DEPARTMENT OF BIOTECHNOLOGY

Faculty Name	Vinayakumar J
Subject Name	Enzyme Technology and Biotransformation
Subject Code	18BT53

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COURSE DETAILS

Faculty Name : Vinayakumar J

Subject Name: **Enzyme Technology and Biotransformation**

Subject Code: **18BT53**

COURSE OUTCOMES

On completion of this course, the students will be able to

	Course Outcomes	**Knowledge** Level
	Describe about the enzyme and its classification, reaction in order t proceed towards various concepts in biotechnology	Ll
	Understand about the enzyme kinetics which will provide the importance and utility of enzyme towards research	L2
	Discuss about the enzyme immobilization techniques and its application in food, pharmaceutical and chemical industries	L2
	Elaborate about production and purification of enzyme at industrial scale	L2
	Explain about the biotransformation application of enzyme	L2

Mapping of Course Outcomes with Program Outcomes and Program Specific **Outcomes**

BT6404	01	PO2	PO3	PO4	PO5	PO6	PO7	PO8	PO9	PO10	POll	PO12	PSO1	PSO2	PSO3	sO4
C213.1	3	1	-	-	-	1	-	-	-	1	1	1	2	1		
C2l3.2	3	-	-	-	-	1	1	-	-	1	1	1	2	1		
C2l3.3	2	1				1	2			1	1		2			
C2l3.4	2	1				1	1				1		2			
C2l3.5	1					1	2				1		2			

BT6404	01	PO2	PO3	PO4	PO5	PO6	PO7	PO8	PO9	PO10	POll	PO12	PSO1	PSO2	PSO3	sO4
C2l3	3	1				1	1			1	1	1	2	1		

LI — Remember; L2 — Understand; L3 — Apply; L4 — Analyse; L5 — Evaluate; L6 - Create

Mapping Relevancy

1: Slight (Low) 2: Moderate (Medium) 3 Substantial (High) - : No correlation

Module 1

INTRODUCTION TO ENZYMES

1. What are enzymes?
   Enzymes are biomolecules that catalyze chemical/biochemical reactions and the rate is increased.
2. **What are the differences between enzymes and chemical catalysts?**
   Enzymes are very efficient catalysts, often far superior to conventional chemical catalysts, Foremost amongst these are their specificity and selectivity not only for particular reactions but also in their discrimination between similar parts of molecules
3. **List the** six **classes of enzymes.**
   Oxidoreductases, Transferases, hydrolases, Lyases, Isomerases and ligases.
4. **What are the general properties of enzymes?**
   (i) They lower the activation energy of reaction,
   (ii) They do not participate in the reaction, and return to their original form at the end of reaction; (iii) they only increase the reaction rat
5. **What are the objectives of Enzyme Engineering?**
   (i) Improved kinetic properties,
   (ii) Elimination of allosteric regulation,
   (iii) Enhanced substrate and reaction specificity,
   (iv) Increasedthermostability,
   (v) Alteration in optimal pH,
   (vi) Suitability for use inorganic solvents,
   (vii) increased/decreased optimal temperature, etc.
6. **What are Isozymes? Give example.**
   Isozymes (also known as isoenzymes) are enzymes that differ in amino acid sequence but catalyze the same chemical reaction. These enzymes usually display different kinetic parameters. An example of an isozyme is glucokinase, a variant of hexokinase
7. **What are Oxidoreductases? Give example.**
   Oxidoreductasesare involved in redox reactions, i.e., transfer of hydrogen or oxygen atoms between molecules. This class includes: dehydrogenases (hydride transfer), oxidases (e- transfer to 02), oxygenase (oxygen atom transfer from 02), and peroxidases (e- transfer to peroxides). Example, glucose oxidase (EC 1.1.3.4).
8. **What is activation energy?**
   The free energy needed to elevate a molecule from its stable ground state to the unstable transition state is known as activation energy (denoted by AG*).
9. What are coenzymes? Give example.
   An organic cofactor is commonly known as coenzyme. Some of these chemicals such as riboflavin, thiamine and folic acid.
10. **What are Transferases? Give example.**
    Transferasescatalyse the transfer of an atom or group of atoms (like acyl-, alkyl- and glycosylgroups) between two molecules. The transferred groups are different from those transferred by the other classes of enzymes like Oxidoreductases, etc. Example, aspartate aminotransferase (EC 2.6.1.1)
11. **Define metallo —enzymes.**
    Enzymes that use a metal in the active site are called metallo-enzymes.
12. **Describe the enzyme specificity.**
    One of the properties of enzymes that makes them so important as diagnostic and research tools is the specificity they exhibit relative to the reactions they catalyze. A few enzymes exhibit absolute specificity; that is, they will catalyze only one particular reaction. Other enzymes will be specific for a particular type of chemical bond or functional group.
13. What are Hydrolases? Give example.
    Hydrolasesare those enzymes, which catalyze hydrolytic reactions (and their reversals); this class includes esterases, glycosidases, proteases and lipases. Example, chymosin or rennin (EC 3.4.23.4).
14. **What do you mean by the active site of an enzyme?**
    One particular portion of the enzyme surface has a strong affinity for the substrate
15. **What are Lyases? Give example.**
    Lyasesare involved in elimination reactions resulting in the removal of a group of atoms from the substrate molecule. This class includes aldolases, decarboxylase, dehydratases and some pectinases. Example, histidine ammonia lyase (EC 4.3.1.3).

16. **What are the assumptions in induced fit model?**
The active site is continually reshaped by interactions with the substrate as the substrate interacts with the enzyme.
17. **What are Isomerases? Give example.**
Isomerasescatalyse the formation of isomers of molecules; they include epimerases, racemases and intramoleculartransferases. Example, xylose isomerase (EC 5.3.1.5).
18. **What is the role of entropy in catalysis.**
Entropy is composed of translational, rotational, and internal entropies. When two molecules react without a catalyst there is a loss of rotational and translational entropies.
19. **What are Ligases? Give example.**
Ligases or synthetases catalyze the formation of covalent bonds between two molecules utilizing the energy obtained from hydrolysis of a nucleoside triphosphate like A TP or GTP. Example, glutathione synthase (EC 6.3.2.3).
20. What is meant by **active site?**
An active site is the part of an enzyme that directly binds to a substrate and carries a reaction. It contains catalytic groups which are amino acids that promote formation and degradation of bonds. By forming and breaking these bonds, enzyme and substrate interaction promotes the formation of the transition state structure.
21. **List the** six classes **of enzymes and brief on the role of** each class.

Class	**Chemical Reaction** Catalyzed	Sample Enzymes
Oxidoreductase	Oxidation-reduction in which oxygen and hydrogen are gained or lost	Cytochrome oxidase, lactate dehydrogenase
Transferase	Transfer of functional groups, such as an amino group, acetyl group, or phosphate group	Acetate kinase, alanine deaminase
Hydrolase	Hydrolysis (addition of water)	Lipase, sucrase
Lyase	Removal of groups of atoms without hydrolysis	Oxalate decarboxylase, isocitratelyase
Isomerase	Rearrangement of atoms within a molecule	Glucose-phosphate isomerase, alanine racemase
Ligase	Joining of two molecules (using energy usually derived from the breakdown of ATP)	Acetyl-CoA synthetase, DNA ligase

.'2. Outline the concept of active site and energetics of ES complex formation

- The enzyme's active site binds to the substrate.
- Increasing the temperature generally increases the rate of a reaction, but dramatic changes in temperature and pH can denature an enzyme, thereby abolishing its action as a catalyst.
- The induced fit model states an enzyme binds to an active site and both change shape slightly, creating an ideal fit for catalysis.
- When an enzyme binds its substrate it forms an enzyme-substrate complex.
- Enzymes promote chemical reactions by bringing substrates together in an optimal orientation, thus creating an ideal chemical environment for the reaction to occur.
- The enzyme will always return to its original state at the completion of the reaction.

23. **Explain the Koshland Induced fit Hypothesis**
Daniel E Koshland formulated this hypothesis in 1959.According to this hypothesis the active site does not have a rigid lock and key conformation. The binding of the substrate moleculeto the enzyme molecule induces to modify the shape of the active site so that it becomes complementary to the

substrate molecule. This is called induced fit. Induced fit is possible because of the flexibility of the protein molecules.

24. What are monomeric and oligomeric enzymes? Give examples

Enzymes having only one polypeptide chain are called monomeric enzymes. eg. DNA Polymerase
Enzymes formed by non covalent bonding of a few monomers are called oligomeric enzymes. eg. pyruvate kimase.

PART B

1. How the enzyme commission developed a system of classification and its recommendations on nomenclature.

The first general principle of these 'Recommendations' is that names purporting to be names of enzymes, especially those ending in -ase, should be used only for single enzymes, i.e. single catalytic entities. They should not be applied to systems containing more than one enzyme. When it is desired to name such a system on the basis of the overall reaction catalysed by it, the word system should be included in the name. For example, the system catalysing the oxidation of succinate by molecular oxygen, consisting of succinate dehydrogenase, cytochrome oxidase, and several intermediate carriers, should not be named succinate oxidase, but it may be called the succinate oxidase system. Other examples of systems consisting of several structurally and functionally linked enzymes (and cofactors) are the pyruvate dehydrogenase system, the similar 2-oxoglutarate dehydrogenase system, and the fatty acid synthase system.

The second general principle is that enzymes are principally classified and named according to the reaction they catalyse. The chemical reaction catalysed is the specific property that distinguishes one enzyme from another, and it is logical to use it as the basis for the classification and naming of enzymes.

Several alternative bases for classification and naming had been considered, e.g. chemical nature of the enzymes (whether it is a flavoprotein, a hemoprotein, a pyridoxal-phosphate protein, a copper protein, and so on), or chemical nature of the substrate (nucleotides, carbohydrates, proteins, etc.). The first cannot serve as a general basis, for only a minority of enzymes have such identifiable prosthetic groups. The chemical nature of the enzyme has, however, been used exceptionally in certain cases where classification based on specificity is difficult, for example, with the peptidases (subclass EC 3.4). The second basis for classification is hardly practicable, owing to the great variety of substances acted upon and because it is not sufficiently informative unless the type of reaction is also given. It is the overall reaction, as expressed by the formal equation, that should be taken as the basis. Thus, the intimate mechanism of the reaction, and the formation of intermediate complexes of the reactants with the enzyme is not taken into account, but only the observed chemical change produced by the complete enzyme reaction. For example, in those cases in which the enzyme contains a prosthetic group that serves to catalyse transfer from a donor to an acceptor (e.g. flavin, biotin, or pyridoxal-phosphate enzymes) the name of the prosthetic group is not normally included in the name of the enzyme. Nevertheless, where alternative names are possible, the mechanism may be taken into account in choosing between them.

A consequence of the adoption of the chemical reaction as the basis for naming enzymes is that a systematic name cannot be given to an enzyme until it is known what chemical reaction it catalyses. This applies, for example, to a few enzymes that have so far not been shown to catalyse any chemical reaction, but only isotopic exchanges; the isotopic exchange gives some idea of one step in the overall chemical reaction, but the reaction as a whole remains unknown.

A second consequence of this concept is that a certain name designates not a single enzyme protein but a group of proteins with the same catalytic property. Enzymes from different sources (various bacterial, plant or animal species) are classified as one entry. The same applies to isoenzymes (see below). However, there are exceptions to this general rule. Some are justified because the mechanism of the reaction or the substrate specificity is so different as to warrant different entries in the enzyme list. This applies, for example, to the two cholinesterases, EC 3.1.1.7 and 3.1.1.8, the two citrate hydro-lyases, EC 4.2.1.3 and 4.2.1.4, and the two amine oxidases, EC 1.4.3.4 and 1.4.3.6. Others are mainly historical, e.g. acid and alkaline phosphatases (EC 3.1.3.1 and EC 3.1.3.2).

A third general principle adopted is that the enzymes are divided into groups on the basis of the type of reaction catalysed, and this, together with the name(s) of the substrate(s) provides a basis for naming individual enzymes. It is also the basis for classification and code numbers.

Special problems attend the classification and naming of enzymes catalysing complicated transformations that can be resolved into several sequential or coupled intermediary reactions of different types, all catalysed by a single enzyme (not an enzyme system). Some of the steps may be spontaneous non-catalytic reactions, while one or more intermediate steps depend on catalysis by the enzyme. Wherever the nature and sequence of intermediary reactions is known or can be presumed with confidence, classification and naming of the enzyme should be based on the first enzyme-catalysed step that is essential to the subsequent transformations, which can be indicated by a supplementary term in parentheses, e.g. acetyl-CoA:glyoxylate C-acetyltransferase (thioester-hydrolysing, carboxymethyl-forming) (EC 2.3.3.9)
he first Enzyme Commission, in its report in 1961, devised a system for classification of enzymes that also serves as a basis for assigning code numbers to them. These code numbers, prefixed by EC, which are now widely in use, contain four elements separated by points, with the following meaning:

(i) the first number shows to which of the six main divisions (classes) the enzyme belongs,

(ii) the second figure indicates the subclass,

(iii) the third figure gives the sub-subclass,

(iv) the fourth figure is the serial number of the enzyme in its sub-subclass.

The subclasses and sub-subclasses are formed according to principles indicated below.

The main divisions and subclasses are:

Class 1. Oxidoreductases.

To this class belong all enzymes catalysing oxidoreduction reactions. The substrate that is oxidized is regarded as hydrogen donor. The systematic name is based on donor:acceptor oxidoreductase. The common name will be dehydrogenase, wherever this is possible; as an alternative, reductase can be used. Oxidase is only used in cases where 02 is the acceptor.

The second figure in the code number of the oxidoreductases, unless it is 11, 13, 14 or 15, indicates the group in the hydrogen (or electron) donor that undergoes oxidation: 1 denotes a -CHOH- group, 2 a -CHO or -CO-COOH group or carbon monoxide, and so on, as listed in the key.

The third figure, except in subclasses EC 1.11, EC 1.13, EC 1.14 and EC 1.15, indicates the type of acceptor involved: 1 denotes NAD(P)+, 2 a cytochrome, 3 molecular oxygen, 4 a disulfide, 5 a quinone or similar compound, 6 a nitrogenous group, 7 an iron-sulfur protein and 8 a flavin. In subclasses EC 1.13 and EC 1.14 a different classification scheme is used and sub-subclasses are numbered from 11 onwards.

It should be noted that in reactions with a nicotinamide coenzyme this is always regarded as acceptor, even if this direction of the reaction is not readily demonstrated. The only exception is the subclass EC 1.6, in which NAD(P)H is the donor; some other redox catalyst is the acceptor.

Although not used as a criterion for classification, the two hydrogen atoms at carbon-4 of the dihydropyridine ring of nicotinamide nucleotides are not equivalent in that the hydrogen is transferred stereospecifically.

Class 2. Transferases.

Transferases are enzymes transferring a group, e.g. a methyl group or a glycosyl group, from one compound (generally regarded as donor) to another compound (generally regarded as acceptor). The systematic names are formed according to the scheme donor:acceptor grouptransferase. The common names are normally formed according to acceptor grouptransferase or donor grouptransferase. In many cases, the donor is a cofactor (coenzyme) charged with the group to be transferred. A special case is that of the transaminases (see below).

Some transferase reactions can be viewed in different ways. For example, the enzyme-catalysed reaction

X-Y + Z = X + Z-Y
may be regarded either as a transfer of the group Y from X to Z, or as a breaking of the X-Y bond by the introduction of Z. Where Z represents phosphate or arsenate, the process is often spoken of as 'phosphorolysis' or 'arsenolysis', respectively, and a number of enzyme names based on the pattern of phosphorylase have come into use. These names are not suitable for a systematic nomenclature, because there is no reason to single out these particular enzymes from the other transferases, and it is better to regard them simply as Y-transferases.

In the above reaction, the group transferred is usually exchanged, at least formally, for hydrogen, so that the equation could more strictly be written as:

X-Y + Z-H = X-H + Z-Y.
Another problem is posed in enzyme-catalysed transaminations, where the -NH2 group and -H are transferred to a compound containing a carbonyl group in exchange for the =O of that group, according to the general equation:

Rl-CH(-NH2)-R2 + R3-CO-R4 [arrow right] R1-CO-R2 + R3-CH(-NH2)-R4.
The reaction can be considered formally as oxidative deamination of the donor (e.g. amino acid) linked with reductive amination of the acceptor (e.g. oxo acid), and the transaminating enzymes (pyridoxal-phosphate proteins) might be classified as oxidoreductases. However, the unique distinctive feature of the reaction is the transfer of the amino group (by a well-established mechanism involving covalent substrate-coenzyme intermediates), which justified allocation of these enzymes among the transferases as a special subclass (EC 2.6.1, transaminases).

The second figure in the code number of transferases indicates the group transferred; a one-carbon group in EC 2.1, an aldehydic or ketonic group in EC 2.2, an acyl group in EC 2.3 and so on.

The third figure gives further information on the group transferred; e.g. subclass EC 2.1 is subdivided into methyltransferases (EC 2.1.1), hydroxymethyl- and formyltransferases (EC 2.1.2) and so on; only in subclass EC 2.7, does the third figure indicate the nature of the acceptor group.

Class 3. Hydrolases.

These enzymes catalyse the hydrolytic cleavage of C-O, C-N, C-C and some other bonds, including phosphoric anhydride bonds. Although the systematic name always includes hydrolase, the common name is, in many cases, formed by the name of the substrate with the suffix -ase. It is understood that the name of the substrate with this suffix means a hydrolytic enzyme.

A number of hydrolases acting on ester, glycosyl, peptide, amide or other bonds are known to catalyse not only hydrolytic removal of a particular group from their substrates, but likewise the transfer of this group to suitable acceptor molecules. In principle, all hydrolytic enzymes might be classified as transferases, since hydrolysis itself can be regarded as transfer of a specific group to water as the acceptor. Yet, in most cases, the reaction with water as the acceptor was discovered earlier and is considered as the main physiological function of the enzyme. This is why such enzymes are classified as hydrolases rather than as transferases.

Some hydrolases (especially some of the esterases and glycosidases) pose problems because they have a very wide specificity and it is not easy to decide if two preparations described by different authors (perhaps from different sources) have the same catalytic properties, or if they should be listed under separate entries. An example is vitamin A esterase (formerly EC 3.1.1.12, now believed to be identical with EC 3.1.1.1). To some extent the choice must be arbitrary; however, separate entries should be given only when the specificities are sufficiently different.

Another problem is that proteinases have 'esterolytic' action; they usually hydrolyse ester bonds in appropriate substrates even more rapidly than natural peptide bonds. In this case, classification among the peptide hydrolases is based on historical priority and presumed physiological function.

The second figure in the code number of the hydrolases indicates the nature of the bond hydrolysed; EC 3.1 are the esterases; EC 3.2 the glycosylases, and so on.

The third figure normally specifies the nature of the substrate, e.g. in the esterases the carboxylic ester hydrolases (EC 3.1.1), thiolester hydrolases (EC 3.1.2), phosphoric monoester hydrolases (EC 3.1.3); in the glycosylases the O-glycosidases (EC 3.2.1), N-glycosylases (EC 3.2.2), etc. Exceptionally, in the case of the peptidyl-peptide hydrolases the third figure is based on the catalytic mechanism as shown by active centre studies or the effect of pH.

Class 4. Lyases.

Lyases are enzymes cleaving C-C, C-O, C-N, and other bonds by elimination, leaving double bonds or rings, or conversely adding groups to double bonds. The systematic name is formed according to the pattern substrate group-lyase. The hyphen is an important part of the name, and to avoid confusion should not be omitted, e.g. hydro-lyase not 'hydrolyase'. In the common names, expressions like decarboxylase, aldolase, dehydratase (in case of elimination of CO2, aldehyde, or water) are used. In cases where the reverse reaction is much more important, or the only one demonstrated, synthase (not synthetase) may be used in the name. Various subclasses of the lyases include pyridoxal-phosphate enzymes that catalyse the elimination of a §- or y-substituent from an o-amino acid followed by a replacement of this substituent by some other group. In the overall replacement reaction, no unsaturated end-product is formed; therefore, these enzymes might formally be classified as alkyl-transferases (EC 2.5.1...). However, there is ample evidence that the replacement is a two-step reaction involving the transient formation of enzyme-bound n,b(or §,y)-unsaturated amino acids. According to the rule that the first reaction is indicative for classification, these enzymes are correctly classified as lyases. Examples are tryptophan synthase (EC 4.2.1.20) and cystathionine b-synthase (EC 4.2.1.22).

The second figure in the code number indicates the bond broken: EC 4.1 are carbon-carbon lyases, EC
4.2 carbon-oxygen lyases and so on.

The third figure gives further information on the group eliminated (e.g. CO2 in EC 4.1.1, H2O in EC 4.2.1).

Class 5. Isomerases.

These enzymes catalyse geometric or structural changes within one molecule. According to the type of isomerism, they may be called racemases, epimerases, cis-trans-isomerases, isomerases, tautomerases, mutases or cycloisomerases.

In some cases, the interconversion in the substrate is brought about by an intramolecular oxidoreduction (EC 5.3); since hydrogen donor and acceptor are the same molecule, and no oxidized product appears, they are not classified as oxidoreductases, even though they may contain firmly bound NAD(P)+.

The subclasses are formed according to the type of isomerism, the sub-subclasses to the type of substrates.

Class 6. Ligases.

Ligases are enzymes catalysing the joining together of two molecules coupled with the hydrolysis of a diphosphate bond in ATP or a similar triphosphate. The systematic names are formed on the system X:Y ligase (ADP-forming). In earlier editions of the list the term synthetase has been used for the common names. Many authors have been confused by the use of the terms synthetase (used only for Group 6) and synthase (used throughout the list when it is desired to emphasis the synthetic nature of the reaction). Consequently NC-IUB decided in 1983 to abandon the use of synthetase for common names, and to replace them with names of the type X-Y ligase. In a few cases in Group 6, where the reaction is more complex or there is a common name for the product, a synthase name is used (e.g. EC 6.3.2.11 and EC 6.3.5.1).

[EC 1.2.3.4 Transferred entry: now EC 5.6.7.8 - common name].

2. Write short note on (i) Development of enzyme (ii) Induced-Fit Model

In 897, Buchner observed the cell extract from yeast which fermented sugar even though no living cells were present

During 1890's Fisher' suggested the lockand key' model of enzyme action while a mathematical model of enzyme action was proposed by Michaelis and Menten in 1913

In 1926 Sumner crystallized for the first time an enzyme (urease)

The transition state theory of enzyme action was put forth by Pauling in 1948 and in 1951 Pauling and Corey discovered the o-helix and b-sheet structures of enzymes.

Sanger in 1953 determined the amino acid sequence of a protein (insulin). In 1986 Cech discovered catalytic RNA while Lerner and Schutlz developed catalytic antibodies

i) Enz mes are amazingl fast at catal zing reactions and without them chemical reactions in the body would be considerably slower than they are More than a century ago in 1894 Emil Fischer proposed that enzymes worked their magic via a model called the lock and key model which is still used today. However, a more precise model proposed by Daniel Koshland in the 1950s, the induced

Da edK h **nd Induced Fit Model**

The induced fit model is an elaboration on the basic idea of the lock and key model In this model though the key and the enzyme active site do not fit perfectly together Instead the substrate interacts with the active site, and both change their shape to fit together. This still means that only

The basis of chemical reactions is a change in atom arrangement and bonds between atoms When the substrate interacts with the enzyme it undergoes a chemical reaction that allows the atoms to move relative to each other the bonds to possibly lengthen or shorten and the most reactive groups to move closer to each other, causing a shape change. This shape change makes the substrate more

amenable to alteration, as it holds the substrate in a transitional state, which helps speed up the reaction that that enzyme catalyzes.

Advantages of the Induced Fit Model

With the induced fit model, the way that the substrate has to change its structure may be useful in terms of the catalysis itself. It may represent the beginning of the reaction that the enzyme is catalyzing. Conversely, in the lock and key model, the catalysis follows after the substrate fits into the enzyme.

3. Mathematically explain in detail about the transition and collision theory and Compare.

Collision theory basically states that reactants need to do two things for a reaction to occur.

They need to slam into one another with sufficient force

They also need to be at the correct angle

Turns out that getting a reaction to occur is a little like trying to stuff a shopping cart into a long row of shopping carts. If you don't line them up properly, then they won't fit together. If you don't shove your cart in hard enough, then your cart will not fit inside the other carts. And, of course, there also have to be other carts around.

