifunctional reagents (glutaraldehyde or carbodiimide) act as the bridge between surface and enzyme via covalent bonding. While the first group matches the immobilization surface and forms a so-called self-assembled monolayer (SAM), the second ground bound to preactivated support then forms a covalent bond with the enzyme. Different linkers are used for different surfaces (inorganic material, natural or synthetic polymer, membranes) and immobilization protocols (directly onto the transducer surface or onto a thin membrane fixed onto the transducer).

Covalent immobilization provides strong bindings between enzymes and support matrix and therefore little leakage of enzyme from the support may occur. In addition, high uniformity of the SAM layer and good control of the immobilized enzyme amount are the other advantages. In covalent attachment, there is a high risk of enzyme denaturization when most enzymes must go through chemical modifications to possess functional group. In addition, the method requires high volume of bioreagent but only small amounts of enzymes may be immobilized (~0.02 grams per gram of matrix). The immobilization procedure largely increases enzyme stability but decreases enzyme activity in affinity reaction and is poorly reproducible. In comparison to adsorption, covalent bonding requires longer incubation time, since the formation of the SAM and the subsequent linkage of the enzymes to it take several hours. The process is also more complex and care has to be taken to ensure chemical purity so that the SAM is obtained in high homogeneity.

ENTRAPMENT

In entrapment immobilization, enzyme is not directly attached to the support surface but entrapped within a polymeric network which allows only the traverse of substrate and products but retains the enzyme hence enzyme diffusion is constrained. Entrapment immobilization process is conducted through two steps: (1) mixing enzyme into a monomer solution, followed by (2) polymerization of monomer solution by a chemical reaction or changing experimental conditions. As an enzyme is physically confined within a polymer lattice network, the enzyme does not chemically interact with the entrapping polymer. The method thus could improve enzyme stability and minimize enzyme leaching and denaturation. Another advantage of the method is the capability to optimize microenvironment for the enzyme by modifying the encapsulating material to have the optimal pH, polarity or amphilicity. However, a limitation of the method is the mass transfer resistance occurred as polymerization extension tends to increase the gel matrix thickness, substrate for this reason cannot diffuse deep into the gel matrix to reach the enzyme active site. Furthermore, the entrapped enzymes are likely to suffer from leakage if the pores size of the support matrix is too large. The method also has low enzyme loading capacity and the support material could be corrupted as effects of polymerization. There is a variety of procedures used in entrapment immobilization depending on type of entrapment such as electropolymerization, photopolymerization, sol-gel process for lattice or fiber type and microencapsulation for microcapsule type.

CROSS LINKING

Enzyme immobilization by cross-linking is an irreversible method performed by the formation of intermolecular cross-linkages between the enzyme molecules by covalent bonds. The process is carried out with the assistance of a multifunctional reagent which acts as linkers to connect enzyme molecules into three dimensional cross linked aggregates. The immobilized enzyme is present in the reaction mixture and not bound to any support. There are two approaches in cross linking immobilization which are the uses of cross linking enzyme aggregate (CLEA), and cross linking enzyme crystal (CLEC). Both methods require the use of a cross linking agent such as glutaraldehyde to cross-links enzyme molecules via the reactions of the free amino groups of lysine residues on the reactive site of neighboring enzyme molecules. In CLEC-based method, glutaraldehyde is added to cross-link enzyme crystals after crystallization. Enzymes immobilized by CLEC usually possesses significant improvements in mechanical properties thus immobilized enzyme in CLECs are usually stable and having higher efficiency than the untreated forms. CLEA is an improved version of CLEC production which could work in aqueous solutions while CLEC requires the formation of crystals. In CLEA-based method, the addition of salts, organic solvents or non-ionic polymers results in the formation of enzyme aggregates which retain enzyme catalytic properties.

Immobilization by crosslinking is a simple method which based on the strong chemical binding of enzyme biomolecules thus enzyme leakage is minimal. Another advantage of the method is the possibility to adjust microenvironment for enzyme by using suitable stabilizing agents through surface complementarity which helps increase stability. However, the use of glutaraldehyde could result in severe enzyme modifications and possibly lead to enzyme conformational changes and loss of activity. For this reason, inert proteins like gelatin, bovine serum albumin (BSA) may be added during the immobilization process to minimize this drastic modification of enzymes.

1. G = $\frac{t}{n}$

t = 10 hours (600 mins)

n = 3.3 log $\frac{15,000,000}{15,000}$ (9.9)

G = $\frac{600}{9.9}$

G = 60.60 minutes / 1.01 hour