Row of Shopping Carts
Uh, who's driving this thing?

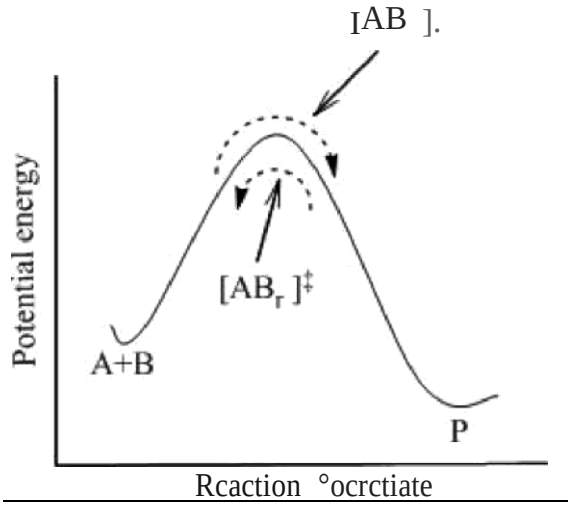
(Source)Let's discuss this a little more scientifically. We can calculate how often molecules or atoms slam into one another, and it's described as the collision frequency (Z). We can also estimate the fraction (II of the total number of collisions that will overcome the activation energy needed to get a reaction started. Finally, we can estimate how many collisions will happen with atoms or molecules lined up properly (1).

Using these factors, chemists have found that collision theory can be expressed mathematically. The rate constant k = Z ^ f 1. Just to be clear, the rate constant, k, is not the same as the rate of the reaction. Rather, the rate constant is used to calculate the rate of the reaction.

One of the things that collision theory helps to explain is why increasing temperatures result in an increase in reaction rates. Collision frequency increases with increasing temperature because atoms and molecules move more rapidly at higher temperatures. Temperature also increases the energy of collisions, which increases the value of f. Essentially, increasing temperature is a two-for-one deal for increasing the rate constant.

Transition-state theory goes hand-in-hand with collision theory. Transition-state theory states that a reaction follows a distinct reaction path that involves bonds being formed and being broken simultaneously. It's basically like watching a reaction in super-slow-motion, where atoms or molecules move and change position. Bond breaking and formation require energy, which is the reason that reactions have an **activation energy** to overcome. Remember, activation energy is the minimum amount of energy needed for a reaction to occur.

Another important concept in transition-state theory is the idea of an **activated complex.** Basically, the activated complex is a weird hybrid thing that is formed which is neither reactants nor products. As two reactants come closer and closer together, the atoms in each molecule start to move in response. Bonds between the reactants may start to break and new product bonds may start to form.



Activated complexes are highly unstable and are not observed *becausethe* y are unstable. The activated complex is the point in a reaction where its **potential energy** is highest.

4. **(i) Explain any three reactions involving oxidoreductase.**

Oxidation and reduction. Enzymes that carry out these reactions are called oxidoreductases. For example, alcohol dehydrogenase converts primary alcohols to aldehydes.

$$H_3CCH_2OH + NAD \rightleftharpoons H_3CCHO + NADH + H^+$$

In this reaction, ethanol is converted to acetaldehyde, and the *cofactor,* NAD, is converted to NADH. In other words, ethanol is oxidized, and NAD is reduced. (The charges don't balance, because NAD has some other charged groups.) Remember that in redox reactions, one substrate is oxidized and one is reduced.

Accepted **name:** alcohol dehydrogenase

Reaction: (1) a primary alcohol + NAD“ an aldehyde + NADH + H“ (2) a secondary alcohol + NAD“ = a ketone + NADH + H“

Other name(s): aldehyde reductase; ADH; alcohol dehydrogenase (NAD); aliphatic alcohol dehydrogenase; ethanol dehydrogenase; NAD-dependent alcohol dehydrogenase; NAD-specific aromatic alcohol dehydrogenase; NADH-alcohol dehydrogenase; NADH-aldehyde dehydrogenase; primary alcohol dehydrogenase; yeast alcohol dehydrogenase

Systematic name: alcohol:NAD“ oxidoreductase

Comments: A zinc protein. Acts on primary or secondary alcohols or hemi-acetals with very broad specificity; however the enzyme oxidizes methanol much more poorly than ethanol. The animal, but not the yeast, enzyme acts also on cyclic secondary alcohols.

Accepted name: aldehyde dehydrogenase (NAD)

Reaction: an aldehyde + NAD“ HzO = a carboxylate + NADH + H“

Other name(s): CoA-independent aldehyde dehydrogenase; m-methylbenzaldehyde dehydrogenase; NAD-aldehyde dehydrogenase; NAD-dependent 4-hydroxynonenal dehydrogenase; NAD-dependent aldehyde dehydrogenase; NAD-linked aldehyde dehydrogenase; propionaldehyde dehydrogenase; aldehyde dehydrogenase (NAD)

Systematic name: aldehyde:NAD“ oxidoreductase

Comments: Wide specificity, including oxidation of D-glucuronolactone to D-glucarate. Formerly EC 1.1.1.70.

Accepted **name:** dihydropyrimidine dehydrogenase (NAD“)

Reaction: (1) 5,6-dihydrouracil + NAD+ = uracil + NADH + H+
(2) 5,6-dihydrothymine + NAD“ = thymine + NADH + H“

Other name(s): dihydropyrimidine dehydrogenase; dihydrothymine dehydrogenase; pyrimidine reductase; thymine reductase; uracil reductase; dihydrouracil dehydrogenase (NAD“)

Systematic name: 5,6-dihydropyrimidine:NAD“ oxidoreductase

Comments: An iron-sulfur flavoenzyme. The enzyme was originally discovered in the uracil-fermenting bacterium, *Clostridium uracilicum,* which utilizes uracil and thymine as nitrogen and carbon sources for growth . Since then the enzyme was found in additional organisms including *Alcaligene eutrophus , Pseudomonas* strains and *Escherichia coli* .

(ii) Explain the Strain and transition state Mechanism of enzyme action (Nov **2016)**

This is the principal effect of induced fit binding, where the affinity of the enzyme to the transition state is greater than to the substrate itself. This induces structural rearrangements which strain substrate bonds into a position closer to the conformation of the transition state, so lowering the energy difference between the substrate and transition state and helping catalyze the reaction.

However, the strain effect is, in fact, a ground state destabilization effect, rather than transition state stabilization effect. Furthermore, enzymes are very flexible and they cannot apply large strain effect.

In addition to bond strain in the substrate, bond strain may also be induced within the enzyme itself to activate residues in the active site.

For example:

Substrate, bound substrate, and transition state conformations of lysozyme.

The substrate, on binding, is distorted from the half chair conformation of the hexose ring (because of the steric hindrance with amino acids of the protein forcing the equatorial c6 to be in the axial position) into the chair conformation

5. Explain with neat diagram the various models of enzyme action on substrate.

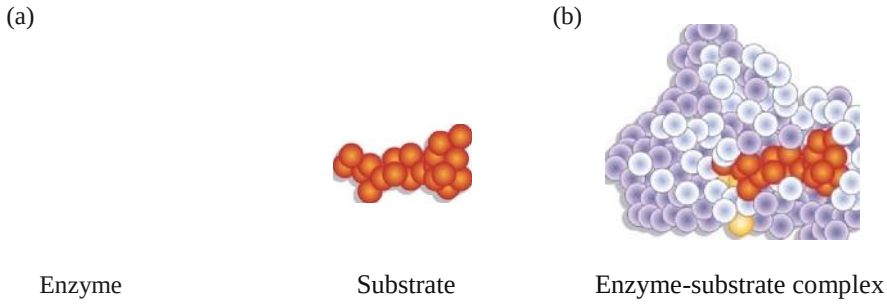
Enzyme-catalyzed reactions occur in at least two steps. In the first step, an enzyme molecule (E) and the substrate molecule or molecules (S) collide and react to form an intermediate compound called the *enzyme-substrate* (E S) *complex.* (This step is reversible because the complex can break apart into the original substrate or substrates and the free enzyme.) Once the E S complex forms, the enzyme is able to catalyze the formation of product (P), which is then released from the enzyme surface:

S + E E—SE—S P + E

Hydrogen bonding and other electrostatic interactions hold the enzyme and substrate together in the complex. The structural features or functional groups on the enzyme that participate in these interactions are located in a cleft or pocket on the enzyme surface. This pocket, where the enzyme combines with the substrate and transforms the substrate to product is called the active site of the

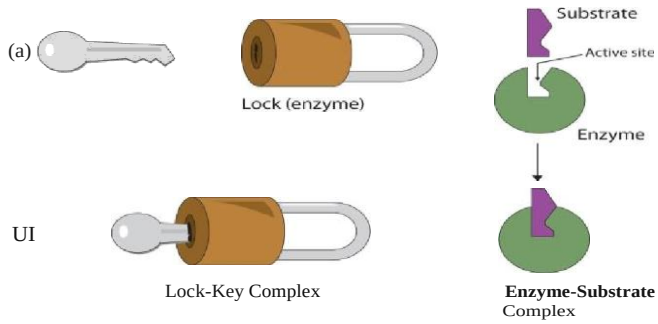
enzyme. It possesses a unique conformation (including correctly positioned bonding groups) that is complementary to the structure of the substrate, so that the enzyme and substrate molecules fit together in much the same manner as a key fits into a tumbler lock. In fact, an early model describing the formation of the enzyme-substrate complex was called the lock-and-key model . This model portrayed the enzyme as conformationally rigid and able to bond only to substrates that exactly fit the active site.

lsubstrate Binding to the ***Active Site of*** *an* ***Enzyme***



The enzyme dihydrofolate reductase is shown with one of its substrates. NADP" (a) unbound and (b) bound. The NADP" (shown in red) binds to a pocket that is complementary to it in shape and ionic properties.

The Lock-and-Key Model ofEnzyme Action

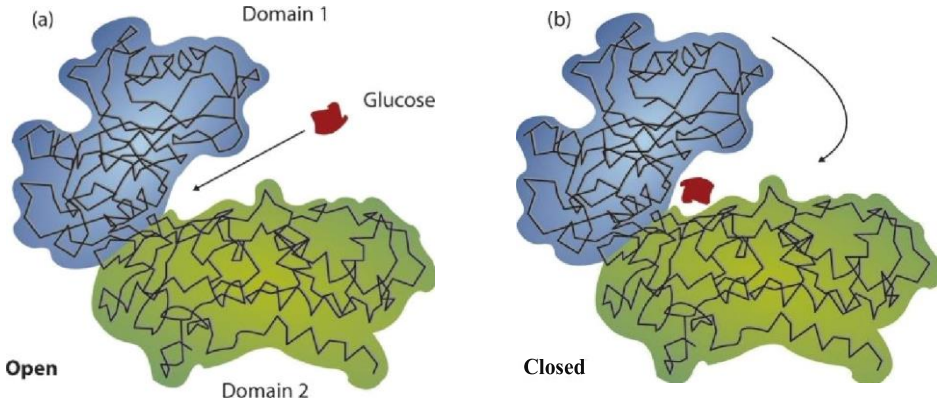


(a) Because the substrate and the active site of the enzyme have complementary structures and bonding groups, they fit together as a key fits a lock. (b) The catalytic reaction occurs while the two are bonded together in the enzyme-substrate complex.

Working out the precise three-dimensional structures of numerous enzymes has enabled chemists to refine the original lock-and-key model of enzyme actions. They discovered that the binding of a substrate often leads to a large conformational change in the enzyme, as well as to changes in the structure of the substrate or substrates. The current theory, known as the induced-fit model, says that

enzymes can undergo a change in conformation when they bind substrate molecules, and the active site has a shape complementary to that of the substrate only *after* the substrate is bound, as shown for hexokinase in Figure. After catalysis, the enzyme resumes its original structure.

The Induced-Fit Model of Enzyme Action



(a) The enzyme hexokinase without its substrate (glucose, shown in red) is bound to the active site. (b) The enzyme conformation changes dramatically when the substrate binds to it, resulting in additional interactions between hexokinase and glucose.

The structural changes that occur when an enzyme and a substrate join together bring specific parts of a substrate into alignment with specific parts of the enzyme's active site. Amino acid side chains in or near the binding site can then act as acid or base catalysts, provide binding sites for the transfer of functional groups from one substrate to another or aid in the rearrangement of a substrate. The participating amino acids, which are usually widely separated in the primary sequence of the protein, are brought close together in the active site as a result of the folding and bending of the polypeptide chain or chains when the protein acquires its tertiary and quaternary structure. Binding to enzymes brings reactants close to each other and aligns them properly, which has the same effect as increasing the concentration of the reacting compounds.

6. Write detailed note on Active site in enzymes. ACTIVE SITE:

The region which contains the binding and catalytic sites is termed the active site or active center of the enzyme. This comprises only a small proportion of the total volume of the enzyme and is usually at or near the surface, since it must be accessible to substrate molecules. In some cases, X — ray diffraction studies have revealed a clearly defined pocket or elect in the enzyme molecule into which the whole or part of each substrate can fit.

SALIENT FEATURES OF ACTIVE SITE:

The substrate molecules are usually much smaller than the enzyme molecules. They bind to a specific region or site of the enzyme molecule. Such sites are referred to as active site or catalytic site, which possess the following common features.

- ❖ The existence of active site is due to the tertiary or quaternary structure of the enzyme — protein molecules. Loss of native configuration leads to alterations of the active site.
- ❖ The active site of the enzyme consists of a very small portion or part of the enzyme molecule.
- ❖ The active sites are usually in the form of grooves or cervices or pockets occupying a small region in the outer surface of the enzyme molecule.

s• The active site made up of amino acids– the common amino acids found at the active site are serine, asparate, histidine, lysine, cysteine, arginine, glutamate and tyrosine. Among these amino acids, serine is the most frequently found.
â• The arrangement of side chains in the active site is well defined. It provides marked specifically to the enzyme molecule.
s• Water molecules are usually excluded from the active site.
â• The active site often includes both polar and non—polar amino acid residues, creating an arrangement of hydrophilic and hydrophobic microenvironment not found elsewhere on an enzyme molecule. Thus, the function of an enzyme may depend not only on the spatial arrangement of binding and catalytic sites, bus also on the environment in which these sites occur.
•• Co — enzymes or cofactors are present as a part of the active sites in some enzymes.
â• Active site consists of two parts, namely, the substrate binding site and the catalytic site.
â• Only weak forces are used for binding of the substrate with its active site.
s• The configuration of the active site changes only slightly when a substrate approaches it for equilibrium.

The following functional groups present at the active site of the enzyme molecule take part in catalysis:

- —COOH groups of discarboxylic amino acid and terminal —COOH group of a polypeptide chain.
- — NH_2 groups of lysine and terminal– NH_2 groups of a polypeptide chain.
- Guanidine group of arginine
- Imidazole group of histidine.
- —OH group of serine and threonine
- — SH group of cystein and disulfide group of cystine
- Phenolic group of tyrosine, etc.

7. Write detail note on

i) Specificity of enzymes ii)Salient features of Enzymatic reactions

1) One of the properties of enzymes that makes them so important as diagnostic and research tools is the specificity they exhibit relative to the reactions they catalyze. A few enzymes exhibit absolute specificity; that is, they will catalyze only one particular reaction. Other enzymes will be specific for a particular type of chemical bond or functional group. In general, there are four distinct types of specificity:

Absolute specificity - the enzyme will catalyze only one reaction.
Group specificity - the enzyme will act only on molecules that have specific functional groups, such as amino, phosphate and methyl groups.
Linkage specificity - the enzyme will act on a particular type of chemical bond regardless of the rest of the molecular structure.
Stereochemical specificity - the enzyme will act on a particular steric or optical isomer.
Though enzymes exhibit great degrees of specificity, cofactors may serve many apoenzymes. For example, nicotinamide adenine dinucleotide (NAD) is a coenzyme for a great number of dehydrogenase reactions in which it acts as a hydrogen acceptor. Among them are the alcohol dehydrogenase, malate dehydrogenase and lactate dehydrogenase reactions.

Reaction specificity • The same substrate can undergo different types of reactions, each catalysed by a separate enzyme and this is referred to as reaction specificity. • An amino acid can undergo transamination, oxidative deamination, decarboxylation, racemization etc. • The enzymes however, are different for each of these reactions.
Substrate specificity • Absolute substrate specificity: • Certain enzymes act only on one substrate e.g. glucokinase acts on glucose to give glucose 6 - phosphate, urease cleaves urea to ammonia and carbon dioxide • Relative substrate specificity: • Some enzymes act on structurally related substances, • May be dependent on the specific group or a bond present. • The action of trypsin is a good example for group specificity

Bond Specificity: • Most of the proteolytic enzymes are showing group (bond) specificity. • E.g. trypsin can hydrolyse peptide bonds formed by carboxyl groups of arginine or lysine residues in any proteins

Group Specificity: • One enzyme can catalyse the same reaction on a group of structurally similar compounds, • E.g. hexokinase can catalyse phosphorylation of glucose, galactose and mannose.

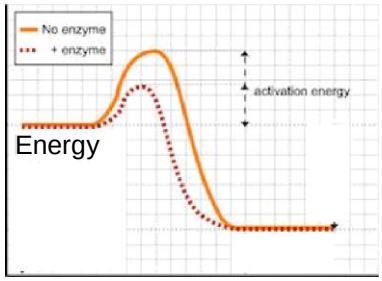
ii) Characteristics of enzymes are as follows:

- Enzymes possess great catalytic power.
- Enzymes are highy specific.
- Enzymes show varying degree of specificities.
- Absolute specificity where the enzymes react specifically with only one substrate.
- Stereo specificity is where the enzymes can detect the different optical isomers and react to only one type of isomer.
- Reaction specific enzymes, these enzymes as the name suggests reacts to specific reactions only.
- Group specific enzymes are those that catalyze a group of substances that contain specific substances.
- The enzyme activity can be controlled but the activity of the catalysts can not be controlled.
- All enzymes are proteins.
- Like the proteins, enzymes can be coagulated by alcohol, heat, concentrated acids and alkaline reagents.
- At higher temperatures the rate of the reaction is faster.
- The rate of the reaction invovlving an enzyme is high at the optimum temperature.
- Enzymes have an optimum pH range within which the enzymes function is at its peak.
- If the substrate shows deviations larger than the optimum temperature or pH, required by the enzyme to work, the enzymes do not function such conditions.
- Increase in the concentration of the reactants, and substrate the rate of the reaction increase until the enzyme will become saturated with the substrate; increase in the amount of enzyme, increases the rate of the reaction.
- Inorganic substances known as activators increase the activity of the enzyme.
- Inhibitors are substances that decrease the activity of the enzyme or inactivate it.
- Competitive inhibitors are substances that reversibly bind to the active site of the enzyme, hence blocking the substrate from binding to the enzyme.
- Incompetitive inhibitors are substances that bind to any site of the enzyme other than the active site, making the enzyme less active or inactive.
- Irreversible inhibitors are substances that from bonds with enzymes making them inactive.

8. Discuss in detail about the mechanisms of enzyme action.(May 2015, May 2017)

Enzymes speed up chemical reactions in the body, making things go faster than they normally would. But how do they accomplish this feat? Well, every reaction has an initial barrier called **activation energy.** Activation energy is like the hump the reaction has to get over before it can get started. Even reactions that net a production of energy still need to break this barrier. Think of it like pushing a car that broke down. It's really hard to get started, but once you and your friends get some momentum going the car starts to roll and you can ease it to the side of the road.

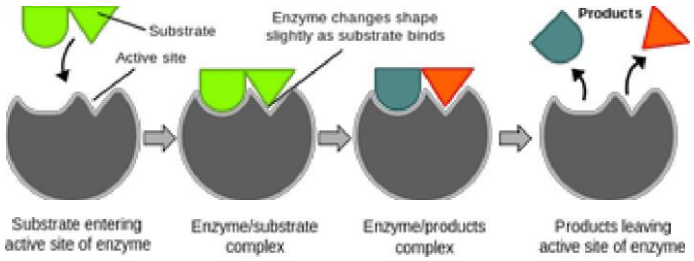
Enzymes lower the activation energy of a reaction, which helps it go faster. Some enzymes, like carbonic anhydrase, which converts carbon dioxide to bicarbonate in the blood, make the reaction proceed nearly a million times faster than without the enzyme just by lowering activation energy. How does the enzyme do this? The answer is in the way the enzyme binds the reactants it works with, called the **substrate.**



Energy used with and without an enzyme

Lock and Key Model

Enzymes and substrates are thought to bind together in a model called lock and key. In this model, the enzyme is considered the lock and the right key - the substrate - fits in it perfectly. Each enzyme is specific to only one or two substrates, giving the enzyme **specificity.** When the enzyme binds the substrate there is a slight change in the shape of the enzyme. It shifts slightly to fit with the substrate better. This is called **induced fit** and is thought of as an extension to the earlier lock and key model. The lock and key not only fit together, but need each other to achieve the final goal.



Induced fit model

When the enzyme binds the substrate, it holds it in a way that orients it for the reaction. For example, if two molecules are being attached by the enzyme, the enzyme holds them in a way that the sites that should be connected are easily accessible. Without the enzyme, the reactants would randomly have to land together this way, which is unlikely.

9. What are the chemical mechanisms that enzymes use to make it easier to get to the transition state?

Enzymologists have determined that a number of mechanisms seem to operate, including:

1. Proximity. Enzymes can bring two molecules together in solution. For example, if a phosphate group is to be transferred from ATP to glucose, the probability of the two molecules coming close together is very low in free solution. After all, there are many other molecules that the ATP and the sugar could collide with. If the ATP and the sugar can bind separately and tightly to a third component—the enzyme's active site—the two components can react with each other more efficiently.
2. Orientation. Even when two molecules collide with enough energy to cause a reaction, they don't necessarily form products. They have to be oriented so that the energy of the colliding molecules is transferred to the reactive bond. Enzymes bind substrates so that the reactive groups are steered to the direction that can lead to a reaction.

3. Induced fit. Enzymes are flexible. In this regard, they are different from solid catalysts, like the metal catalysts used in chemical hydrogenation. After an enzyme binds its substrate(s), it changes conformation and forces the substrates into a strained or distorted structure that resembles the transition state. For example, the enzyme hexokinase closes like a clamshell when it binds glucose. In this conformation, the substrates are forced into a reactive state.

4. Reactive amino acid groups. The side chains of amino acids contain a variety of reactive residues. For example, histidine can accept and/or donate a proton to or from a substrate. In hydrolysis reactions, an acyl group can be bound to a serine side chain before it reacts with water. Having enzymes with these catalytic functions close to a substrate increases the rate of the reactions that use them. For example, a proton bound to histidine can be donated directly to a basic group on a substrate.

5. Coenzymes and metal ions. Besides their amino acid side chains, enzymes can provide other reactive groups. Coenyzmes are biomolecules that provide chemical groups that help catalysis. Like enzymes themselves, coenzymes are not changed during catalysis. This distinguishes them from other substrates, such as ATP, which are changed by enzyme action. Coenzymes, however, are not made of protein, as are most enzymes. Metal ions can also be found in the active sites of a number of enzymes, bound to the enzyme and sometimes to the substrate.

10. What are enzymes? How will you classify it? What are the rules for classification and nomenclature?

Except for some of the originally studied enzymes such as pepsin, rennin, and trypsin, most enzyme names end in "ase". The International Union of Biochemistry (I.U.B.) initiated standards of enzyme nomenclature which recommend that enzyme names indicate both the substrate acted upon and the type of reaction catalyzed. Under this system, the enzyme uricase is called urate: Oz oxidoreductase, while the enzyme glutamic oxaloacetic transaminase (GOT) is called L-aspartate: 2-oxoglutarate aminotransferase.

Enzymes can be classified by the kind of chemical reaction catalyzed.

1. Addition or removal of water

A. Hydrolases - these include esterases, carbohydrases, nucleases, deaminases, amidases, and proteases

B. Hydrases such as fumarase, enolase, aconitase and carbonic anhydrase

2. Transfer of electrons

A. Oxidases

B. Dehydrogenases

Transfer of a radical
Transglycosidases - of monosaccharides
Transphosphorylases and phosphomutases - of a phosphate group
Transaminases - of amino group
Transmethylases - of a methyl group
Transacetylases - of an acetyl group
Splitting or forming a C-C bond
Desmolases
Changing geometry or structure of a molecule
Isomerases
Joining two molecules through hydrolysis of pyrophosphate bond in ATP or other tri-phosphate
Ligases

11. Explain the following (i) Principle of catalysis (ii) Role **of entropy in catalysis(May 2015)**

(i) Catalysis is the study of materials that can control chemical transformations. The ideal catalyst for a given chemical reaction satisfies two general criteria:

- It converts the starting chemicals (reactants) to the desired products with no production of undesired byproducts (in other words, it is perfectly selective)
- It enables the reaction to proceed at a very high rate (it is very active)

In practice, real catalysts must compromise on one or both of these criteria, and the challenge of catalyst design is to find materials that will come as close to optimal performance as possible.

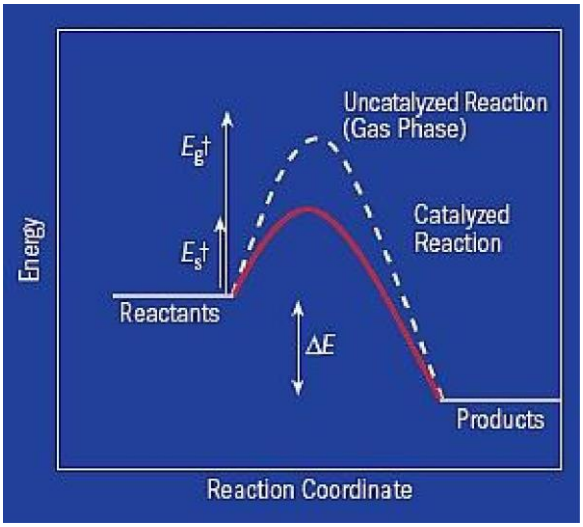


Figure 2. Potential energy surface illustrating how catalysts reduce the activation barriers of reactions.

Complex chemical reactions generally comprise many interrelated elementary reaction steps. Catalysts function by altering the kinetics and thermodynamics of the various elementary steps. By lowering the activation barrier for elementary steps that lead to desired products, for example, the catalyst can increase the rate of formation of those products (figure 2). Conversely, by raising the activation barrier for steps leading to undesired products, the catalyst can suppress the formation of those unwanted chemicals. The catalyst is thus seen to be a sort of master puppeteer, controlling the intricate interactions between different chemical species and elementary reactions to produce desired chemical products with a high degree of activity and selectivity.

Catalysts can take many forms. Among the forms most actively investigated are homogeneous catalysts, wherein isolated metal atoms and associated ligands are suspended in solution, and heterogeneous catalysts, wherein metal clusters or nanoparticles are immobilized on support materials. Both types of catalysts find broad application in the fundamental sciences and industry

Module 2

ENZYME ASSAY AND CO- ENZYMES

What are the factors that determine the rate of the enzymatic reaction?

Concentration of substarte, presence of inhibitors, temperature, pH etc.

1. Define **Km and** Vmax.
   Km and Vmax are MichaelisMenton Parameters. Km is Michaelismenton constant, Km is equal to S at Vamx/2. It is related to affinity of enzyme towards substrate. Vmax is the reaction rate when S is very high.
2. What is uncompetitive **inhibition?**
   Uncompetitive inhibition occurs when the inhibitor binds only to the enzyme—substrate complex, not to the free enzyme; the EIS complex is catalytically inactive. This mode of inhibition is rare and causes a decrease in both max and the *K* value
3. **What is E-H plot?**
   The plot between Vo and Vo/[So] is called E-H plot.
4. Define **turnover number and** kcatal. (Nov **2016,** May **2017)**
   In enzymology, turnover number (also termed kcat) is defined as the maximum number of molecules of substrate that an enzyme can convert to product per catalytic site per unit time and can be calculated as follows: kcat' V g/[E]T.

The katal (symbol: kat) is the SI unit of catalytic activity. For example, is that amount of trypsin which breaks a mole of peptide bonds per second under specified conditions.

5. **What is the importance of Michaelis-Mentonquation?**
   M-M equation describes the kinetics of single substarte single active site enzymatic reactions. The importance of Initial velocity of the reaction for kinetics study was first insisted by M-M.
6. **What are the disadvantages of** M-M **graphical evaluation of** K and V «,?
   The M-M pasrameters from M-M graph is highly significant because of hyperbolic shape of M-M graph.
7. **What is competitive inhibition?**
   In competitive inhibition, the substrate and inhibitor cannot bind to the enzyme at the same time, as shown in the figure on the left. This usually results from the inhibitor having an affinity for the active site of an enzyme where the substrate also binds; the substrate and inhibitor *compete* for access to the enzyme's active site.
8. **Differentiate the sequential and ping-pong bi substrate** enzyme **reactions with an example.**
   The sequential model of allosteric regulation holds that subunits are not connected in such a way that a conformational change in one induces a similar change in the others.
9. What is Hans plot?
   The plot between [o] o and [o] is called Hans plot.
10. What is L-B Plot?
    The plot between 1/V and 1/[S] is called L-B plot.
11. **What is non-competitive inhibition?**
    Non-competitive inhibition is a form of mixed inhibition where the binding of the inhibitor to the enzyme reduces its activity but does not affect the binding of substrate. As a result, the extent of inhibition depends only on the concentration of the inhibitor.
12. **Write the assumptions of Michaelis-Menton kinetics.**
    An equilibrium between enzyme, substrate and ES complex was was instantly setup and maintained, the breakdown of ES complex to product is too slow to disturb the equilibrium.
13. **What is quasi-first order reaction?**
    A single substrate reaction where the substrtate concentration is maintained very higher, therefore the reaction is independent of substrate concentration.
14. **What is substrate inhibition? Explain.**
    Substrate inhibition is where the substrate of an enzyme reaction inhibit the enzyme's activity.
15. **What is denaturation and renaturation of Enzyme?**
    Denaturation is a process in which proteins lose their 3D-structure by application of some external stress or compound for example, treatment of proteins with strong acids or bases, high concentrations of inorganic salts, organic solvents (e.g., alcohol or chloroform), or heat. The original structure of some proteins can be regenerated upon removal of the denaturing agent and restoration of conditions favouring the native state. Proteins subject to this process, called renaturation, include serum albumin from blood, hemoglobin (the oxygen-carrying pigment of red blood cells), and the enzyme ribonuclease. Explain the effect of pH on enzyme action.
16. **Explain the effect of pH on** enzyme action.
    The pH at which the rate or a suitable parameter is a maximum is called the *pH optimum* and the plot of rate or parameter against pH is called a *pH profile*
17. **What are the units to measure enzyme activity?**
    The katal (symbol: kat) is the SI unit of catalytic activity. Non-SI unit is Unit of Enzyme acyivity.
18. **What is the assumption of Briggs-Haldane on Michaelis-Menton Enzyme kinetics?**
    Steady state assumption is that the rate of change of ES is negligible compared to rate of change of product over the initial period of reaction, except during the very brief period.
19. **What is E-C-B Plot?**
    The plot between Vmax and Km at fixed concentrations of [S] is called E-C-B plot.
20. **What are allosteric enzymes?**
    Allosteric enzymes, have the binding of one ligand enhances the attraction between substrate molecules and other binding sites. Example the binding of oxygen molecules to hemoglobin.
21. **Explain the** irreversible **inhibition.**
    Irreversible inhibitors usually covalently modify an enzyme, and inhibition cannot therefore be reversed. Irreversible inhibitors often contain reactive functional groups such as nitrogen mustards, aldehydes, haloalkanes or alkenes.

22. Write the unit of Vmax and Km.
Unit of Vmax — mmol/mL.sec and unit of Km is mmol/mL or mg/ml.
23. What are the different types of reversible enzyme inhibition?
Reversible inhibitors bind to enzymes with non-covalent interactions such as hydrogen bonds, hydrophobic interactions and ionic bonds. Competitive inhibition, uncompetitive inhibition, non-competitive inhibition and mixed inhibition are some types of reversible inhibition.
24. What is enzyme kinetics? (May 2015)
Enzyme kinetics is the study of the chemical reactions that are catalysed by enzymes. In enzyme kinetics, the reaction rate is measured and the effects of varying the conditions of the reaction are investigated. Studying an enzyme's kinetics in this way can reveal the catalytic mechanism of this enzyme, its role in metabolism, how its activity is controlled, and how a drug or an agonist might inhibit the enzyme.
25. What is enzyme **inhibition?**
Enzyme inhibition refers to the decrease of enzyme-related processes. The term seems to be used for two different situations:
- In enzyme production (see protein biosynthesis), inhibition refers to the halting or reduction of the production of an enzyme. This is the opposite of enzyme induction, which triggers or increases production.
- In enzyme activity, inhibition refers to the decrease of an enzyme's activity, caused by a substance called an enzyme inhibitor. The opposite of an enzyme inhibitor is called an enzyme activator.

26. Define allosteric inhibitor. State the effect of it on enzyme binding. (Nov 2016)
Allosteric inhibitor is an effector molecule which binds to a site other than enzyme's active site. Thus decreasing enzymatic activity.

27. Why does Vm but not Km depend on the amount of enzyme used in an enzyme reaction? (May **2017)**
According to MM equation Km is the substrate concentration when Vo- Vol./2. But Vm is proportional to enzyme concentration

$$V = \frac{k_2 E_0 \times S}{Km + S}$$

28. Given the reaction of an enzyme that follows MichaelisMenten kinetics:

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} ES \overset{kp}{\Rightarrow} E + P$$

If K **=30mM** and V«,= 60pMmin '. What is the **initial reaction** velocity at a substrate concentration of **30mM.** (Nov **2015)**

$$\frac{Vmaxx\ S}{Km+S} \qquad \frac{60 \times 10^3 \times 30}{30+30} = 30\text{pM/min}$$

PART B

1. Briefly describe the kinetics of single substrate reactions **and multi** substrate enzyme reactions.

In 1913, the German biochemist Leonor Michaelis (1875 1949) and the Canadian biochemist Maud L. Menten (1879-1960), building on the work of the French chemist Victor Henri (1872-1940), proposed a mechanism to explain thedependence of the initial rate of enzyme-catalyzed reactions on concentration. They considered the following scheme, in which ES is the enzyme—substratecomplex:

$$E + S \underset{}{\overset{k_1}{\rightleftharpoons}} ES \overset{bat}{\longrightarrow} E + P \qquad (1)$$

In this model, the substrate S reversibly associates with the enzyme E in a first step, and some of the resulting complex ES is allowed to break down and yield the product P and the free enzyme back.

We would like to know how to recognize an enzyme that behaves according to this model. One way is to look at the enzyme's kinetic behavior -- at how substrate concentration affects its rate. So we want to know what rate law such an enzyme would obey. If a newly discovered enzyme obeys that rate law, then we can assume that it acts according to this model. Let's derive a rate law from this model.

For this model, let vo be the initial velocity of the reaction. The latter stands for the appearance of the product P in solution (^ d[P]/dt) whose **phenomenological rate equation** (first-order) is given by

ve R ‹[ES] (2),

containing an experimentally measurable (dependent) variable - vo, a kinetic parameter - fat, and another variable unknown to us - [ES].

Before proceeding, one should state (and remember) some implicite assumptions:

• As long as initial velocity is considered, the concentration of product can be neglected (compared to that of the substrate, thus [P] << [S]), and

• The concentration of substrate is in large excess over that of the enzyme ([E] << [S]).

These assumptions, which hold in most kinetic experiments performed in test tubes at low enzyme concentration, are convenient when considering the mass conservation equations for the reactants

[ ]0' [S]free + [ES] + [P] which now approximates to [S]o = [S],

while that for the enzyme is

[E]total = [E]free + [ES] (the possible formation of a complex EP is not considered here).

We want to express vo in terms of measurable (experimentally defined, independent) variables, like [S] and [E]total , so we can see how to test the mechanism by experiments in kinetics. So we must replace the unknown [ES] in (2) with measurables.

During the initial phase of the reaction, *as long as the reaction velocity remains constant,* the reaction is in a steady state, with ES being formed and consumed at the same rate. During this phase, the rate of formation of [ES] (one second order kinetic step) equals its rate of consumption (two first order kinetic steps). According to model(1),

Rate of formation of [ES] = ki[E][S].

Rate of consumption of [ES] = k.}[ES] + fat [ES].

So in the **steady state,**

k.][ES] + at[ES] k][E][S] (3)

Remember that we are trying to solve for [ES] in terms of measurables, so that we can replace it in (2). First, collect the kinetic constants, and the concentrations (variables) in (3):

$$(k + knoi)[ES] = ki[E][S],$$

and $$(l + fat)/k] = [E][S]/[ES] \quad (4)$$

To simplify (4), first group the kinetic constants by defining them as K<:

$$K\% = (k\text{-}1 + kcat)/k1 \quad (5)$$

and then express [E] in terms of [ES] and [E]total, to limit the number of unknowns:

$$[E] — [E]total - [ES] \quad (6)$$

Substitute (5) and (6) into (4):

$$K\% = ([E]total - [ES])[S]/[ES] \quad (7)$$

Solve (7) for [ES]:

First multiply both sides by [ES] :

$$[ES]\ Kjy' \quad [E]total[S] \bullet [ES][S]$$

Then collect terms containing [ES] on the left:

$$[ES]\ K + [ES][S] = [E]total[S]$$

Factor [ES] from the left-hand terms:

$$[ES](Kg + [S])' \quad [E]total[S]$$

and finally, divide both sides by (K+ + [S]):

$$[ES] = [E]total[S]/(Key + [S]) \quad (8)$$

Substitute (8) into (2):

$$vo' \quad kcat[E]total[S]/(Kg + [S]) \quad (9)$$

The maximum velocity Vmax occurs when the enzyme is saturated -- that is, when all enzyme molecules are tied up with S, or [ES]' [E]tota1. Thus,

$$V_{max} = k_{cat}\,[E]_{total} \qquad (10)$$

Substitute Vmax into (9) for kcat [E]total:

$$vo' \quad Vmax[S]/(K\% + [S]) \qquad (11)$$

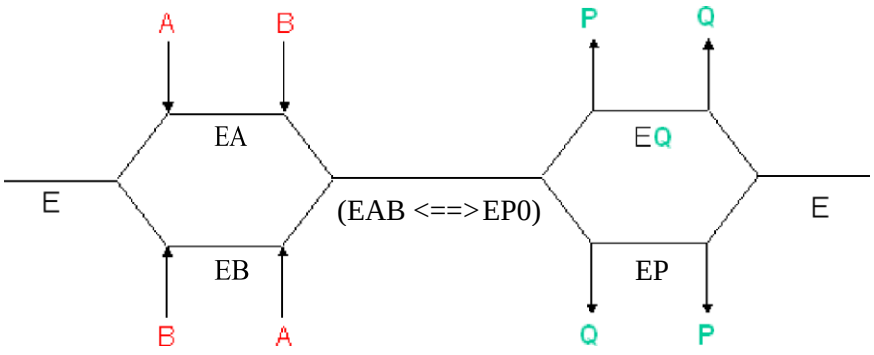
This equation expresses the initial rate of reaction in terms of a measurable quantity, the **initial** substrate concentration. The two kinetic parameters, Va, and K> , will be different for every enzyme-substrate pair.

Sequential Mechanism

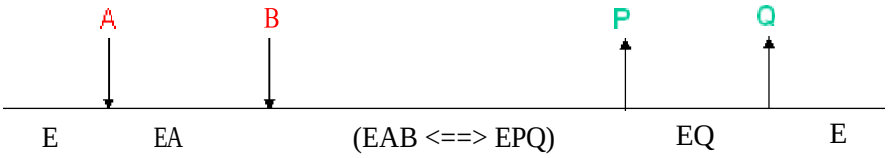
In this mechanism, both substrates must bind to the enzyme before any products are made and released.The substrates might bind to the enzyme in a random fashion (A first then B or vice-versa) or in an ordered fashion (A first followed by B). An abbreviated notationscheme developed by W.W. Cleland is shown below for the sequential random and sequential ordered mechanisms. For both mechanisms, Lineweaver-Burk plots at varying A and different fixed values of B give a series of intersecting lines. Derivative curves can be solved to obtain appropriate kinetic constants.

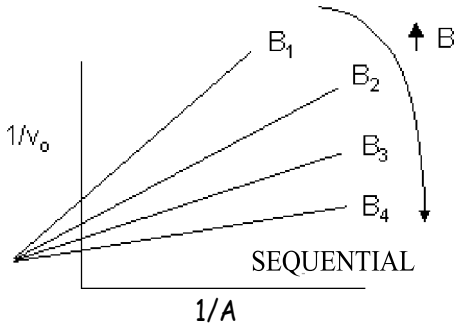
Sequential: R eactants (A,B) both bind before both products (P,Q) are r eleased

A. Random Sequential: random order of reactants binding and products leasing



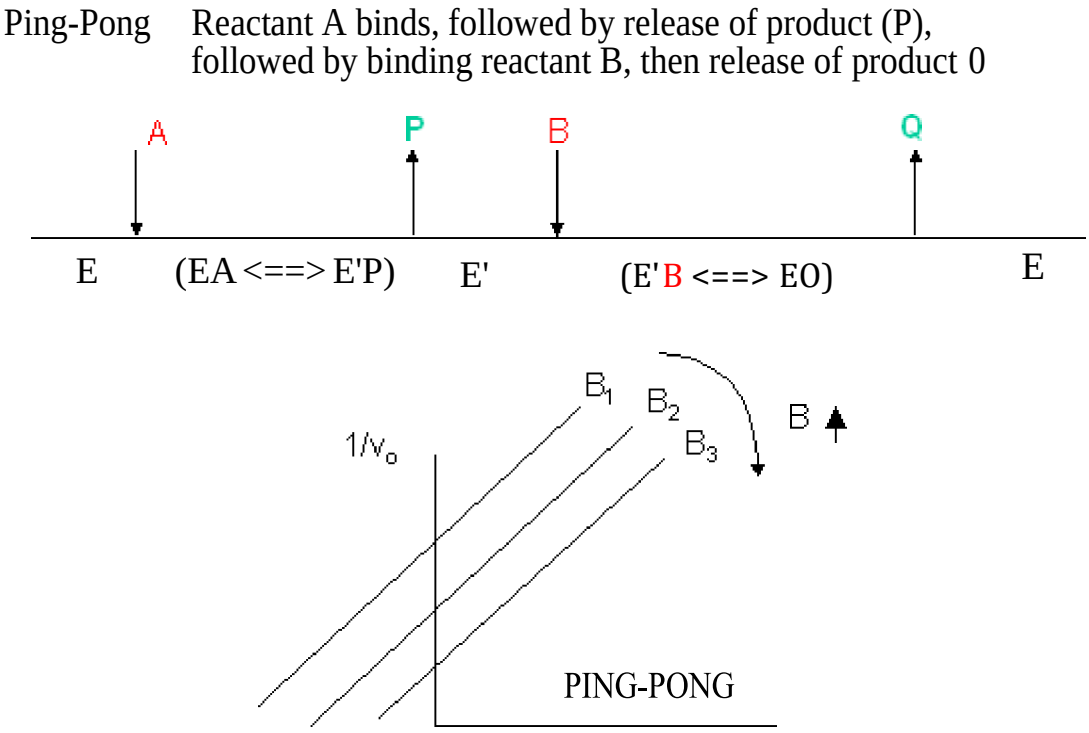
B. **Ordered Sequential:** specific order of reactants binding and products leaving





Ping-Pong Mechanism

In this mechanism, one substrate bind first to the enzyme followed by product P release. Typically, product P is a fragment of the original substrate A.The rest of the substrate is covalently attached to the enzyme E, which is designated as E'. Now the second reactant, B, binds and reacts with the enzyme to form a covalent adduct with the A as it is covalentattached to the enzyme to form product Q. This is now released and the enzyme is restored to its initial form, E. This represents a ping-pong mechanism. An abbreviated notation scheme is shown below for the ping-pong mechanisms. For this mechanism, Lineweaver-Burk plots at varying A and different fixed values of B give a series of parallel lines. An example of this type of reaction might be low molecular weight protein tyrosine phosphatase against the small substrate p-initrophenylphosphate(A) which binds to the enzyme covalently with the expulsion of the product P, the p-nitrophenol leaving group. Water (B) then comes in and covalently attacks the enzyme, forming an adduct with the covalently bound phosphate releasing it as inorganic phosphate. In this particular example, however, you cannot vary the water concentration and it would be impossible to generate the parallel Lineweaver-Burk plots characteristic of ping-pong kinetics.



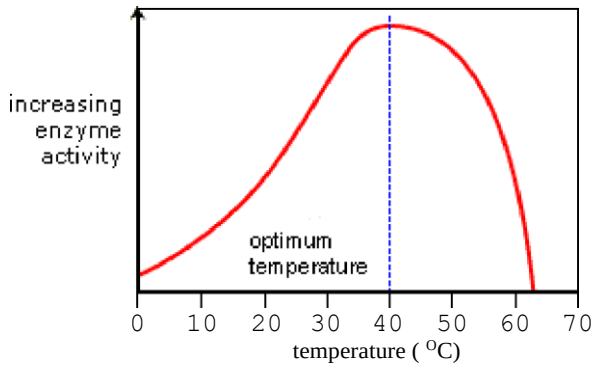
2. Describe the various factors that affect enzyme activity.

Factors affecting Enzvme Activitv

- The activity of an Enzyme is affected by its **environmental conditions.** Changing these **alter** the **rate of reaction** caused by the enzyme. In nature, organisms **adjust** the **conditions** of their enzymes to produce an **Optimum rate of reaction,** where **necessary,** or they may have enzymes which are **adapted** to **function well** in **extreme conditions** where they live.

Temperature

- Increasing temperature increases the Kinetic Energy that moleculespossess. In a fluid, this means that there are more random collisionsbetween molecules per unit time.
- Since enzymes catalyse reactions by randomly colliding with Substrate molecules, increasing temperature increases the rate of reaction, forming more product.
- However, increasing temperature also increases the Vibrational Energythat molecules have, specifically in this case enzyme molecules, which putsstrain on the bonds that hold them together.
- As temperature increases, more bonds, especially the weaker Hydrogenand Ionic bonds, will break as a result of this strain. Breaking bonds within the enzyme will cause the Active Site to change shape.
- This change in shape means that the Active Site is less Complementary to the shape of the Substrate, so that it is less likely to catalyse the reaction. Eventually, the enzyme will become Denatured and will no longer function.
- As temperature increases, more enzymes' molecules' Active Sites' shapes will be less Complementary to the shape of their Substrate, andmore enzymes will be Denatured. This will decrease the rate of reaction.
- In summary, as temperature increases, initially the rate of reaction willincrease, because of increased Kinetic Energy. However, the effect ofbond breaking will become greater and greater, and the rate of reaction will begin to decrease.



- The temperature at which the maximum rate of reaction occurs is called the enzyme's Optimum Temperature. This is different for different enzymes. *Most enzymes in the human body have an Optimum Temperature of around 37.0 °C.*

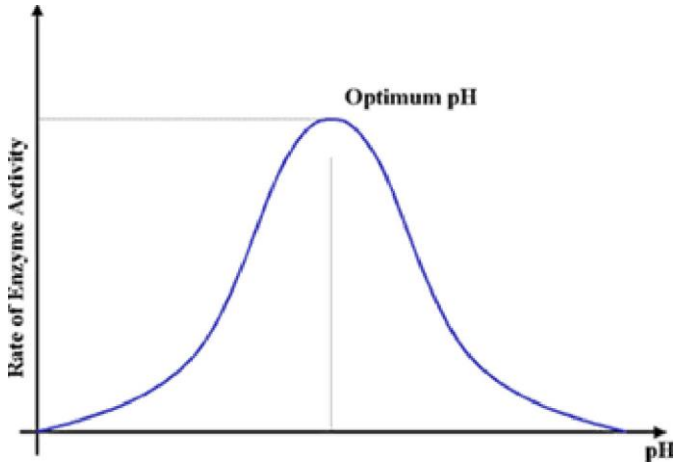
pH - Acidity and Basicity

- pH measures the Acidity and Basicity of a solution. It is a measure of theHydrogen Ion (H') concentration, and therefore a good indicator of theHydroxide Ion (OH) concentration. It ranges from pH1 to pH14. Lower pHvalues mean higher H" concentrations and lower OH concentrations.
- Acid solutions have pH values below 7, and Basic solutions (alkalis are bases) have pH values above 7. Deionised water is pH7, which is termed 'neutral'.

- H“ and OH Ions are charged and therefore interfere with Hydrogen andIonic bonds that hold together an enzyme, since they will be attracted orrepelled by the charges created by the bonds. This interference causes achange in shape of the enzyme, and importantly, its Active Site.

- Different enzymes have different Optimum pH values. This is the pH value at which the bonds within them are influenced by H“ and OH Ions in such a way that the shape of their Active Site is the most Complementary to theshape of their Substrate. At the Optimum pH, the rate of reaction is at an optimum.

- Any change in pH above or below the Optimum will quickly cause adecrease in the rate of reaction, since more of the enzyme molecules will have Active Sites whose shapes are not (or at least are less)Complementary to the shape of their Substrate.



- Small changes in pH above or below the Optimum do not cause apermanent change to the enzyme, since the bonds can be reformed. However, extreme changes in pH can cause enzymes to Denature andpermanently lose their function.

- Enzymes in different locations have different Optimum pH values since their environmental conditions may be different. *For example, the enzyme Pepsin functions best at around pH2 and is found in the stomach, which contains Hydrochloric Acid (pH2).*

Concentration

- Changing the Enzyme and Substrate concentrations affect the rate of reaction of an enzyme-catalysed reaction. Controlling these factors in acell is one way that an organism regulates its enzyme activity and so itsMetabolism.

- Changing the concentration of a substance only affects the rate of reaction if it is the limiting factor: that is, it the factor that is stopping a reaction from preceding at a higher rate.

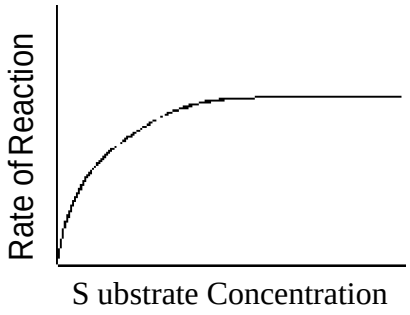
- If it is the limiting factor, increasing concentration will increase the rateof reaction up to a point, after which any increase will not affect the rate of reaction. This is because it will no longer be the limiting factor andanother factor will be **limiting** the maximum rate of reaction.

- As a reaction proceeds, the rate of reaction will decrease, since thesubstrate will get used up. The highest rate of reaction, known as thelnitial Reaction Rate is the maximum reaction rate for an enzyme in anexperimental situation.

Substrate Concentration

- **Increasing Substrate Concentration increases** the **rate** of reaction. This is because **more substrate** molecules will be colliding with enzyme molecules, so more product will be formed.

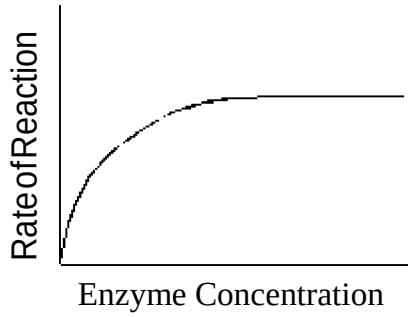
- However, after a **certain** concentration, any increase will have no effecton the **rate** of reaction, since Substrate Concentration will no longer be the **limiting** factor. The enzymes will effectively become saturated, and will be working at their maximum possible rate.



Enzyme Concentration

- **Increasing** Enzyme Concentration will increase the **rate** of reaction, asmore enzymes will be **colliding** with **substrate** molecules.

- However, this too will only have an effect up to **a certain concentration,** where the Enzyme Concentration is no longer the **limiting factor.**



3. Derive an expression for Competitive **inhibition.**

Enzymes play central roles in life processes. It holds for most enzymes that their function is needed only in certain conditions. When those conditions do not apply, the activity of a given enzyme can be futile or even harmful. Accordingly, the activity of most enzymes is under strict control. Enzymes can be regulated at multiple levels, ranging from transcriptional regulation of the expression of the enzyme-encoding gene through the direct regulation of the activity of the enzyme molecule by effector molecules to the controlled proteolytic decomposition of the enzyme. In this chapter, only those inhibitors will be reviewed that reversibly and specifically bind to enzymes through non-covalent interactions and inhibit the substrate-binding and/or catalytic apparatus of the given enzyme. These inhibitors can be classified into three mechanistic groups based on their mechanism of action: competitive, uncompetitive and mixed inhibitors. The type of inhibition can be determined through enzyme kinetic measurements.

Competitive inhibition

Competitive inhibitors compete for the substrate-binding site of the enzyme with the substrate, because the substrate and the inhibitor bind to identical or overlapping sites. Due to the overlapping nature of the binding sites, a ternary complex—in which the substrate and the inhibitor would simultaneously bind to the enzyme—cannot form. Accordingly, in the enzyme-inhibitor complex, the enzyme is completely inactive.

By popular—but quite misleading—terminology, these inhibitors are said to "displace" the substrate from the enzyme. While this term is aimed to be expressive, it is totally inadequate to explain the mechanism of this type of inhibition. The popular term suggests that the inhibitor would bind to the ES complex and would thus somehow actively force the substrate to dissociate. As already mentioned, no ternary complex is formed—not even temporarily. This inhibitory mechanism simply obeys a thermodynamic principle: two equilibria exist in parallel, one between the enzyme and the inhibitor and another between the enzyme and the substrate. More precisely, the latter one is a quasi-equilibrium because, during the measurement, the enzyme and the substrate are in a steady-state (as shown in Figure 9.4). The equilibrium concentrations of the free compounds and those of the complexes are dictated by the total concentrations of the individual compounds and the affinity of their interactions.

The equilibrium between the enzyme and the inhibitor is described by Equation 1 in which the Ki term represents a dissociation constant:

$$K = \frac{[E][I]}{[EI]} \qquad (1)$$

The two equilibria are not independent as the complexes, ES and EI, equilibrate with the same free enzyme pool, E. Increasing El concentration by increasing the inhibitor concentration can be achieved only through a decrease in ES concentration, and vice versa: an elevated substrate concentration can increase the concentration of the ES complex only at the expense of the EI complex.

This mechanism is illustrated in Figure 1.

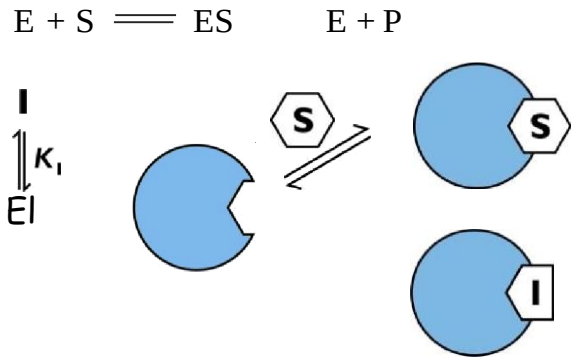


Figure 1. The scheme of competitive inhibition

When solving the Michaelis-Menten equation, we made use of the simple fact that the total enzyme concentration can be expressed as follows: [E]T [E]+[ES]. On the other hand, in the presence of a competitive inhibitor, [E]T- [E]+[ES]+[EI]. Solving the Michaelis-Menten equation such that this difference is taken into consideration leads to Equation 2. (For brevity, the intermediate steps that yield this equation are not shown.)

$$V_0 = \frac{V_{max}[S]}{(\alpha K_M + [S])} \qquad (2)$$

The meaning of the term o in Equation 2 is explained in Equation 3:

$$n - 1 + \frac{I]}{} \qquad (3)$$

It is readily apparent that, in the absence of inhibitor, the value of n is one and, as expected, we get the original equation. In the presence of inhibitor, the value of o exceeds one. The higher the concentration of the inhibitor compared to the value of the KI dissociation constant, the higher the value of o. Equation 2 clearly indicates that the measured Vmax will be invariant, regardless of the presence of the inhibitor. On the other hand, in the presence of a competitive inhibitor, the measured Kg will be higher than in the absence of the inhibitor. As in the case of this inhibitor type the substrate competes with the inhibitor, it is intuitively comprehensible that, at "infinitely" high substrate concentrations, the presence of the inhibitor should not affect the measurements, i.e. the maximal rate of the reaction should be unchanged. However, as in the presence of a competitive inhibitor higher than normal substrate concentration is needed to achieve a (half-) maximal rate, the value of Kv must be higher than in the uninhibited case. That is exactly what Equation 2 expresses.

When Equation 2 is rearranged according to the double reciprocal transformation, we get Equation 4, which is analogous to the uninhibited case:

$$\frac{1}{V_0} = \frac{\alpha K_M}{V_{max}[S]} + \frac{1}{'max} \qquad (4)$$

Equation 4 is graphically illustrated by the plots shown in Figure 2.The double reciprocal plots clearly show that, in the presence of a competitive inhibitor, the lines are steeper than in the uninhibited case; but the intercept on the y axis, which refers to the 1/Vmax value, remains the same. The plot nicely illustrates the didactical strength of double reciprocal data analysis to demonstrate the mechanism of a reversible inhibitor.

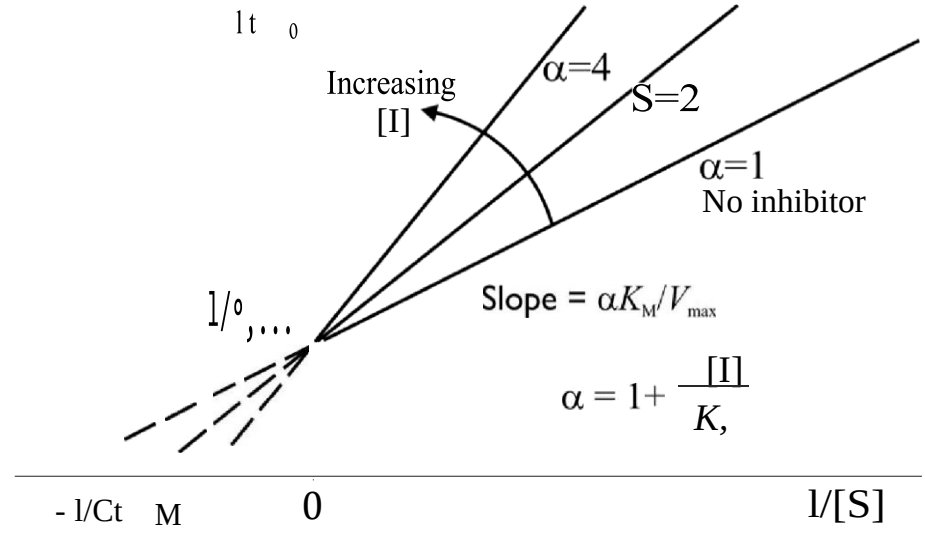


Figure 2. Double reciprocal Lineweaver-Burk analysis of competitive inhibition

As competitive inhibitors compete with the substrate for overlapping binding sites on the enzyme, it is not surprising that competitive inhibitors often resemble the substrate in terms of chemical structure, shape and polarity pattern. Due to this, competitive inhibitors are often used as useful reagents to study the substrate binding mechanism of enzymes. Comparative analysis of the structure of the substrate and that of a set of different competitive inhibitors can help in identifying the functionally most important parts of the substrate—those that provide the most binding energy in the ES complex. Note that such indirect approaches are important because direct analysis of the short-lived ES complex is a demanding scientific challenge.

4. **Derive the Michaelis-Menten equation for single substrate enzyme catalysed reactions. Write the significance Michaelis-Menton parameters. (May 2016)**

Ref.Question No.1

Significance of Michaelis-Menten Constant:

There are many advantages of knowing the Km values of enzyme-substrate systems:

(i) By knowing the Km value of a particular enzyme-substrate system, one can predict whether the cell needs more enzymes or more substrate to speed up the enzymatic reaction.

(ii) If an enzyme can catalyse a reaction with two similar substrates (e.g., glucose and fructose) in the cell, it will prefer that substrate for which the enzyme has lower Km value.

(iii) Km value gives an approximate measure of the concentration of substrate of the enzyme in that part of the cell where reaction is occurring. For instance, those enzymes which catalyse reactions with relatively more concentrated substrates (such as sucrose), usually have relatively high Km value. On the other hand, the enzymes that react with substrates which are present in very low concentrations (such as hormones) have comparatively lower Km values for the substrates

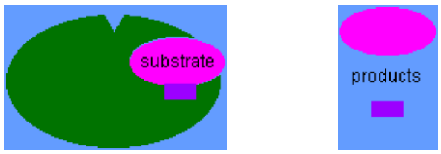
5. **Describe elaborately the different types of enzyme inhibition? Compare the competitive inhibition with uncompetitive and noncompetitive inhibition. (May 2015, May 2016)**

Specific Inhibitors:

Specific Inhibitors exert their effects upon a single enzyme. Most poisons work by specific inhibition of enzymes. Many drugs also work by inhibiting enzymes in bacteria, viruses, or cancerous cells and will be discussed later.

Competitive Inhibitors:

A competitive inhibitor is any compound which closely resembles the chemical structure and molecular geometry of the substrate. The inhibitor competes for the same active site as the substrate molecule. The inhibitor may interact with the enzyme at the active site, but no reaction takes place. The inhibitor is "stuck" on the enzyme and prevents any substrate molecules from reacting with the enzyme. However, a competitive inhibition is usually reversible if sufficient substrate molecules are available to ultimately displace the inhibitor. Therefore, the amount of enzyme inhibition depends upon the inhibitor concentration, substrate concentration, and the relative affinities of the inhibitor and substrate for the active site.



inhibitor

inhibitor

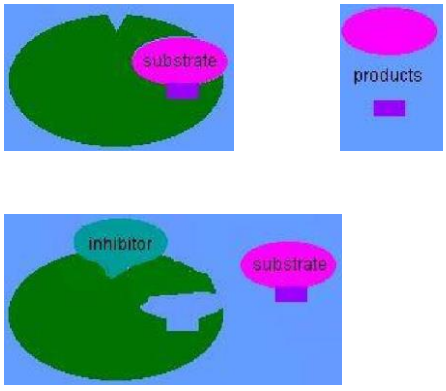
Non competitive Inhibitors:

A noncompetitive inhibitor is a substance that interacts with the enyzme, but usually not at the active site. The noncompetitive inhibitor reacts either remote from or very close to the active site. The net effect of a non competitive inhibitor is to change the shape of the enzyme and thus the active site, so that the substrate can no longer interact with the enzyme to give a reaction. Non competitive inhibitors are usually reversible, but are not influenced by concentrations of the substrate as is the case for a reversible competive inhibitor. See the graphic on the left.

Irreversible Inhibitors form strong covalent bonds with an enzyme. These inhibitors may act at, near, or remote from the active site. Consequently, they may not be displaced by the addition of excess substrate. In any case, the basic structure of the enzyme is modified to the degree that it ceases to work.

Since many enzymes contain sulfhydral (-SH), alcohol, or acid groups as part of their active sites, any chemical which can react with them acts as an irreversible inhibitor. Heavy metals such as Ag+, Hg2+, Pb2+ have strong affinities for -SH groups.

Nerve gases such as diisopropylfluorophosphate (DFP) inhibit the active site of acetylcholine esterase by reacting with the hydroxyl group of serine to make an ester.



Uncompetitive inhibition

Some inhibitors bind only to the ES complex without binding to the free enzyme. This interaction scheme is illustrated in Figure3.

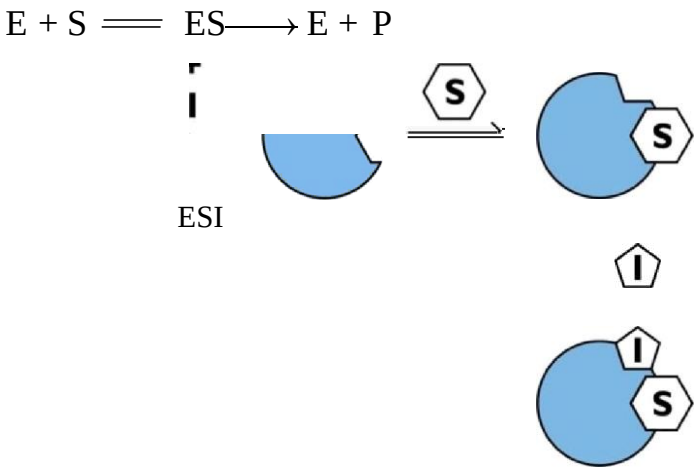


Figure 3. Uncompetitive inhibition

The kinetic equation of this type of inhibition can also be expressed as a modified version of the uninhibited case, as shown in Equation 9.57:

$$\text{'0} \quad \frac{V_{\max}[S]}{(K_M + \alpha'[S])} \tag{1}$$

The meaning of the Ki' term is shown in Equation 2:

$$K'_I = \frac{[ES][I]}{[ESI]} \tag{2}$$

However, as shown in Equation 1, o' is associated with Ki' instead of Ki:

$$\alpha' = 1 + \frac{\quad}{K';} \tag{3}$$

Double reciprocal transformation of Equation 1 results in Equation 4:

$$\frac{1}{V_0} = \frac{K_M}{V_{\max}[S]} + \frac{\text{«'}}{V_{\max}} \tag{4}$$

A graphical illustration of Equation 4 is presented in Figure 4.

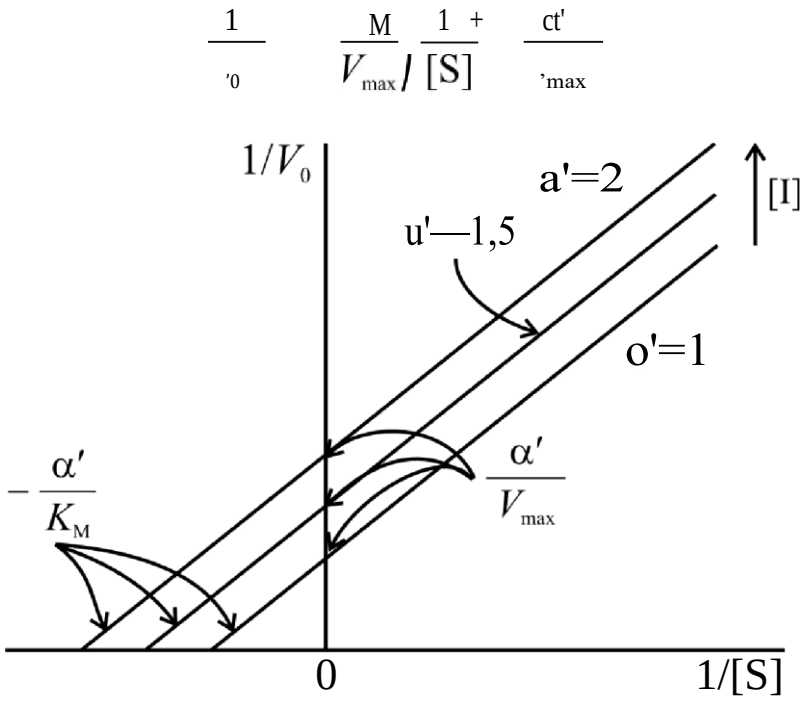


Figure 4. Double reciprocal Lineweaver-Burk analysis of uncompetitive inhibition

The plot clearly illustrates that both the KM and Vmax values are divided by the value of o' (i.e. their reciprocal value is multiplied by the value of e'). This means that, unlike in the case of competitive inhibitors, the presence of an uncompetitive inhibitor results in a decreased Vmax value. Moreover, the KM (i.e. the substrate concentration at which the reaction rate reaches its half maximum) also decreases, and it does so to exactly the same extent as the Vmax. As both kinetic parameters decrease to the same degree, the slopes of the lines do not change. This type of inhibition is dramatically different from the competitive one. Namely, the effect of an uncompetitive inhibitor, although its binding is reversible, cannot be abolished by increasing substrate concentration.

6. The following results were obtained at fixed total enzyme concentration

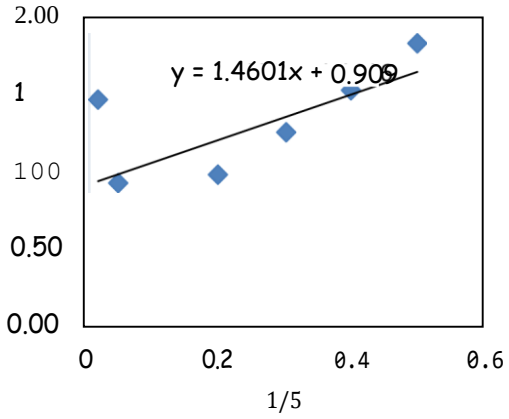
Sub. Cone. (mmol/L)	Initial velocity (absorbance/min)		
	Uninhibited	With 1 mmol/L of **inhibitor** 'A'	With 1 mmol/L of **inhibitor 'b'**
50	0.684	----	----
20	1.08	----	----
10	**1.43**	**1.01**	**0.653**
5.0	**1.02**	**0.649**	**0.468**
3.3	**0.798**	**0.476**	**0.363**
2.5	**0.657**	**0.374**	**0.296**
2.0	**0.549**	**0.311**	**0.250**

Find the type inhibition by inhibitor 'A' and 'B' and also calculate their apparent Vmax and apparent Km.

Sub. Conc. (mmol/L)	Initial velocity (absorbance/min)						
	Uninhibited	With l mmol/L of inhibitor 'A'	With 1 mmol/L of inhibitor 'b'	1/S	I/V for uninhibited	I/V for inhibitor A	I/V for inhibitor B
o	0•684			0.02	1.46	#VALUE!	#VALUE!

0	1.08	----	- ---	0.05	0.93	#VALUE!	#VALUE!
0	1.43	1.01	0.653	0.1	0.70	0.99	1.53
	1.02	0.649	0.468	0.2	0.98	1.54	2.14
.3	0.798	0.476	0.363	0.30 303	1.25	2.10	2.75
.5	0.657	0.374	0.296	0.4	1.52	2.67	3.38
	s0.549	0.311	0.25	0.5	1.82	3.22	4

1/S vs 1/V for uninhibited rn



For uninhibited Reaction,
Km/Vm= 1.4601
1/Vm=0.9096 ieVm= 1.0994
ie Km=l.4601*1.0994= 1.605

7. Derive the Monod changeux wyman model.

The MWC scheme is very simple to implement at the molecular level and there are many natural molecular systems that employ this scheme. While the model is easy to implement on the molecular level, understanding how this model leads to cooperative behavior is rather tricky.

Here are the five assumptions that define the MWC model:
1) Identical subunits occupy equivalent positions in a protein. The contacts and environment of each of these subunits is identical.
2) Each subunit contains a unique receptor site for a ligand.
3) At least two conformational states are reversibly accessible for the protein and the microscopic binding constant for the two states differ. (To keep things simple we will assume that only one of the two forms can bind ligands i.e. the binding constant for the R state is infinitely small)
4) The conformational switching between these two states is concerted; the subunits are either all in one conformation, or they are all in the other conformation i.e. there are only two types of interfaces.
5) The microscopic binding constant for the ligand depends only on the conformational state of the protein, but not on the binding state of the other subunits.

The model
First lets look at the model then we can work through the math and see if we get the expected results. Finally we can then try to understand why the MWC model gives rise to cooperative behavior. Our system has five equilibria. The first is between the R state and the T state. R stands for relaxed and T for tense, but you could give them any other name if you want.
The other four equilibira are between the ligand bound forms. As the model states, the microscopic binding constant k between each of the monomers and the ligand is identical. However we have to apply statistical prefactors. For example there are 3 open binding sites on TX. So for each receptor

we have 3 chances to go from TX to TX2 but there are only two ligand-bound monomers in TX2 giving us only 2 ways to go from TX2 back to TX. To correct for this we have to apply the statistical p :f r t th il d il
tant K2 '322o nd(ng]o]) Ake w hten d n u enctmnsch be nndhdentu ibg'u

statistical prefactors that relate our equilibrium constants to the microscopic binding constant k we can now write down all our equilibria. The goal as always is to write end up with an equation that relates the fraction receptor sites.

$$L = \frac{[\cdot\cdot\cdot]}{[T]} \quad \Rightarrow \quad [R] = [T]L$$

$$2\,\frac{k}{[\wedge *]][*]} \quad [TX_2] = \frac{3}{2}k[TX][X] = [T]\cdot\frac{3}{2}\cdot 4\cdot k^2[X]^2 = [T]\cdot 6\cdot k^2[X]^2$$

$$\frac{2}{3}k = \frac{[TX_3]}{[TX_2][X]} \quad \Rightarrow \quad [TX_3] = \frac{2}{3}k[TX_2][X] = [T]\cdot\frac{2}{3}\cdot\frac{3}{2}\,4\;k'''[X]' = [T]\;*\;k'''[X$$

$$\frac{1}{4}k = \frac{[TX_4]}{[TX_3][X]} \quad \Rightarrow \quad [TX_4] = \frac{1}{4}k[TX_3][X] = [T]\cdot\frac{1}{4}\cdot\frac{2}{3}\cdot\frac{3}{2}\cdot 4\cdot k^4[X]^4 = [T]\cdot k^4[X]^4$$

nowlets write down the

$$\bar{v} = \frac{ligand\ sites\ occupied}{total\ \#\ of\ receptors} = \frac{[TX] + 2[TX_2] + 3[TX_3] + 4[TX_4]}{[R] + [T] + [TX] + [TX_2] + [TX_3] + [TX_4]}$$

the goal as always is to express our average number of ligands bound in terms of only the free ligand concentration and the binding and conformation equilibrium constants. Once again we do this by substituting our [TXi] by the appropriate expressions we derived above.

$$\bar{v} = \frac{\text{ligand sites occupied}}{\text{total \# of receptors}} = \frac{[T]4k[X] + 2[T]\cdot 6\cdot k^2[X]^2 + 3[T]\cdot 4\cdot k^3[X]^3 + 4[T]\cdot k^4[X]^4}{[T]L + [T] + 4k[T][X] + [T]\cdot 6\cdot k^2[X]^2 + [T]\cdot 4\cdot k^3[X]^3 + [T]\cdot k^4[X]^4}$$

dividr bs- [T]

r=4 1[X) + 3 1'(X]' + 3 1'[X]' + k' [X]'
1 + J + -4 kt X]+ -6 1' [X]' + -4 k"tX) ' + -1'(X)'

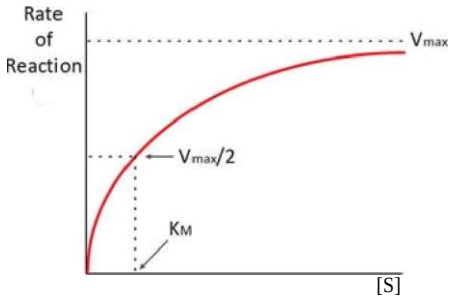
$$\bar{v} = 4\frac{k[X](1 + 3\cdot k[X] + 3\cdot (k[X])^2 + (k[X])^3)}{L + 1 + 4\cdot k[X] + 6\cdot (k[X])^2 + 4\cdot (k[X])^3 + (k[X])^4} = 4\frac{k[X](1 + k[X])^3}{L + (1 + k[X])^4}$$

8. The following results were obtained for an enzyme catalyzed reaction.

Substrate conc. mmol/L	5	6.67	10	20	40
Initial velocity pmol/L.min	147	182	233	323	400

Calculate Michaelis-Menton parameters using (i) graph for M-M equation (ii) LB plot (iii) E-H plot (iv) Hanes Plot

(i) Graph of M-M equation graph:



(ii) LB Plot

Plot the graph 1/S Vs 1/V-
Slope= Km/Vm
Intercept= 1/Vm

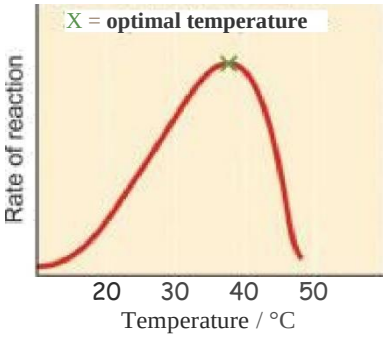
(iii) E-H Plot

Plot the graph 1/S Vs l/V-
Slope= —Km
Intercept= Vm

(iv) Hanes Plot
Plot the graph 1/S Vs l/V-
Slope= 1/Vm
Intercept= Km/Vm

9. Explain the pH and temperature effect on enzymes & its deactivation kinetics.(Nov 2015, May 2016)

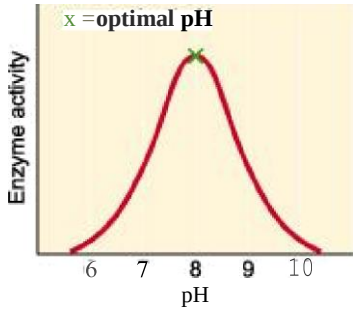
Temperature



As the temperature rises, reacting molecules have more and more kinetic energy. This increases the chances of a successful collision and so the rate increases. There is a certain temperature at which an enzyme's catalytic activity is at its greatest (see graph). This optimal temperature is usually around human body temperature (37.5 °C) for the enzymes in human cells.

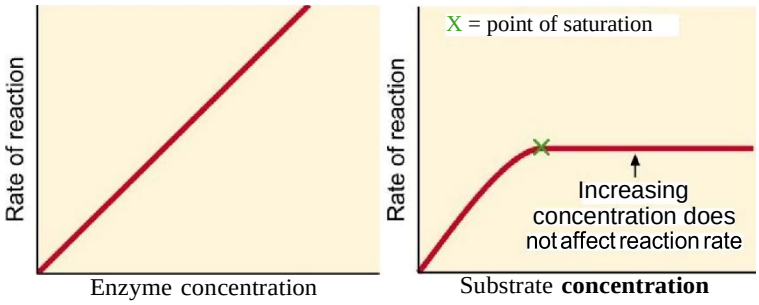
Above this temperature the enzyme structure begins to break down **(denature)** since at higher temperatures intra- and intermolecular bonds are broken as the enzyme molecules gain even more kinetic energy.

pH



Each enzyme works within quite a small pH range. There is a pH at which its activity is greatest (the optimal pH). This is because changes in pH can make and break intra- and intermolecular bonds, changing the shape of the enzyme and, therefore, its effectiveness.

Concentration of enzyme and substrate



The rate of an enzyme-catalysed reaction depends on the concentrations of enzyme and substrate. As the concentration of either is increased the rate of reaction increases (see graphs).

For a given enzyme concentration, the rate of reaction increases with increasing substrate concentration up to a point, above which any further increase in substrate concentration produces no significant change in reaction rate. This is because the active sites of the enzyme molecules at any given moment are virtually saturated with substrate. The enzyme/substrate complex has to dissociate before the active sites are free to accommodate more substrate. (See graph)

Provided that the substrate concentration is high and that temperature and pH are kept constant, the rate of reaction is proportional to the enzyme concentration. (See graph)

b) The thermal denaturation of an enzyme may be modelled by the following serial deactivation scheme:

$$E \xrightarrow{k_{d1}} E_1 \xrightarrow{k_{d2}} E_2$$

where Ai and fiz are the first-order deactivation rate coefficients, E is the native enzyme which may, or may not, be an equilibrium mixture of a number of species, distinct in structure or activity, and Ei and Ez are enzyme molecules of average specific activity relative to E of Ai and Az. Ai may be greater or less than unity (i.e. Ei may have higher or lower activity than E) whereas Az is normally very small or zero. This model allows for the rare cases involving free enzyme (e.g. tyrosinase) and the somewhat commoner cases involving immobilised enzyme (see Chapter 3) where there is a small initial activation or period of grace involving negligible discernible loss of activity during short incubation periods but prior to later deactivation. Assuming, at the beginning of the reaction:

$$[E] = [E]_{0(2)}$$

and:

$$[c,] = [c,] = o \quad 3)$$

At time t,

$$[Ej + [*i] + [Ei] = [*d, 4$$

It follows from the reaction scheme [1],

$$-\frac{d[E]}{dt} = k_{d1}[E] \quad (5)$$

Integrating equation 1.25 using the boundary condition in equation 1.22 gives:

$$[Ej = [\quad , e(\ ``) \quad (6)$$

From the reaction scheme 1],

$$-\frac{d[E_1]}{st} = k_{d2}[E_1] - k_{d1}[E] \quad (7)$$

Substituting for [E] from equation 6,

$$-\frac{d[E_1}{st} = \ 'd2[\ \ 1]" \ 'd1\ \ \ 0\ \ '—"') \quad (8)$$

Integrating equation 7 using the boundary condition in equation 3 gives:

$$[\quad 1]' \quad \frac{k_{d1}[TO}{k_{,\ 3} - k_{d1}}\left(e^{(-k_{d}t)} - e^{(-k_{d2}t)}\right) \tag{9}$$

If the term 'fractional activity' (A') is introduced where,

$$A^{r} = [E] + A_1[E_1] + A_2[E_2] \tag{10}$$

then, substituting for [E2] from equation 4, gives:

$$if \quad \_\ [\quad\quad [\ 1]\ \underline{4([*d0 - [*d"\ [\ 1])} \tag{11}$$

therefore:

$$A^{r} = A_2 + \left(1 + \frac{A_1 k_{d1} - A_2 k_{d2}}{k_{d2}" \quad k_{d}\backslash}\right) e^{(-k_{d}t)} - \frac{(A_1 - A_2)k_{d1}}{k_{d2}" \quad k_{d1}}\ e^{(-k_{d2}t)} \tag{12}$$

When both Ai and A2 are zero, the simple first order deactivation rate expression results

$$P' - S'^{*''''} \tag{13}$$

The **half-life** (t 2) of an enzyme is the time it takes for the activity to reduce to a half of the original activity (i,e. A' = 0.5). If the enzyme inactivation obeys equation 1.33, the half-life may be simply derived,

$$*\ (*/*) = \quad dll@ \tag{14}$$

therefore:

$$!1' \quad \frac{0.693}{"} \tag{15}$$

10. **Derive the Michaelis —Menten equation** for single substrate enzyme catalyzed reactions (Nov **2015)**
    Ref Question no.1
11. Derive **the kinetics** for **bisubstrate** enzyme catalysed **reaction by sequential** mechanism. (Nov **2016)**

Cleland Nomenclature for Enzymes

- Cleland has devised a standardized way of referring to bisubstrate (Bi-Bi) enzymatic reactions, which make up 60% of all enzymatic transformations. The substrates, products and stable enzyme forms are denoted as follows:
  - Substrates are lettered A, B, C and D, in the order that they are added to the enzyme
  - Products are lettered P, Q, R and S, in the order that they leave the surface of the enzyme
    Stable enzyme forms are lettered E, F and G, in the order that they occur

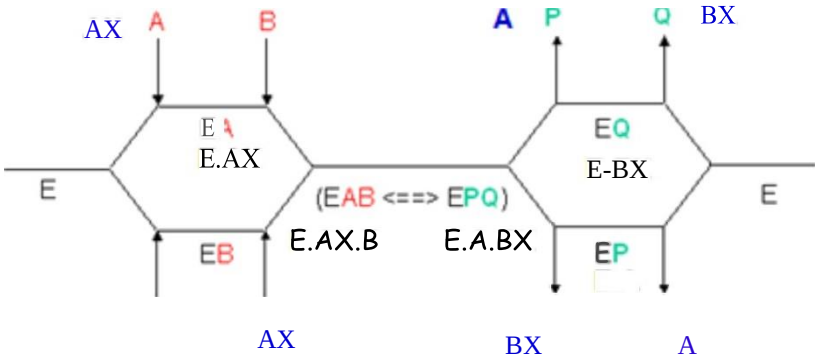
The number of reactants in the reaction are designated by the terms Uni, Bi, Ter and Quad

These are transfer reactions so can be presented as

$AX + B \leftrightarrow AB + X$

- The first important type of bi-bi reaction is known as sequential, which means that all substrates must add to the enzyme before any reaction takes place
- The sequential bi-bi can be
  - random, any substrate can bind first to the enzyme and any product can leave first
  - ordered, meaning that the substrates add to and products leave the enzyme in a specific order
  - A ternary complex (E + both substrates) is formed in both cases

A Random Sequenti al r am‹i:›nJ ‹: r‹Jer ..f r+-a‹:tarits bm ‹Min .J on ‹1 §ir‹. ‹k i.ts l e a'.'in '1



B. **Ordered Sequential**: specific order of reactants binding and products leaving

The general rate equation of Alberty (1953)

- Many two-substrate reactions obey the MM equation with respect to one substate at constant concentration

$$v = \frac{V_{max}[AX][B]}{K_m^B[AX] + K_m^{AX}[B] + [AX][B] + K_s^{AX}K_m^B}$$

- V max vo When both AX and B are saturating
  = . [AX] which gives 1/2V when B is saturating
  £ • [B] which gives 1/2V when AX is saturating
  £"t . dissociation constant for E + AX EAX

$$v = \frac{V_{max}[AX][B]}{K_m^B[AX] + K_m^{AX}[B] + [AX][B] + K_s^{AX}K_m^B}$$

At very large [B]: *'' ——.t+———

At constant but non saturating [B]: ›' = $\frac{V_{max}K_1[AX]}{[AX] + K_2}$

$$K \quad —\frac{[B]}{K_m^B + [B]} \qquad K_{\text{،}} = \frac{K_s^{AX}K_m^B + K_m^{AX}[B]}{K_m^B + [B]}$$

It works well for reactions using 1 or 2 substrate and producing 1 or 2 products but for more complex reactions, other approaches are used

General rate equation of Dalziel

(1957)

$$\frac{[E]}{v} = \phi_0 + \frac{\phi_{AX}}{[.W]} + \frac{\phi_B}{[B]} + \frac{\phi_{AXB}}{[\,.\quad\,][B]}$$

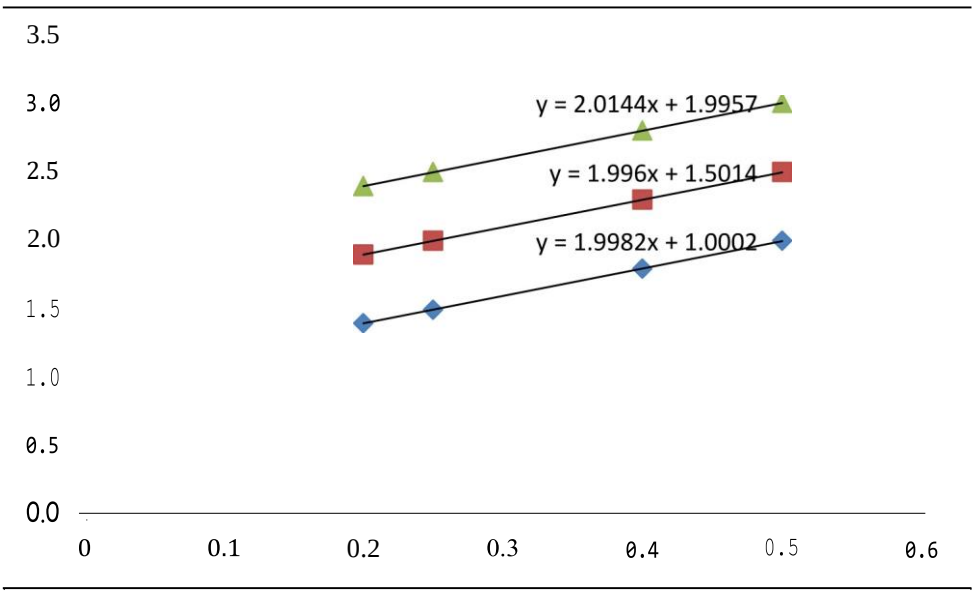
terms: kinetic coefficients found from primary and secondary plots

- Primary plots of [E]/v versus 1/[AX] at constant [B] are drawn for series of different [Bj
- Secondary plots
  - Slope vs 1/[B]4 intercept: , slope: »B
  - Intercepts vs 1/[B]4 intercept: $. slope: gB

12. Explain defferent types of reversible enzyme inhibition. How do you distinguish them using kinetic data (May 2017)
Refer Question No.5

13. A single substrate enzyme catalysed reaction was investigated at series of inhibitor concentrations at fixed pH, temperature and enzyme concentration. Determine the type of inhibition (Nov 2016)

Initial Substrate conc. (mmol/L)	Initial velocity (pmol/L.min) Io = 0	I = 2 mmol/L	I= 4 mmol/L
2	500	400	333
2.5	556	435	357
4	667	500	400
5	714	526	417



For Uninhibited reaction
Km/Vm = **1.998**
1/Vm = 1

For I=2
Km/Vm = **1.996**
1/Vm = 1.501

For 1=4
Km/Vm = 2.014
1/Vm = 1.995

Module 3

ENZYMATIC TECHNIQUES

Define enzyme **immobilization**
Enzyme immobilization may be defined as confining the enzyme molecules to a distinct phase from the one in which the substrates and the products are present.

1. **What are the methods available for the** enzyme **immobilization?**
   The various methods used for immobilization of enzymes may be grouped into the following 5 types:
   (i) adsorption,
   (ii) covalent bonding,
   (iii) entrapment, and
   (iv) membrane confinement.
2. **What are the inert supports used for the covalent immobilization?**
   The most commonly employed matrices are agarose, celluloses and polyacrylamides. Sepharose, an agarose, is available commercially as beads, is highly hydrophilic and is generally inert to microbial attack. Sepharose is activated by treating it with chloroformates, carbodiimides, glutaraldehyde or other compounds.
3. **What is meant by encapsulation?**
   Encapsulation is another approach in enzyme immobilization by entrapping method. In this approach enzyme can be immobilized within capsules prepared from organic polymers, so that the enzyme can not escape, although low molecular weight substrates and products can enter and leave the capsule by diffusion through the membrane.
4. Why **immobilized or whole cells are predominantly used rather than pure** enzymes **in industrial** scale enzymatic conversions
   Enzymes are costly items. Immobilization permits their repeated use.
   The product is readily freed from the enzyme.
   Immobilized enzymes can be used in nonaqueous systems as well.
   Continuous production systems can be used, which is not possible with free enzymes.
   Thermostability of some enzymes may be increased.
5. **What** are the **types** of **matrixes available** for enzyme **immobilization?**
   Some of the commonly used matrices are ion exchange matrices, porous carbon, clays, hydrous metal oxides, glasses, and polymeric aromatic resins.
6. **Define** cross **linking.**
   Cross-linking is the bonding that link one polymer chain to another. They can be covalent bonds or ionic bonds.
7. Give **industrial** applications of immobilized enzyme
   Glucoamylase - Dextrins production
   Penicillin amylases - Penicillin G and penicillin V
   Lactase - Milk and whey
   Glucose isomerase - D-glucose in glucose syrup
8. State the general application of immobilization.
   Used of drug delivery and development of biosensors
9. **Physical method of immobilization technique is** best, **justify**

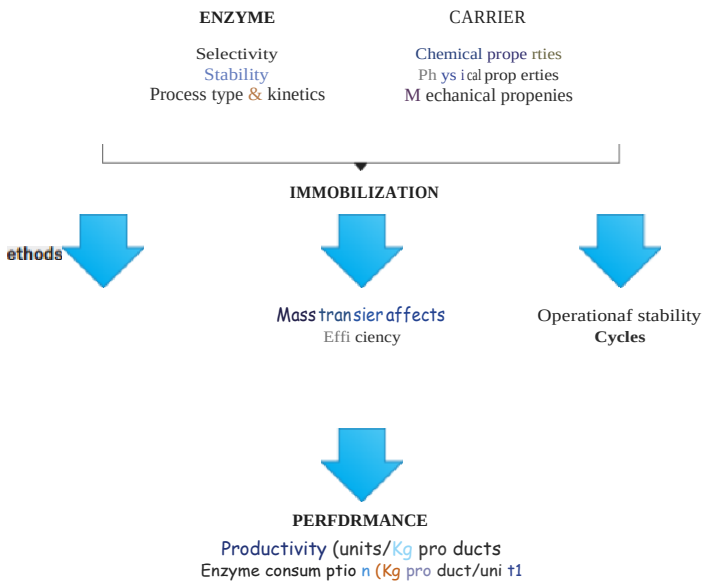
Enzymes are attached by weaker forces and it surface of the matrix, Therefore there is no mass transfer resistance and enzyme characteristics are not affected by immobilization.

10. What are **the** process factors to be considered **for immobilization of** enzyme?
    Type of reactor PBR are FBR, substrate and product molecular size and liquid viscosity
11. **Can the enzyme immobilized on polymeric matrix, influence reaction rate? Explain**
    Yes, increased mass transfer resistance affects the reaction rate in enzyme immobilized on polymeric matrix
12. **Explain the entrapment** method of **immobilization?**
    Enzymes can be entrapped inside a cross linked gel matrix by allowing the gel to be formed in an aqueous solution containing one or more enzymes. The polymerization of the gel is carried out in the presence of enzyme(s). The enzyme is physically entrapped within the matrix and cannot escape by permeation.
13. **List** out **some** solid **matrices & polymeric materials used** for enzyme **immobilization?**
    Alumina, amberlite CG-50, bentonite, calcium phosphate gels, carbon, car boxymethyl cellulose, carboxymethylsephadex, collagen, DEAE-cellulose, DEAE-sephadex, glass, silica gel titania (ceramics).
14. **What are the reagents** used to **activate the functional groups on support material?**
    cyanogen bromide, acyl isourea and NaNO2 + HCL.
15. Give **a list of support materials for adsorbtion immobilization**
    Calcium phosphate gels, carbon, car boxymethyl cellulose, glasses, and polymeric aromatic resins
16. **What are the disadvantages of immobilization though cross linking?**
    High binding force and possibility of binding on active site alters the characteristics of the enzyme
17. **How the** active site **protected during immobilization?**
    Active site is protected by activation of matrices to bind the enzymes in non active sites.
18. **Explain the mechanism of covalent bonding?**
    An enzyme can be covalently bound to support materials by different methods. The enzyme forms a covalent link with active groups of support material
19. **How are polymeric matrices** activated for covalent **immobilization?**
    The reactants required to activate the support are with -OH group. Such supports can be activated for covalent linking by treating with either triazines or cyanogen bromide. The reaction with enzyme protein in each case involves the -NH2 group of the lysine.
20. **What are major criteria for selecting support material?**
    The ideal carrier matrix has the following properties:
    (i) low cost,
    (ii) inertness,
    (iii) physical strength,
    (iv) stability,
    (v) regenerability after the useful lifetime of the immobilized enzyme,
    (vi) enhancement of enzyme specificity, and
    (vii) reduction in product inhibition
21. Give **applications of immobilized enzymes in medical diagnosis**
    Glucose oxidase – Blood glucose level
    Urease – Urea
22. Give the reaction mechanism in polyacrylamide gel preparation

23. What are the difference between gelentrapment and encapsulation
    Both gelentrapment and encapsulation may be viewed as putting the enzyme molecule in a molecular cage. However, diffusion of the substrate to the enzyme and of the product away from the enzyme creates difficulties in gelentrapement
24. How enzymes are immobilized by cross linking?
    Three types of basic approaches have been used in immobilizing enzymes by cross linking:
    (1) Cross linking of enzyme with glutaraldehyde to form an insoluble aggregate; e.g. papain.
    (2) Adsorption of enzyme onto a surface followed by cross linking; for instance, cross linking trypsin adsorbed to the surface of colloidal silica particles.
    (3) Impregnation of porous material with the enzyme followed by cross linking of the enzyme in the pores; for instance papain in collodion membrane.
25. What are biosensors?(Nov 2015)
    A biosensor is an analytical device, which employs a biological material to specifically interact with an analyte; this interaction produces some detectable physical change, which is measured and converted into an electrical signal by a transducer. Finally, the electrical signal is amplified, interpreted and displayed as analyte concentration in the solutiorrpreparation.
26. Give the classification of biosensors.
    Types of biosensors
    (i) Calorimetric biosensors
    (ii) potentiometric biosensors
    (iii) amperometric biosensors
    (iv) Optical biosensors
    (v) piezo-electric biosensors
27. What are an enzyme electrode and its application in health care? (Nov 2016)
    The enzyme electrode is a combination of any electrochemical probe (amperometric, potentiometric or conductimetric) with a thin layer (10 - 200mm) of immobilised enzyme.
28. What is meant by hybrid Biosensor?
    The hybrid Biosensor relates to an apparatus and method for monitoring cells and to a method for monitoring changes in cells upon addition of an analyte to the cell's environment, comprising a device which includes an array of microelectrodes disposed in a cell culture chamber, upon which array a portion of cells adhere to the surfaces of the microelectrodes.
29. Give an example on the use of enzymes in leather and detergent industry
    Lipase — Leather Industry
    Alkaline protease - detergent industry
30. State the application of biosensors in diagnosis
    Glucose oxidase biosensor — Blood glucose
    Urease biosensor — Blood Urea
31. State the applications of enzymes in food and pharmaceutical industries
    Rennet - milk industry
    Amylase — Starch industry
    Pencillin G acylase — Pencillin Industry
32. What are the main components of biosensors?
    i. Biological component ii. Tranducer iii. Signal processor
33. What are the physical changes accompanying with the reaction in biosensor
    Analyte reacts in ensy,me system and converted into product, the changes in concentration of substrate or product is converted into electrical signal by transducer ,processed and calibrated to concentration of analyte.
34. What is the function of Transducer in biosensors?
    Transducer converts the changes in concentration of substrate or product into electrical.
35. What is the function of Amplifier in biosensors?
    The signal produced in biological system and converted by transducer will be very low, amplifier increases the amplitude of a signal.
36. What are the three types of ion selective electrodes used in biosensors?
    Glass electrodes for cations, Glass pH electrodes and Solid-state electrodes
37. Write the principle of Amperometric biosensors
    Amperometric biosensors function by the production of a current when a potential is applied between two electrodes.

38. **Write the principle of Calorimetric biosensors**
Calorimetric biosensors measure the change in temperature of the solution containing the analyte following enzyme action and interpret it in terms of the analyte concentration in the solution.
39. **Write the principle of Optical biosensors**
The principle involves determining changes in light absorption between the reactants and products of a reaction, or measuring the light output by a luminescent process.
40. **Write the principle of Immunobiosensors**
Biosensors may be used in conjunction with enzyme-linked immunosorbent assays (ELISA). ELISA is used to detect and amplify an antigen-antibody reaction; the amount of enzyme-linked antigen bound to the immobilised antibody being determined by the relative concentration of the free and conjugated antigen and quantified by the rate of enzymic reaction.
41. **Write the principle of Potentiometric biosensors**
Potentiometric biosensors make use of ion-selective electrodes in order to transduce the biological reaction into an electrical signal.
42. **Write the principle of Piezo-electric biosensors**
Piezo-electric crystals (e.g. quartz) vibrate under the influence of an electric field. The frequency of this oscillation (f) depends on their thickness and cut, each crystal having a characteristic resonant frequency.
43. **What are the advantages of micro-biosensors?**
Decreased the detection time, more portability and these can detect pathogens rapidly and inexpensively.
44. **What are the beneficial features of biosensors?**
The biocatalyst must be highly specific for the purpose of the analyses, be stable under normal storage conditions and, except in the case of colorimetric enzyme strips and dipsticks (see later), show good stability over a large number of assays (i.e. much greater than 100). The reaction should be as independent of such physical parameters as stirring, pH and temperature as is manageable. This would allow the analysis of samples with minimal pre-treatment. If the reaction involves cofactors or coenzymes these should, preferably, also be co-immobilised with the enzyme. The response should be accurate, precise, reproducible and linear over the useful analytical range, without dilution or concentration. It should also be free from electrical noise. The complete biosensor should be cheap, small, portable and capable of being used by semi-skilled operators.
45. **Give example for calorimetric biosensors**
Cholesterol oxidase biosensor — Cholesterol
Amylase biosensor - Starch
46. Give **example for potentiometric biosensors**
Lipase - Lipids
peroxidase — H2Oi
urease - urea
47. **Give example for amperometric biosensors**
Alcohol oxidase — Alcohol
Cholesterol oxidase - cholesterol
Glucose biosensor (based on glucose oxidase)
48. **Give example for immunobiosensors**
Immunosensors coupled with biological syatem used to detect TNT and RDX in soil and groundwater
49. **List the disadvantages of entrapment technique used for enzyme immobilization(May 2016)**
The disadvantages of entrapment technique used for enzyme immobilization are
    - Regeneration of enzyme is impossible
    - Preparation is difficult
    - Lack of control over microenvironmental conditions
    - The enzyme may leak from the pores.
50. **Give example for Piezoelectric biosensors**
Formaldehyde - formaldehyde dehydrogenase
Diagnosis of Tularemia in brown

51. What are the various factors that affect the performance of immobilized enzyme systems?



52. What **are the limitations of** enzyme **biosensors?**

- Heat sterilization is not possible because of denaturaziation of biological material,
- Stability of biological material (such as enzyme, cell, antibody, tissue, etc.), depends on the natural properties of the molecule that can be denaturalized under environmental conditions (pH, temperature or ions)
- The cells in the biosensor can become intoxicated by other molecules that are capable of diffusing through the membrane.

53. What are solid matrices commonly **used** for covalent **binding of enzymes?** How do you **activate them?**

Some of the commonly used matrices for covalent binding are cellulose, sephadex, sepharose, gluteraldehyde etc. Covalent bonding is directed to a specific group on the surface of the enzyme. differnt methods of covalent bonding are diazotation, formation of peptide bond, group activation (use of cyanogen bromide to a support containing glucol group.) and use of polufunctional reagents.

54. What is Damkohler **number?** State **its significance.**

The Damköhler numbers (Da) are dimensionless numbers used in chemical engineering to relate the chemical reaction timescale (reaction rate) to the transport phenomena rate occurring in a system.
Da is associated with characteristic diffusion and reaction times therefore scaling is necessary.

- For Da >>1 the reaction rate is much greater than the diffusion rate distribution is said to be diffusion limited (diffusion is slowest so diffusion characteristics dominate and the reaction is assumed to be instantaneously in equilibrium)
- For Da <+1 diffusion occurs much faster than the reaction, thus diffusion reaches an 'equilibrium' well before the reaction is at equilibrium.

PART B

1. Describe the cross **linking** method of enzyme **immobilization** with **its merits** and **demerits**
(Cross linking (copolymerization):

Cross Linking (Copolymerization)
Enzyme Immobilization

This method is also called as copolymerization. In this method of immobilization enzymes are directly linked by covalent bonds between various groups of enzymes via polyfunctional reagents. Unlike other methods, there is no matrix or support involved in this method. Commonly used polyfunctional reagents are glutaraldehyde and diazonium salt. This technique is cheap and simple but not often used with pure enzymes. This method is widely used in commercial preparations and industrial applications. The greatest disadvantage or demerit of this method is that the polyfunctional reagents used for cross linking the enzyme may denature or structurally modify the enzyme leading to the loss of catalytic properties.

2. Give an overview **of applications of immobilized** enzymes

Applications of enzyme immobilization:

(1). Industrial production: Industrial production of antibiotics, beverages, amino acids etc. uses immobilized enzymes or whole cells.

(2). Biomedical applications: Immobilized enzymes are widely used in the diagnosis and treatment of many diseases. Immobilized enzymes can be used to overcome inborn metabolic disorders by the supply of immobilized enzymes. Immobilization techniques are effectively used in drug delivery systems especially to oncogenic sites.

(3). Food industry: Enzymes like pectinases and cellulases immobilized on suitable carriers are successfully used in the production of jams, jellies and syrups from fruits and vegetables.

(4). Research: A Research activity extensively uses many enzymes. The use of immobilized enzyme allow researcher to increase the efficiency of different enzymes such as Horse Radish Peroxidase (HRP) in blotting experiments and different Proteases for cell or organelle lysis.

(5). Production of bio-diesel from vegetable oils.

(6). Waste **water management:** treatment of sewage and industrial effluents.

(7). Textile industry: scouring, bio-polishing and desizing of fabrics.

(8). Detergent industry: immobilization of lipase enzyme for effective dirt removal from cloths.

3. i) List out the major advantages and disadvantages of immobilized enzymes over free enzymes(8)

ii) Compare physical and chemical methods of immobilization (8)

Advantages of immobilized enzymes:

(1). Increased functional efficiency of enzyme
(2). Enhanced reproducibility of the process they are undertaking
(3). Reuse of enzyme
(4). Continuous use of enzyme
(5). Less labour input in the processes
(6). Saving in capital cost and investment of the process
(7). Minimum reaction time
(8). Less chance of contamination in products
(9). More stability of products
(10). Stable supply of products in the market
(11). Improved process control
(12). *High enzyme substrate ratio*

Disadvantages of enzyme **immobilization:**

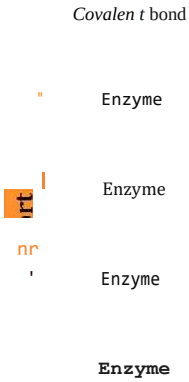
(1). Even though there are many advantages of immobilized enzymes, there are some disadvantages also.
(2). High cost for the isolation, purification and recovery of active enzyme (most important disadvantage)
(3). Industrial applications are limited and only very few industries are using immobilized enzymes or immobilized whole cells.
(4). Catalytic properties of some enzymes are reduced or completely lost after their immobilization on support or carrier.
(5). Some enzymes become unstable after immobilization.
(6). Enzymes are inactivated by the heat generated in the system

4. What is immobilization of enzymes? Explain covalent immobilization with its merits and demerits

What is enzyme immobilization?

Immobilization is defined as the imprisonment of cell or enzyme in a distinct 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 Aspergi/fosoryZae for the production of L-amino acids in Japan.

Covalent bonding:



Covalent Bonding
Jnzyirie Immobilization)

This method involves the formation of covalent bonds between the chemical groups in enzyme and to the chemical groups on the support or carrier. It is one of the widely used methods of enzyme immobilization. Hydroxyl groups and amino groups of support or enzyme form covalent bonds more easily. Chemical groups in the support or carrier that can form covalent bonds with support are amino groups, imino groups, hydroxyl groups, carboxyl groups, thiol groups, methylthiol groups, guanidyl groups, imidazole groups and phenol ring.

Important functional groups of the enzyme that provide chemical groups to form covalent bonds with support or carrier are:

1. Alpha carboxyl group at 'C' terminal of enzyme
2. Alpha amino group at 'N' terminal of enzyme
3. Epsilon amino groups of Lysine and Arginine in the enzyme
4. § and y carboxyl groups of Aspartate and Glutamate
5. Phenol ring of Tyrosine
6. Thiol group of Cysteine
7. Hydroxyl groups of Serine and Threonine
8. Imidazole group of Histidine
9. Indole ring of Tryptophan

Carriers or supports commonly used for covalent bonding are:

(a). **Carbohydrates:** Eg. Cellulose, DEAE cellulose, Agarose
(b). **Synthetic agents:** Eg. Polyacrylamide
(c). **Protein carriers:** Collagen, Gelatin
(d). **Amino group bearing carriers:** Eg. amino benzyl cellulose
(e). **Inorganic carriers:** Porous glass, silica
(f). Cyanogen bromide (CNBr)-agarose and CNBrSepharose

Methods of covalent bonding

(1). Diazoation: Bonding between amino group of support and tyrosil or histidyl group of enzyme.
(2). Peptide bond: Bonding between amino or carboxyl groups of the support and that of the enzyme.
(3). Poly **functional reagents:** Use of a bi-functional or multifunctional reagent (glutaraldehyde) which forms covalent bonds between the amino group of the support and amino group of the enzyme.

Advantages of covalent bonding:

(a). Strong linkage of enzyme to the support
(b). No leakage or desorption problem
(c). Comparatively simple method
(d). A variety of support with different functional groups available

(e). Wide applicability

Disadvantages of covalent bonding (major problem with covalent bonding):

(a). Chemical modification of enzyme leading to the loss of functional conformation of enzyme.

(b). Enzyme inactivation by changes in the conformation when undergoes reactions at the active site. This can be overcome through immobilization in the presence of enzyme's substrate or a competitive inhibitor.

5. Describe the ionic adsorption method of enzyme immobilization with its merits and demerits

(1). Adsorption

Adsorption is the oldest and simplest method of enzyme immobilization. Nelson & Griffin used charcoal to adsorb invertase for the first time in 1916. In this method enzyme is adsorbed to external surface of the support. The support or carrier used may be of different types such as:

*(1). Mineral suppo*rt (Eg. aluminum oxide, clay)

(2). Organic support (Eg. starch)

(3). Modified sepharose and ion exchange resins

There is no permanent bond formation between carrier and the enzyme in adsorption method. Only weak bonds stabilize the enzymes to the support or carrier. The weak bonds (low energy bonds) involved are mainly:

(a). Ionic interaction

(b). Hydrogen bonds

(c). Van der Waal forces

For significant surface bonding the carrier particle size must be small (500 & to 1 mm diameter). The greatest advantage of adsorption method is that there will not be "pore diffusion limitations" since enzymes are immobilized externally on the support or the carrier.

Methods of adsorption:

(1). Static process: Immobilization to carrier by allowing the solution containing enzyme to contact the carrier without stirring.

(2). Dynamic batch process: Carrier is placed in the enzyme solution and mixed by stirring or agitation.

(3). Reactor loading process: Carrier is placed in the reactor, and then the enzyme solution is transferred to the reactor with continuous agitation.

(4). Electrode position process: Carrier is placed near to an electrode in an enzyme bath and then the current is put on, under the electric field the enzyme migrates to the carrier and deposited on its surface.

Advantages of adsorption method:

(a). No pore diffusion limitation

(b). Easy to carry out

(c). No reagents are required

(d). Minimum activation steps involved

(e). Comparatively cheap method of immobilization

(f). Less disruptive to enzyme than chemical methods

Disadvantages of adsorption method:

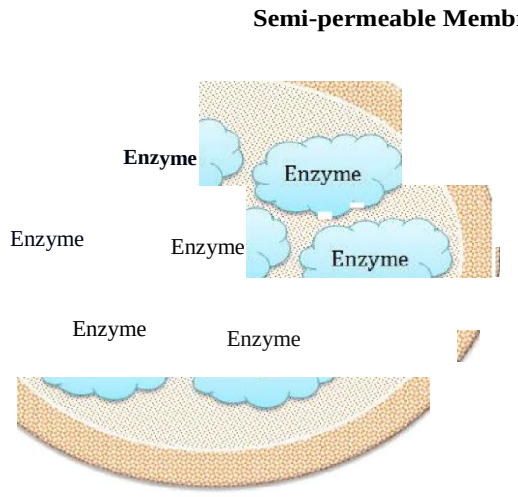
(a). Desorption of enzymes from the carrier

(b). Efficiency is less

6. Discuss the microencapsulation of enzymes. Give its merits and demerits(May 2017)

Encapsulation:

This type of immobilization is done by enclosing the enzymes in a membrane capsule. The capsule will be made up of semi permeable membrane like nitro cellulose or nylon. In this method the effectiveness depends upon the stability of enzymes inside the capsule.



Advantages of encapsulation:

(a). Cheap and simple method

(b). Large quantity of enzymes can be immobilized by encapsulation

Disadvantages of encapsulation:

(a). Pore size limitation

(b). Only small substrate molecule is able to cross the membrane

7. Discuss the methods of immobilization of enzymes using membranes with advantages and examples

Membrane confinement of enzymes may be achieved by a number of quite different methods, all of which depend for their utility on the semipermeable nature of the membrane. This must confine the enzyme whilst allowing free passage for the reaction products and, in most configurations, the substrates. The simplest of these methods is achieved by placing the enzyme on one side of the semipermeable membrane whilst the reactant and product stream is present on the other side. Hollow fibre membrane units are available commercially with large surface areas relative to their contained volumes ($> 20\,m^2\,L'$) and permeable only to substances of molecular weight substantially less than the enzymes. Although costly, these are very easy to use for a wide variety of enzymes (including regenerating coenzyme systems, without the additional research and development costs associated with other immobilisation methods. Enzymes, encapsulated within small membrane-bound droplets or liposomes may also be used within such reactors. As an example of the former, the enzyme is dissolved in an aqueous solution of 1,6-diaminohexane. This is then dispersed in a solution of hexanedioic acid in the immiscible solvent, chloroform. The resultant reaction forms a thin polymeric (Nylon-6,6) shell around the aqueous droplets which traps the enzyme. Liposomes are concentric spheres of lipid membranes, surrounding the soluble enzyme. They are formed by the addition of phospholipid to enzyme solutions. The micro-capsules and liposomes are washed free of non-confined enzyme and transferred back to aqueous solution before use.

Table: Generalised comparison of different enzyme immobilisation techniques.

Characteristics	Adsorption	Covalent binding	Entrapment	Membrane confinement
Preparation	Simple	Difficult	Difficult	Simple
Cost	Low	High	Moderate	High
Binding force	Variable	Strong	Weak	Strong
Enzyme leakage	Yes	No	Yes	No

Applicability	Wide	Selective	Wide	Very wide
Running Problems	High	Low	High	High
Matrix effects	Yes	Yes	Yes	No
Large diffusional barriers	No	No	Yes	Yes
Microbial protection	No	No	Yes	Yes

8. Glucose oxidase is substrate in an exothermic reaction. With this fact in mind outline the design of biosensor to measure the concentration of glucose in blood stream

The first generation glucose biosensors estimated glucose concentration in the sample based on hydrogen peroxide production by glucose oxidase (GOx) utilizing dissolved oxygen as given below

Cilucose + O — • Ulucunic acid + H•O•

A negative potential is applied to the Pt working electrode for a reductive detection of the oxygen consumption as

0, + 4H+ + 4c- 2H,O

The key point of above reaction lies in the redox center of the GOx (FAD) which performs the function of the initial electron acceptor. The interaction of glucose molecule with flavin adeninedinucleotiede (FAD) of GOx results in its reduction.

$$\text{Glucose} + \text{GOx (FAD)} \longrightarrow \text{Gluconate} + \text{GOx (FADH}_2\text{)}$$

The rejuvenation of the cofactor of enzyme GOx occurs in the presence of molecular oxygen, resulting in the formation of hydrogen peroxide (H2O2) as,

$$\text{GOx (FADH}_2\text{)} + \text{O}_2 \longrightarrow \text{GOx (FAD)} + \text{H}_2\text{O}_2$$

Thus, the rate of reduction of oxygen is directly proportional to the glucose concentration that is enumerated by either measuring the reduced oxygen concentration or increased concentration of hydrogen peroxide.

Hydrogen peroxide thus produced as a byproduct is oxidized at platinum (Pt) anode. The electrons transferred are recognized by electrode and thus the number of electrons transferred is directly proportional to the number of glucose molecules present.

H, —> O,+2H +2+

Major drawbacks of first generation glucose biosensor:

- Interference from electroactive species present in blood, such as uric acid, ascorbic acid and other constituents of blood, at the high operational potential (+0.6V) required for amperometric measurement of hydrogen peroxide. This limits the high selectivity of the analyzer and results in inaccurate measurements of glucose concentration.
- Oxygen deficit — Sensors involving natural oxygen as the electron acceptor due to presence of oxidase enzyme, generally face errors resulting from fluctuations in oxygen tension due to the limited solubility of oxygen in biological fluids. This reduces the linear range of the biosensor.

9. Discuss the applications of enzymes biosensors in analytical, medical and environmental monitoring

A biosensor has a wide range of applications in different fields.

Medicinal Application: biosensors have been used in various diagnostic procedures to determine various tests.

Industrial application: various manufacturing processes can be monitored by biosensors to provide assistance with regard to increase the quality and quantity of product obtained.

Environmental application: it helps in measuring the toxicity of water bodies, microbial contamination of natural resources helping in developing steps towards a cleaner environment.

Military application: it helps to detect explosives, drugs etc., aiding in defence of the people.

Another breakthrough in the field of biosensors was the production of a product called 'smart skin'. It is a kind of biosensor which detects any chemical or biological attack nearby and warns the person using the same.

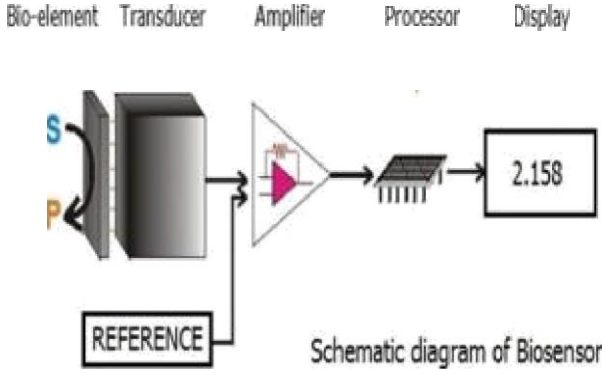
Drug development: a biosensor called 'nano sensors' has been developed which detects and analyse the binding of proteins to its targets which has proved very useful in drug designing. This also helps to monitor certain side effects caused by some medicines.

10. What are the major components of biosensor? Explain the working principle of biosensor with neat diagram

A biosensor mainly consists of two parts

(i) a biological part: this constitutes of enzymes antibodies etc., which mainly interacts with the analyte particles and induce a physical change in these particles.

(ii) a transducer part: which collects information from the biological part, converts, amplifies and display them. In order to form a biosensor, the biological particles are immobilized on the transducer surface which acts as a point of contact between the transducer and analyte. When a biosensor is used to analyse a sample, the biological part specific to the analyte molecules, interacts specifically and efficiently. This produces a physicochemical change of the transducer surface. This change is picked up by the transducer and gets converted into electric signals. These then undergo amplification, interpretation and finally display of these electric units accounting to the amount of analyte present in the sample.

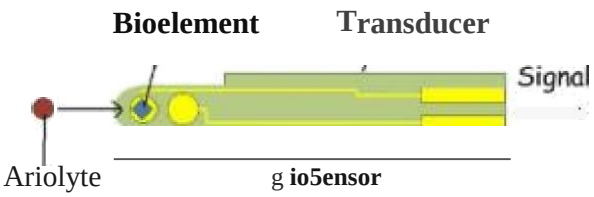


Schematic diagram of Biosensor

Bio Sensor

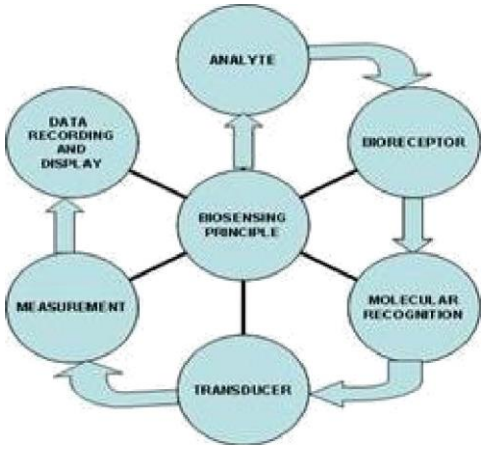
The Biosensor is used to detect the analyte so the Biosensor is an analytical device and it gathers the biological components with a Physicochemical detector. The sensing biological elements are biometric components interact with the recognize and analyze the study and the components like tissue, microorganisms, antibodies, nucleic acids and etc. The sensitive elements of biological can also generate by the biological engineering. The detector elements transform the signals from the interface of analyte with the biochemical elements into other signals like transducer and it can be measured more easily and qualified. The Biosensor devices are associated with the electronics and the signal processors and they are generally responsible for the display of the results and they are user-friendly. The Biosensor research has a significant role in the development of modern electronics. This article discusses about different types of Biosensors working and applications.

A Biosensor is an analytical device. The sensor which integrates the biological elements with the Physiochemical transducer to produce an electronic signal is proportional to a single analyte and which is fetched into a detector.



Working of Biosensors

The preferred biological material like enzyme is preferred for conventional methods like physical or membrane entrapment and non covalent or covalent binding. The preferred biological material is in contact with the transducer. To produce a bound analyte through the analyte binds to the biological material which produces the electrical response to be measured. In some cases the analyte changed to a product and have some probability to associate with the release of heat, gases like oxygen, electrons or hydrogen ions.



11. Explain the different types of biosensor and give their advantage and disadvantages of biosensors

A biosensor is a device for the detection of an analyte that combines a biological component with a physicochemical detector component.

An analytical device which functions to analyse a sample for the presence of a specific compound is known as sensor. A sensor which utilizes biological material to specifically interact with an analyte is known as **biosensor.** An **analyte** refers to the compound which has to be 'sensed' or the presence of which has to be determined. The interaction of analyte and biosensor is measured and converted to signals, which are again amplified and displayed. A biosensor thus involves converting a chemical flow of information into electrical signals. The biological materials used in biosensors are mostly enzymes, antibodies, nucleic acids, lectins, a cell as a whole etc.
According to the mode of interaction biosensors are of two types:
Catalytic biosensor: The interaction of biological material in the biosensor and the analyte result in modification of analyte into new chemical molecule. The biological material used is mainly enzymes.
Affinity biosensor: Here, upon interaction, the analyte binds to the biomolecule on the biosensor. These are mainly composed of antibodies, nucleic acids etc.
Essential **properties** of a biosensor:
(i) *Specificity:* a biosensor should be specific to the analyte which it interact.
(ii) *Durability:* it should withstand repeated usage.
(iii) *Independent nature:* It should not be affected by variations in the environment like temperature, pH etc.
(iv) *Stability* in results: the results produced by interaction should be corresponding to the concentration of analyte.
(v) *Ease* of use and transport: it should be small in size so that it can be easily carried and used.
Types of biosensors:
(i) *Calorimetric* biosensor: some enzyme- analyte reactions are exothermic and releases heat into the sample. This change in temperature is detected by the transducer. The amount of heat generated is proportional to the analyte concentration present and is processed likewise.
(ii) *Potentiometric* biosensor: an electric potential is produced as a result of interaction which is detected by the transducer
(iii) *Amperometric* biosensor: analyte when comes in contact with biological material induces a redox reaction. This results in movement of electrons which is picked up by transducer.
(iv) *Optical* biosensors: in this, a biosensor reacts with analyte to absorb or release light which is identified by the transducer and interpreted.

(v) *Acoustic wave* biosensors: biological component of biosensor undergoes a biomass change ascertained by transducer.

The **advantages** of biosensors include accuracy in results, minute detection capability, ease of use, versatile and continuous monitoring available.

12. Write in detail about the design of the enzyme electrode

Electrode

Electrochemical electrodes (orEnzyme eletrodes) are a new type of detector *orbiosensor* that have been exclusively designed for thepotentiometricoramperometricassay of substrates, for instance : alcohol, amino acids, glucose, andlactic ac i d.

The enzyme electrode is a combination of any electrochemical probe(amperometric, potentiometric or conductimetric) with a thin layer (10 - 200mm) of immobilised enzyme.

History

Enzyme electrodes are a type of biosensor that have enzyme as a biologicalcomponent. The history of biosensors started in the year 1962 with the development of amperometric enzyme electrode for glucose by the scientist Leland C. Clark.The year1969 marks first potentiometric biosensor:urease immobilized on an ammoniaelectrode to detect urea.During the year 1972-75, first commercial glucose biosensor.

Biosensors are called enzymatic electrodes when an enzyme is the biological component and transduction is based on electrochemical principles. This type of biosensor now plays an important role, in particular, for biomedical and technological applications.Enzymesare proteins which present an outstanding ability for molecular recognition. The biochemical changes following this process are finally electrochemically transduced.Many enzymes have been characterized, particularly with respect to tertiary structure and are also commercially available. Therefore, it is not surprising that a great majority of the biosensors, so far described, are based on enzymes2.

Redox enzymes, called oxido-reductases, are probably the most frequently employed. They are classified with regard to the prosthetic group, a molecule tightly bound to the protein structure: flavin, quinone, heme or copper center.More than 80 flavin enzymes are known with FAD (flavin adenine dinucleotide) or FMN (flavin adenine mononucleotide) covalently or tightly attached. The active redox site of this molecule corresponds to the isoalloxacine group and the redox process depends strongly on pH.The redox potential of FAD has been studied in different enzymes and depending on the protein structure, it varies between -730 and -50 mV vs. SCE.

The enzymes which contain quinones as prosthetic group, called quinoenzymes, are less common. The redox active group is 2,7,9-tricarboxy- lH- pyrrolo- (2,3-quinoline-4,5-dione) known as PQQ and since oxygen does not reoxidise it, they are dehydrogenases. Quino enzymes used in electrochemical studies include alcohol dehydrogenase and glucose dehydrogenase.

13. Explain in detail about

Calorimetric Biosensors (8) ii) Potentiometric Biosensors (8)

Potentiometric Biosensors

The basic principle behind potentiometric sensor measurements is the development of a voltage related to the analyte activity (concentration) [A] in the sample through the Nernst relation:

$$E = E^o + \frac{RT}{n} \ln [A]$$

Potentiometric sensors will generally require a reference electrode as well as the indicator (working) electrode to be in contact with the test sample solution. The use of ion-selective membranes can make these sensors sensitive to various ions (e.g, hydrogen, fluorine, iodine, chlorine ions) in addition to gases such as carbon dioxide and ammonia. Enzyme systems, that change the concentration of any of these ions or gases, can also be incorporated into the sensor in order to be able to measure enzyme

substrate concentrations, or to detect inhibitors (e.g., heavy metal ions, insecticides) or modulators of the enzyme.

Ideally, the potential difference between the indicator and reference electrode is proportional to the logarithm of the ion activity or gas fugacity. However, this is only the case when:

• The membrane or indicator electrode surface layer is 100% selective for the test analyte, or

• There is a constant or low enough concentration of interfering ions, and

• Potential differences at various phase boundaries (junction potentials) are either negligible or constant, except at the membrane-sample solution interface.

Many of these sensors take the form of a pH electrode to measure the activities of enzymes (and hence the concentration of the specific substrate for that enzyme) which produce or consume protons as a result of catalysis. Examples of enzymes that can be used in this way are urease, glucose oxidase, penicillinase and acetylcholinesterase - to monitor the concentrations of urea, glucose, penicillin, and the neurotransmitter acetylcholine (or some pesticides that inhibit acetylcholinesterase) respectively.

Strengths:

• A wide concentration range for detection of ions (typically 1 qM to 0.1 M).

• Can perform continuous measurements (ideal for clinical/environmental use).

• Inexpensive and portable.

Weaknesses:

• pH buffers are often required to maintain optimum enzyme activity, and this can limit the dynamic range of detection of the analyte for enzyme-based sensors.

14. Explain in detail about

Amperometric Biosensors (8) ii) Optical Biosensors (8)

Amperometric Biosensors

These are the most commonly reported class of biosensor. They typically rely on an enzyme system that catalytically converts electrochemically non-active analytes into products that can be oxidized or reduced at a working electrode. This electrode is maintained at a specific potential with respect to a reference electrode. The current produced is linearly proportional to the concentration of the electroactive product, which in turn is proportional to the nonelectroactive enzyme substrate. Enzymes typically used in amperometric biosensors are oxidases that catalyze the following class of reactions.'

$$\text{Substrate} + 02 \text{ —+ Product} + H2O2$$

As a result of the enzyme-catalyzed reaction, the substrate (analyte) concentration can be determined by amperometric detection of oxygen or hydrogen peroxide (H2O2). An example of this configuration would be an oxygen-consuming enzyme coupled to an oxygen-sensing electrode. The ambient oxygen concentration is then continuously monitored as it diffuses through a semi-permeable membrane and is reduced at a platinum (Pt) electrode. Other common configurations include the use of oxidases specific to various substrates to produce H2O2

During measurement, the working electrode may act as an anode or a cathode, according to the nature of the analyte. For example, a glucose-sensitive biosensor that uses glucose oxidase could detect the H2O2 produced by the enzymatic reaction by polarising the working electrode to a positive potential (+0.6V vs. SCE), or by polarising the working electrode to a negative potential (-0.65V vs. SCE) to monitor oxygen.

These sensors which use an oxidase enzyme and detect the products electrochemically are considered to be first generation devices which have a number of problems.

Strengths:

• The use of low-cost and disposable electrodes.

• High degree of reproducibility that is possible for these (one-time use) electrodes eliminates the cumbersome requirement for repeated calibration.

• The instrumentation for these biosensors is inexpensive and compact, allowing for the possibility of on-site measurements.

Weaknesses:

• Tend to have a small dynamic range due to saturation kinetics of the enzyme.

• Potential interference to the response if several electroactive compounds can generate false current values. (These effects have been eliminated, for clinical applications, through the use of selective

membranes, which carefully control the molecular weight of the charge of compounds that have access to the electrode.)
• Require oxygen for the enzyme activity to transfer electrons. If the oxygen content of the measured solution is too low then the reaction rate will depend on this rather than the glucose concentration.

One method to get round these issues involves the use of membranes as mentioned above.
The outer membrane can control the flux of oxygen and glucose between the sensed environment and the immobilised enzyme layer. This can prevent the sensor being dependent on 02 concentration or on the diffusion barrier in an unstirred solution. The inner barrier is designed to prevent interferents which are electroactive at the same potential as H2O2 from reaching the electrode. Most of these interferents are larger molecules which enables this discrimination.
Other solutions to cross tolerance with interferants in H2O2 sensing may involve the use of the materials, enzymes etc. to lower the potentials used and avoid other electroactive substances. The paper mentioned on glucose sensing earlier in the course uses "Prussian Blue" immobilised in chitosan which has a dual effect of catalysing the peroxide sensing and blocking other molecules.
Amperometric biosensors operate by measuring the current generated by oxidation or reduction of redox species at an electrode surface, which is maintained at an appropriate electrical potential. The current observed has (hopefully) a linear relationship with the concentration of the analyte. However, the direct electron transfer between the redox-active site of an enzyme immobilized at an electrode surface is normally prohibited by an intervening, insulating, part of its polypeptide structure. Some kind of charge carrier has then to be used as an intermediate between the enzyme redox centre and the electrode. In glucose 4 oxidase the redox-active site is a flavin adenine dinucleotide (FAD) molecule at the heart of the protein

Optical Biosensors

There are two main areas of development in optical biosensors. These involve determining changes in light absorption between the reactants and products of a reaction, or measuring the light output by a luminescent process. The former usually involve the widely established, if rather low technology, use of colorimetric test strips. These are disposable single-use cellulose pads impregnated with enzyme and reagents. The most common use of this technology is for whole-blood monitoring in diabetes control. In this case, the strips include glucose oxidase, horseradish peroxidase (EC 1.11.1.7) and a chromogen (e.g. o-toluidine or 3,3',5,5'-tetramethylbenzidine). The hydrogen peroxide, produced by the aerobic oxidation of glucose ,oxidising the weakly colouredchromogen to a highly coloured dye.

$$\text{chromogen(2H)} + \text{H2Oz} \xrightarrow{\text{Peroxidase}} \text{dye} + 2\text{HzO}$$

The evaluation of the dyed strips is best achieved by the use of portable reflectance meters, although direct visual comparison with a coloured chart is often used. A wide variety of test strips involving other enzymes are commercially available at the present time.A most promising biosensor involving luminescence uses firefly luciferase (Phofioos-luciferin 4-monooxygenase (ATP-hydrolysing), EC 1.13.12.7) to detect the presence of bacteria in food or clinical samples. Bacteria are specifically lysed and the ATP released (roughly proportional to the number of bacteria present) reacted with D-luciferin and oxygen in a reaction which produces yellow light in high quantum yield.

$$\text{ATP} + \text{D-luciferin} + \text{O2} \xrightarrow{\text{luciferase}} \text{oxyluciferin} + \text{AMP} + \text{pyrophosphate} + \text{CO2} + \text{light(562 nm)}$$

The light produced may be detected photometrically by use of high-voltage, and expensive, photomultiplier tubes or low-voltage cheap photodiode systems. The sensitivity of the photomultiplier-containingsystems is, at present, somewhat greater ($< 10^4$ cells ml^{1}, $< 10'°$ M ATP) than the simpler photon detectors which use photodiodes. Firefly luciferase is a very expensive

enzyme, only obtainable from the tails of wild fireflies. Use of immobilised luciferase greatly reduces the cost of these analyses.

15. Discuss in details about the applications of immobilized enzymes in biosensors with example

Biosensors are electrical, optical, chemical or mechanical devices with the capability to detect biological species selectively. They are often modified with biological entities to enhance their selectivity. Examples of biological recognition molecules include enzymes, antibodies and oligonucleotides. The ideal biosensor not only has to respond to low concentrations of analytes but also must have the ability to discriminate among species according to the recognition molecules that are immobilized on its surface. Biosensors have wide applications including biomarker detection for medical diagnostics and pathogen and toxin detection in food and water (Leung et al., 2007). Analytical technology based on biosensors is an extremely broad field which impacts on many major industrial sectors such as the pharmaceutical, healthcare, food and agricultural industries as well as environmental monitoring. Because of their exceptional performance, capabilities which include high specificity and sensitivity, rapid response, low cost, relatively compact size and user-friendly operations, these properties of biosensors make them an important tool for detection of various chemical and biological components (Amine et a1., 2006). The development of biosensors based on immobilized enzymes came out to solve several problems such as loss of enzyme, maintainace of enzyme stability and shelf life of biosensors and additionally to reduce the time of enzymatic response and offer disposable devices which can be easily used in stationary or in flow system.

Biosensors based on principle of enzyme inhibition have by now been applied for a wide range of significant analytes such as Organophosphorus Pesticides (OP) organochlorine pesticides, derivatives of insecticides, heavy metals and glycoalkaloids. The choice of enzyme/analyte system is based on the fact that these toxic analytes inhibit normal enzymatic function. Typically, the percentage of inhibited enzyme (1%) that results after exposure to inhibitor is quantitatively related to the inhibitor (i.e., analyte) concentration (Ivanov et al., 2003a, b).

Malitesta and Guascito (2005) have described the application of biosensors based on glucose oxidase immobilized by electropolypmerization for heavy metal determination. Similarly, urease has been entrapped in both Polyvinyl Chloride (PVC) and cellulose triacetate layers on the surface of pH-sensitive iridium oxide electrodes and used for the determination of mercury. The immobilization of polyphenol oxidase during the anodic electropolypmerization of polypyrrole has been also reported.

The biosensor has been used for the determination of atrazine concentration in low ppm level. The determination of pesticides with the help of biosensors have become increasingly important in recent years because of the widespread use of these compounds (El-Kaoutit et a1., 2004).

16. Write a short note on

Piezo-electric Biosensors (8) Immunosensors (8)

Piezoelectric Biosensors:

Piezoelectric biosensors are based on the principle of acoustics (sound vibrations), hence they are also called as acoustic biosensors. Piezoelectric crystals form the basis of these biosensors. The crystals with positive and negative charges vibrate with characteristic frequencies. Adsorption of certain molecules on the crystal surface alters the resonance frequencies which can be measured by electronic devices. Enzymes with gaseous substrates or inhibitors can also be attached to these crystals.

A piezoelectric biosensor for organophosphorus insecticide has been developed incorporating acetylcholine esterase. Likewise, a biosensor for formaldehyde has been developed by incorporating formaldehyde dehydrogenase. A biosensor for cocaine in gas phase has been created by attaching cocaine antibodies to the surface of piezoelectric crystal.

Limitations of Piezoelectric Biosensors:

It is very difficult to use these biosensors to determine substances in solution. This is because the crystals may cease to oscillate completely in viscous liquids.

Immuno-Biosensors:

Immuno-biosensors or immunochemical bio-sensors work on the principle of immunological specificity, coupled with measurement (mostly) based on amperometric or potentiometric bio-sensors.

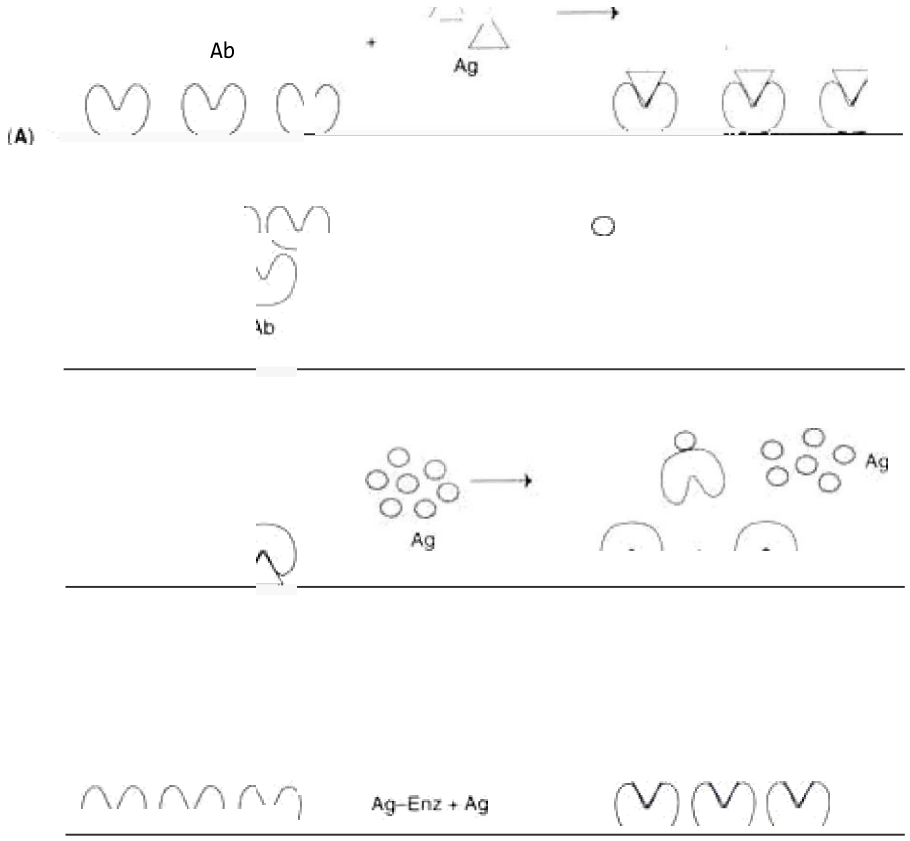


Fig. 21.18 : *Diagrammatic representation of selected immunobiosensors (**A**) Direct binding of antigen to immobilized antibody, (**B**) Antigen–antibody sandwiches (immobilized antigen binds to antibody and then to a*

Immunobiosensors

1. An immobilized antibody to which antigen can directly bind
2. An immobilized antigen that binds to antibody which in turn can bind to a free second antigen
3. An antibody bound to immobilized antigen which can be partially released by competing with free antigen.
4. An immobilized antibody binding free antigen and enzyme labeled antigen in competition.

For the biosensors 1-3, piezoelectric devices can be used. The immuno-biosensors using enzymes are the most commonly used. These biosensors employ thermometric or amperometric devices. The activity of the enzymes bound to immuno-biosensors is dependent on the relative concentrations of the labeled and unlabeled antigens. The concentration of the unlabeled antigen can be determined by assaying the enzyme activity.

17. What are micro-biosensors? Explain in detail with example

Whole Cell Biosensors:

Whole cell biosensors are particularly useful for multi-step or cofactor requiring reactions. These biosensors may employ live or dead microbial cells. A selected list of some organisms along with the analytes and the types of biosensors used is given in Table

T4szz A1.a A sdjected lfst of organ	along Bi the aaa	zzid the ”ef biosensors
Orj;anism	*Ana!yt‹•*	*Tyfi›e od biosensor*
Escherichia coli	Glu[a*aie	Polenliomettic {CON)
Sarella f/aya	Glvlamine	Po:enlòme[ric (NHy)
Proteus *mo riii*	Cy‹1aine	Po'eniiome[nc (H/]
K'/rosman+s sp	Ammo.nia	Amperornelric (Oy)
Lar/odzci/is /s'rrtterifi	Tfiiamine	Ampeomelric (rnad1a\edl
Lactobacillus arabinosus	Nm1iniC ael	Poterrliomelric (H“)
Peas//ou”dré desu/fur/¢ans	Sulfdie	Polentónt+ifi¢ (SD})
Gyanobaclen,a	Heròicides	Ampeiometric (media1ed)
Many organisms	eiologicai oxygen	Ampe emeiri6 (0)

Organisms along with the Analytes and the Types of Biosensors

Advantages of microbial cell biosensors:
The microbial cells are cheaper with longer half-lives. Further, they are less sensitive to variations in pH and temperature compared to isolated enzymes.

Limitations of microbial cell biosensors:
The whole cells, in general, require longer periods for catalysis. In addition, the specificity and sensitivity of whole cell biosensors may be lower compared to that of enzymes.

18. Explain the physical and chemical techniques of enzyme immobilization(May 2015)
PhysicalMethods
Adsorption
Entrapping
Membrane confinement
- ChemicalMethods
Covalent Bonding
Cross Linking
Complexation&Chelation

19. Outline the various methods of enzyme immobilization with their major advantage and disadvantage. Explain how the immobilizing matrices affect the enzyme activity (May 2016, May 2017)
Different methods of enzyme immobilization are
- PhysicalMethods
Adsorption
Entrapping
Membrane confinement
- ChemicalMethods
Covalent Bonding
Cross Linking
Complexation&Chelation
Advantages of immobilization are:
- Recoveredat the end of the reactiontherebycanbereused.
- Economy of the reactionisimproved.
- Easyseparationof enzyme from the productsoccurs.
- Stabilityof immoblilised enzymeincreases.
- Enhancedenzyme properties.

- Efficiency of the catalyticreactionisbetter in a few cases.
- **Better control** of reactioncanbeachieved.
- Catalyticprocesscanbeoperatedcontinuously.
- **Multi** enzyme reaction possible.
- **Potentialin** industrial&medicinal use.

Disadvantages of enzyme **immobilization:**

(1). Even though there are many advantages of immobilized enzymes, there are some disadvantages also.

(2). High cost for the isolation, purification and recovery of active enzyme (most important disadvantage)

(3). Industrial applications are limited and only very few industries are using immobilized enzymes or immobilized whole cells.

(4). Catalytic properties of some enzymes are reduced or completely lost after their immobilization on support or carrier.

(5). Some enzymes become unstable after immobilization.

(6). Enzymes are inactivated by the heat generated in the system

20. **Design a enzyme electrode based biosensor for measuring ethanol in the fermentation** process.

Alcohol content in fermentation broths can be realized by many conventional methods, for example hydrometry and gas chromatography. Considered the error limit or high expense or time-costing procedures of them, biosensor is a good alternative.

A colorimetric biosensor was proposed by Kuswandi et al. The sensor was constructed by polyaniline film immobilized alcohol oxidase. When ethanol is in presence, a color change from green to blue can be observed due to the oxidation of polyaniline by the enzyme reaction product H2O2. Through the computer processing software, the method can determine alcohol quantitatively range between 0.01 and 0.8% . Gotoh et al. devised an amperometric alcohol sensor based on co-immobilized alcohol dehydrogenase and coenzyme NAD+, the enzyme electrode shown linear response to solution contains ethanol between 0.05 and 10v/v%. As a reagentless enzyme sensor, it can stand at least weeks of continual detections without addition of the coenzyme .

Module -4

ENZYME ENGINEERING AND MEDICAL IMPORTANCE

What are the major plant sources of enzymes? Give examples. Papain—Papaya, Bromelin—pineapple, Actinidin - Kiwi fruit

1. **What are the major bacterial sources of enzymes?** Give **examples.**

Beta-Amylase — *Bacillus,* Pullulanase -*Klebsiella ,* Penicillin amidase - *Bacillus*

2. **What are the major fungal** sources of enzymes? Give **examples.**

Catalase — Aspergillus, Cellulase — Trichoderma, Dextranase - Penicillium

3. **What are the major** yeast **sources of enzymes? Give examples.**

Invertase– *Saccharomyces,* Lactase— *Kluyveromyces,* Lipase - *Candida*

4. **What** are **the** major **animal** sources of enzymes? Give examples.

Catalase—Liver, Chymotrypsin—Pancreas, Lipase—Pancreas

5. How are enzymes **extracted?**

The molarity and pH of the (solvent) buffer is suitably adjusted to achieve maximum solubility and activity of the enzyme. EDTA is often included in the extraction medium to remove heavy metals, and for disrupting the membranes of cells and cell organelles. Detergents such as Triton-X are also used sometimes to solubilise the membranes.

6. **What are the different methods available for enzyme purification?**

Enzyme purification involves three steps, electrophoresis, Dialysis and Chromatography.

7. **What is coupled** assay?

Even when the enzyme reaction does not result in a change in the absorbance of light, it can still be possible to use a spectrophotometric assay for the enzyme by using a coupled assay. Here, the product

of one reaction is used as the substrate of another, easily detectable reaction. For example, figure 1 shows the coupled assay for the enzyme hexokinase, which can be assayed by coupling its production of glucose-6-phosphate to NADPH production, using glucose-6-phosphate dehydrogenase.

8. **Define fold purification.**
   The enrichment of concentration of enzyme in each step of purification is known as fold purification.
9. **What is intracellular enzyme?**
   An enzyme that remains within the cell in which it is formed
10. **Explain briefly the principle involved in purification of enzymes?**
    Enzymes are proteinaceous in nature, standard extraction and purification procedures for enzymes are the same as those used for proteins except that the activity of the enzyme is assayed at each of the following four steps of extraction and purification.
11. **Name the methods used to characterizing the molecular weight** of enzymes.
    SDS-electrophoresis, Size exclusion chromatography
12. **What is extracellular enzyme?**
    An enzyme which is secreted outside the cell from which it originates
13. Give **example for chromatographic methods used in**enzyme **purification.**
    (i) Adsorption or column chromatography; (ii) ion exchange chromatography; (iii) gel filtration chromatography and (iv)affinity chromatography.
14. **State the principle** of **electrophoresis.**
    Electrophoresis is a technique in which molecules (enzymes, proteins, amino acids, nucleotides and nucleic acids) are separated by differences in their net charge in the presence of an externally applied electric field.
15. **What is crude enzyme?**
    Enzyme present in unpurified fermentation broth is called crude enzyme
16. State **the principle of chromatography.**
    Chromatography is a process whereby a mixture of solutes may be resolved into its components by exploiting differences in affinity of the solutes for particles of an insoluble matrix over which a solution of the components is passing. The insoluble matrix is called the stationary phase, while the solution which passes through it is called the mobile phase.
17. **What are primary factors that affect the separation process?**
    The process used will mainly depend on the purity needed, Physico-chemical characteristics of fermentation broth and the cost, which is acceptable.
18. **Name any four general protein isolation technique.**
    Adsorption, Precipitation, Extraction and Membrane separation processes
19. **What is isoelectric precipitation** .
    When, an acid or base is added, the enzyme protein can be brought to its isoelectric pH. At this pH, there is no net charge on enzyme molecules and electrostatic repulsion between them is low so that they tend to aggregate. Therefore, adjusting the pH to the isoelectric point of a protein causes its precipitation.
20. **What are the two methods used for protein precipitation.**
    Salting-out and solvent precipitation
21. **What is salting-out in protein purification?**
    The salt competes with the protein for solvent molecules and thereby lowers its solvation. Salts can change the structure of the solvent, which can lead to large changes in protein conformation by altering the electrostatic interactions between charged groups on the protein surface and get precipitated.
22. **What are the major unit operation used in enzyme isolation** .
    Filtration, centrifugation, extraction, precipitation, crystallization and drying
23. **Name any two methods used for cell disruption.**
    Ultrasonication and alkali treatment
24. **Microbes are preferred source of** enzyme, why?
    Microbes are preferred source of enzyme because in can be applied easily for Large scale production, enzyme characteristics can be increased and free or less regulation from government agencies .
25. **Mention any two methods available to protect the target enzymes from protease attack during extraction from crude source**
    - Use of protease enzyme inhibitors during extraction
    - Temperature at which extraction is carried out

26. Write a short note on dialysis.

The separation of particles in a liquid on the basis of differences in their ability to pass through a membrane.

27. **Design an assay procedure for an oxidoreductase which utilizes NADH as cofactor.** Spectrophotometric method can be used for the estimation. UV light is often used, since the common coenzymes NADH and NADPH absorb UV light in their reduced forms, but do not in their oxidized forms. An oxidoreductase using NADH as a substrate could therefore be assayed by following the decrease in UV absorbance at a wavelength of 340 nm as it consumes the coenzyme.

28. Microbes are **preferred source of** enzyme,why?

Microbes are preferred to plants and animals as sources of enzymes because:

- They are generally cheaper to produce.
- Their enzyme contents are more predictable and controllable,
- Reliable supplies of raw material of constant composition are more easily arranged, and
- Plant and animal tissues contain more potentially harmful materials than microbes, including phenolic compounds (from plants), endogenous enzyme inhibitors and proteases.

29. Define the enzyme activity units-katal and IU. Which one of the above is considered as SI unit? (Nov 2016)

katal is defined as the amount of enzyme causing loss of 1 mol substrate per second under specified conditions. International units(IU) is defined as as the amount of enzyme causing loss of 1 Mmol substrate per minute under specified conditions.

30. Write any five commercially important enzymes and their sources

Cellulase- Cellulose conteining plant materials, Amylase- saliva, Phospahatase- potato, Lysozyme- Egg white, Protease- Bacillus sp

31. Account on affinity chromatography for purification of enzyme proteins

This technique takes the advantage of the fact that many proteins specifically bind other moolecules as part of their function. Upon passing the protein solution through such a column, only priteins that can bind the ligand will be retained. them the conditions can be adjusted to effect release from the ligand.

1. Describe in detail the different steps involved in isolation, purification and characterization of an industrially important enzyme with example

Isolation and Purification - Isolation and purification is done immediately after terminationof fermentation in a manner that retains the enzyme activity. If the cells are to be used for immobilization, the biomass is isolated and treated to make it ready for use.

The extracellular enzymes are recovered directly from broth, while enzymes localized within cells are isolated by rupturing the cells. Enzyme purification is based on various techniques whose efficacy and cost differ widely; the process used will mainly depend on the purity needed and the cost, which is acceptable.

o - Amylase - This enzyme is an endo hydrolase; it hydrolyzes starch into components, which have three or more linear o -1, 4-gulcan units. It stops hydrolysis when fragments with 2-6 glucose units remain; typically, such fragments contain an o -1,4-6 linked branch point residue.

The end products of starch hydrolysis are dextrins, which are used as adhesives and thickening agents in prepared foods. o -Amylase must be used at high temperatures. The enzyme from Bacillus licheniformis can be used for prolonged periods at 95°C and for a brief period at 105-110°C.

The bacteria are grown on complex media based on maize or potato starch supplemented with soybean meal or corn steep liquor (medium has 20% dry matter). The fermentation is carried out for about 5 days, then the broth is chilled and the cells and solids are removed by flocculation.

The enzyme is extracellular, and is recovered from the broth; it is always stabilized with Ca2+ ions. Its applications are: dextrin production, first stage in glucose manufacture, in brewing and bakery, for removal of starch in textile manufacture, etc.

Amyloglucosidase - Also called glucoamylase, this enzyme is an exohydrolase, and removes terminal glucose residues, one-by-one, from dextrins. This is produced by fungi, such as, Aspergillus or

Rhizopus. The strains used for enzyme production are regulatory mutants (enzyme synthesis not repressed by free glucose), which are grown on o-amylase digested starch (20% w/v) medium.

The fermentation lasts for 4-5 days at pH 4.5, and is N limited. The enzyme is extracellular and is concentrated to about 5% active enzyme. The dextrins obtained by o-amylase digestion of starch are further digested to glucose by glucoamylase.
Glucose Isomerase - Commercial glucose isomerase is, in fact, D-xylose ketol-isomerase, and is produced by several bacteria (Table 10.10). Some improved strains produce the enzyme constitutively. The bacteria are grown in aerated batch cultures at 30°C and 7.0 pH for 2-3 days.
The enzyme is intracellular, and is best used in the form of immobilized cells. The cells are suitably treated to increase their stability and catalytic activity.

Purified glucose syrup is heated to remove dissolved O2 and increase glucose concentration to 40%. pH is adjusted between 7 and 8. The syrup is passed through a column containing immobilized bacterial cells with glucose isomerase activity; the temperature is kept at 60°C.
The enzyme longevity, under practical conditions, is 2,000-4,000 hr optimally; 20 tons or more product can be processed per kg of the catalyst. The end product of glucose isomerase action is a nearly 1 1 mixture of glucose and fructose; this has increased sweetness as compared to glucose and is virtually identical with 'invert sugar obtained from beet or cane sugar (sucrose).
Enzyme Biotechnology - Enzymes are biological catalysts, which initiate and accelerate thousands of biochemical reactions in living cells.
They process reactions which are otherwise not possible under normal conditions found in the cell. For instance, although, hydrolysis of starch in a test tube requires strong acidic medium and high temperature (boiling), in the alimentary canal it is hydrolysed and digested under normal conditions of acidity and temperature.
This is made possible by starch hydrolysing enzymes available in the stomach. In fact, almost all biochemical reactions require one or more enzymes for their completion. Non enzymatic conversions, though are known, but are very few.
Enzymes are proteinaceous in nature; they can be extracted from living tissues, purified and even crystallized. Under controlled conditions of isolation, they retain their original level of activity and in some cases even exhibit an increased activity. Thus, a purified enzyme can be used to carry on a specific biochemical reaction outside the cell.

Isolation and Purification of Enzymes - Enzymes are unstable molecules with a definite physico chemical organization. Even a slight change in this organization reduces the activity of enzyme and sometimes the enzyme is totally inactivated.
Therefore, the enzymes have to be isolated under controlled conditions of pH, ionic strength and temperature.

Since they are proteinaceous in nature, standard extraction and purification procedures for enzymes are the same as those used for proteins except that the activity of the enzyme is assayed at each of the following four steps of extraction and purification.
Preparation of Crude Enzymes - Centrifugation
The enzyme extract is centrifuged to remove cell debris, cell organelles and sometimes other molecular aggregates, leading to partial purification of enzymes.
It also helps in characterization of an enzyme, since, depending upon its mass and shape the enzyme will move through a solution at a definite speed and occupy a characteristic position in the centrifuge tube.
For most cytosolic enzymes, centrifugation at about 30,000 g for 30 minutes is good enough to obtain a fair amount of activity in the supernatant.

However, if the enzyme is located in a specific cell organelle, an extract rich in that organelle is prepared through 'preparative centrifugation'. (Centrifugation for different durations at different velocities allows the cell organelles to sediment according to their sizes. All centrifugation operations are conducted in cold (O-4°C).
Precipitation

Enzymes and other proteins are highly charged molecules, and can be precipitated with appropriate charge neutralizing chemicals. Once their charges are broken, they form aggregates and settle down as precipitate.
When, an acid or base is added, the enzyme protein can be brought to its isoelectric pH. At this pH, there is no net charge on enzyme molecules and electrostatic repulsion between them is low so that they tend to aggregate. Therefore, adjusting the pH to the isoelectric point of a protein causes its precipitation.
Acids and bases, however, often inactivate the enzyme, so that their use for precipitation is not recommended in most cases. Instead ammonium sulphate and other salts are used for precipitation in a process called 'salting out'. Salts can change the structure of the solvent, which can lead to large changes in protein conformation by altering the electrostatic interactions between charged groups on the protein surface.
The salt also competes with the protein for solvent molecules and thereby lowers its solvation. In large scale enzyme precipitation, use of many other neutral salts is preferred over ammonium sulphate, which is corrosive and releases NH3 at higher temperatures.
Some organic solvents like acetone, methanol and ethanol are also used for enzyme precipitation, since water miscible solvents decrease the solubility of proteins, leading to precipitation.
They are cooled upto 40 60°C before their use, and precipitation is carried out at O°C, because precipitation at room temperature causes denaturation of the enzyme, in most cases. Organic solvents are added drop by drop to avoid local concentration.
Water soluble non ionic polymers such as polyethylene glycol, alginate, pectate, carboxymethyl cellulose, polyacrylic and polymeta acrylic acids, etc. also cause enzyme precipitation.

Polyethyleneimine is also widely used as protein precipitant at large scale. They primarily act through the removal of solvent sphere of the enzyme protein.
Extraction of Enzymes - Fresh tissue is crushed into a paste with an extraction medium (often a buffer) in a mortar and pestle, or in a tissue homogenizer, or in a blender or by ultrasonic vibrations (sonication).
The molarity and pH of the buffer is suitably adjusted (which may vary for different enzymes) to achieve maximum solubility and activity of the enzyme. EDTA (ethylene diamine tetra acetic acid) is often included in the extraction medium to remove heavy metals (which otherwise inhibit enzyme activity), and for disrupting the membranes of cells and cell organelles. Detergents such as Triton-X are also used sometimes to solubilise the membranes.
Many enzyme proteins contain disulfide (S-S) bonds due to the presence of cysteine residues, which are easily broken during enzyme extraction leading to loss of enzyme activity. To overcome this problem are added, thiols such as mercaptoethanol whose sulfhydryl (-SH) group is able to maintain the S-S linkage in enzymes.

If the extract is not homogeneous, the homogenate (extract) is filtered to remove cell derbis, fibres etc., otherwise filtration may be avoided. All operations of extraction and purification are generally carried out in cold (O-4°C), since most of the enzymes get inactivated at higher temperatures.

Purification of Enzymes - Enzyme purification involves three steps, electrophoresis. These three techniques described in the following text
1. Dialysis
2. Chromatography.
Dialysis of Enzymes - Dialysis is the process that is used to remove small molecules from enzyme. For this, enzyme precipitate obtained in previous step is dissolved in a small quantity of buffer solution in which the enzyme was originally extracted. The solution is taken in a dialysis bag (may be a cellophane tube) and after sealing securely, the bag is suspended in either distilled water or a buffer of known molarity and ionic composition.
Some other salts or chemicals may have to be added sometimes in the outer solution, to prevent the loss of enzyme activity during dialysis. The dialysis is carried out for a few hours with regular change of the outer solution or distilled water. At large scale enzyme purification 'dialafitration' instead of dialysis is used.

The enzyme solution is filtered .for small molecules through a membrane generally mounted on a fibrous support, by pressure dirven operations.

Chromatography for Enzyme Purification - Chromatographic separation of proteins is the most common method of enzyme purification.
Following four types of chromatography are available for this purpose:
(i) adsorption or column chromatography; (ii) ion exchange chromatography;
(iii) gel filtration chromatography and (iv)affinity chromatography.
Adsorption Chromatography for Enzyme Purification - In adsorption chromatography, the protein or enzyme solution suspected to contain other proteinaceous impurities is passed through a column of inert material packed in a glass or steel tube. Most commonly used column materials include finely divided solids such as charcoal, silica, alumina, calcium phosphate, hydroxyapatite, etc.
The effluent solution is continuously collected in small fractions of 1.0 to 2.0 mi. The protein in each fraction is estimated by measuring the absorption at 280 nm using a UV spectrophotometer. The enzyme is also assayed in each fraction. Various spleen enzymes such as basic RNAase, acidic RNAase, acidic DNAse, phosphodiesterase, phosphomonoesterase, etc. are often separated from each other using adsorption chromatography.
For large scale chromatographic separation of enzymes, the process is accelerated by using motors and other mechanical devices for packing the column, for loading the enzyme on the column and for eluting the enzyme.

Ion Exchange Chromatography for Enzyme Purification - In ion exchange chromatography, generally a cellulosic ion exchange is taken in the column. The proteins are separated according to their charges.

The resolution is quite high and the technique can facilitate large scale protein purification. This has been successfully employed for insulin purification, plasma fractionation and for purification of many other enzymes.

Gel Filtration Chromatography for Enzyme Purification - In this chromatography, various proteins are separated on the basis of differences in their molecular sizes. This type of chromatography is also known as molecular exclusion chromatography or molecular sieve chromatography.
The basic arrangement for gel filtration chromatography is similar to that for adsorption chromatography. A column made up of glass or steel is taken and packed with a gel.
The most commonly used gel is 'sephadex' which is a cross linked dextran produced by certain strains of bacteria. Several types of sephadex, namely G-10, G-30, G-50, G-100, G-150, G-200, etc. are available, which differ according to their pore sizes.

When a mixture of enzymes or proteins is poured on top of the column, different proteins move downwards according to their molecular sizes and come out from the column in order of decreasing sizes; the, larger molecules are eluted first. The elution volume is logarithmically proportional to the molecular size.
Gel filtration chromatography can also be used for determining the molecular weight of the protein by calibrating the column with proteins of known molecular weights.
Affinity Chromatography for Enzyme Purification - In this method, enzymes are purified according to their specificity for a particular substrate or cofactor.
One component of the mixture containing enzyme binds covalently to the solid support of the column, and the other components percolate down through the column. The basic requirements for affinity chromatography are the same as in adsorption or gel filtration chromatography but the packing gel must have some component which can bind with one component of the mixture.

Many commercial gels available for affinity chromatography contain functional groups attached to the 'spacer arms' of the gel. The "spacer arm" is a chemical linkage between the functional group and the gel or matrix proper, so that the binding between functional group and the enzyme is kept away from the gel.
Thus the steric hindrance will be unlikely to prevent binding of the specific enzyme to the column.

Electrophoesis for Enzyme Purification -Electrophoresis is a technique in which molecules (enzymes, proteins, amino acids, nucleotides and nucleic acids) are separated by differences in their net charge in the presence of an externally applied electric field.

This technique is routinely used in enzyme purification and isozymes separation in the laboratories, although it has found only limited application at large scale, since the technique is time consuming and is a bit expensive.

Various types of instrumental approaches have been used to separate and purify charged molecules using electrophoresis. However, the most common method for purifying enzymes is through electrophoresis on polyacrylamide gel.
Polyacrylamide is a polymer of acrylamide and methylene bisacarylamide and when prepared as a ge1 it is transparent, thermostable, non-ionic and extremely regular in structure.
The gel may be taken either in the form of a column or a slab, although the latter is preferred over the former. The protein mixture is loaded in the gel and the components are separated under a direct current of constant voltage. The migration rate of the various components of the mixture is dependent upon their charge and molecular weight.
A variation of the above polyacrylamide gel electrophoresis is the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which is used to determine the molecular weight of proteins. In this method, the separation is caused by the seiving action of the gel.

The proteins migrate through the ge1 depending on their shapes and mass to charge ratio. Gel electrophoresis is also used to separate various isozymes of a given enzyme. Isozymes perform the same catalytic function but differ in their regulatory and some kinetic aspects.

Final Step in Processing Enzymes - Most of the commercially available enzyme preparations, purified as above, are concentrated and sterile filtered, after purification. This is done to reduce both, the volume and the microbial contamination of the sample.
Often, before storage and transport, the sample is freeze dried with additives such as sugar substrates and dextrans.

2. Explain the different types of enzyme assay with example. Mention their major advantages and disadvantages

Types of assay

All enzyme assays measure either the consumption of substrate or production of product over time. A large number of different methods of measuring the concentrations of substrates and products exist and many enzymes can be assayed in several different ways. Biochemists usually study enzyme-catalysed reactions using four types of experiments:

(1) Initial rate experiments. When an enzyme is mixed with a large excess of the substrate, the enzyme-substrate intermediate builds up in a fast initial transient. Then the reaction achieves a steady-state kinetics in which enzyme substrate intermediates remains approximately constant over time and the reaction rate changes relatively slowly. Rates are measured for a short period after the attainment of the quasi-steady state, typically by monitoring the accumulation of product with time. Because the measurements are carried out for a very short period and because of the large excess of substrate, the approximation free substrate is approximately equal to the initial substrate can be made. The initial rate experiment is the simplest to perform and analyze, being relatively free from complications such as back-reaction and enzyme degradation. It is therefore by far the most commonly used type of experiment in enzyme kinetics.

(2) Progress curve experiments. In these experiments, the kinetic parameters are determined from expressions for the species concentrations as a function of time. The concentration of the substrate or product is recorded in time after the initial fast transient and for a sufficiently long period to allow the reaction to approach equilibrium. We note in passing that, while they are less common now, progress curve experiments were widely used in the early period of enzyme kinetics.

(3) Transient kinetics experiments. In these experiments, reaction behaviour is tracked during the initial fast transient as the intermediate reaches the steady-state kinetics period. These experiments are more difficult to perform than either of the above two classes because they require rapid mixing and observation techniques.

(4) Relaxation experiments. In these experiments, an equilibrium mixture of enzyme, substrate and product is perturbed, for instance by a temperature, pressure or pHjump, and the return to equilibrium is monitored. The analysis of these experiments requires consideration of the fully reversible reaction. Moreover, relaxation experiments are relatively insensitive to mechanistic details and are thus not typically used for mechanism identification, although they can be under appropriate conditions.

Enzyme assays can be split into two groups according to their sampling method: **continuous assays,** where the assay gives a continuous reading of activity, and **discontinuous assays,** where samples are taken, the reaction stopped and then the concentration of substrates/products determined.

Enzyme assays measure either the disappearance of substrate over time or the appearance of product over time. Multiple methods have been developed to measure the concentration of substrates or products in a reaction, but all enzyme assays fall into two types: fixed-timed and continuous.

The **fixed-time (discontinuous)** assay measures enzyme concentration in fixed periods of time. A common fixed-time assay method is using a microplate reader to read multiple solution concentrations. Multiple dilutions series are placed into microplate wells: dilution series for the substrate; dilution series for the enzyme; and dilution series for the substrate + enzyme. To start the fixed-time assay a start solution is added to all the wells. After the reactions start, the solutions are incubated for a fixed-period of time: this period of time can be determined from a continuous enzyme assay. To stop the reactions, a stop solution is added to prohibit the enzyme from reacting with the substrate. With fixed-timed assays, one can measure many assays simultaneously.

The **continuous assay** uses a spectrophotometer to measure the appearance of product, or disappearance of substrate in real-time. With continuous assays, one can measure the linearity of the assay which can be used to conduct a fixed-timed assay. For best enzyme activity results, the optimum pH of an enzyme must be determined before conducting a continuous enzyme assay. The disadvantage of a continuous assay is that only one reaction can be measured at a time, but the advantage is the convenience of easily measurable reaction rates.

The **spectrophotometric assay** is the most common method of detection in enzyme assays. The assay uses a spectrophotometer, a machine used to measure the amount of light a substance's absorbs, to combine kinetic measurements and Beer's law by calculating the appearance of product or disappearance of substrate concentrations. The spectrophotometric assay is simple, non-destructive, selective, and sensitive. For example, the NADH/NAD+ molecule is often used in enzymatic oxidatiomreduction reactions. During these reactions NADH is often oxidized to NAD+, or NAD+ is reduced to NADH. NADH absorbs light at 340 nm, however NAD+ does not hold that property. A spectrophotometer can be used to measure the change in absorbance of 340 nm light, thus indicating a change in amount of NADH.

Coupling Reactions
In many reactions, changes in substrates or products are not observable by spectrophotometric methods because they do not absorb light. These reactions can be measured by coupling them to enzymes that can be detected via a spectrophotometer. Light absorbing non-physiological substrates or products are synthesized for enzymes with substrates and products that do not absorb light.

3. What are the methods of determination of molecular weight of enzymes? Explain

There are number of methods for molecular weight determination for proteins. Some methods like light scattering method, osmotic pressure method, depression in freezing point method, diffusion rate method are not in common use whereas gel filtration and ultra-centrifugation methods are commonly used methods. For sub unit molecular weight determination, sodium dodecyl sulfate polyarylamide gel electrophoresis is most commonly used method. The methods of molecular weight determination are described below (in brief):

Light scattering method

Enzymes are proteins and proteins do not make clear solution in aqueous medium. They form colloidal solutions. Colloids have the property of scattering the light called as *Tyndall effect.* If a beam of visible light is passed through a colloidal solution, a part of light is transmitted and other part gets scattered. The fractional decrease in the intensity of the incident light can be

measured and using the following formula, molecular weight of the enzyme protein may be calculated:

$$M' \bullet (lOge\ CIO) / HC1$$

where I is the intensity of the transmitted light, Io is the intensity of incident light, H is the proportionality constant, C is concentration of enzyme protein in gm/ ml, l is the length of the path (in cm) through the scattered solution.

The value of H may be determined using the following formula:

$$H = \frac{32}{3\ N\ d^4} \frac{n^2 (n - no)^2}{c}$$

Where d is the wave length of the incident light, no And n are the indices of refraction of the solvent and solution, respectively, N is the Avogadro number and its value may be taken

x 10^{23} , c is the concentration in gm/ml.

Therefore, one will have to determine the indices of refraction of the solvent and solution separately.

Osmotic pressure method

After determining the osmotic pressure of the enzyme protein solution, molecular weight may be determined using the following formula:

$$cv = nRT$$

where c is the osmotic pressure in atmospheres, v is volume in litres, n is the number of moles of the solute, R is the molar gas constant in litre-atmosphere, T is the absolute temperature, n may be taken as g/M where g is the amount of the solute (enzyme protein) in gm and M is the molecular weight.

Depression in freezing point method

On dissolving the enzyme protein in water, freezing point of water will decrease. The following formula may be used for molecular weight determination:

$$It = KfM$$

where It is the depression in the freezing point, Kr is the molal freezing point or Cryoscopic constant, M is the molar concentration of the enzyme protein. If a solution containing an Avogadro number (6.02 x 10^{23}) of total dissolved particles either as undissociated molecules

or ions or as a mixture of ions and undissociated molecules in 1000 gm of water (one molal solution) freezes at —1.858°C, then it is called cryoscopic constant. Kf for water is found to be —1.858°C.

Diffusion rate method

Diffusion rate is measured by optical methods such as refractive index, light absorption, fluorescence and Tyndall effect which do not disturb the solution.

Diffusion coefficient represents the number of moles of the solute diffusing across unit area per unit time under a concentration gradient of unity. The following formula may be used for molecular weight determination:

$$D = \frac{RT}{Nc6\$} \cdot \frac{4N}{3MV}$$

Where D is the Diffusion Coefficient, R is the gas constant in ergs mole ' deg ', T is the absolute temperature, N is Avogadro number, p is the viscosity of the medium in poises, V is the partial specific volume, M is the molecular weight.

Partial specific volume is the increase in the volume when 1 gm dry substance is added to a large volume of the solvent. For proteins, its value is taken as 0.74.

Ultra-centrifugation method

Using analytical ultracentrifuge, one can determine the sedimentation constant. In fact, it is customary to express sedimentation velocity in terms of sedimentation constant which represents the velocity of particles in a unit cm-gm-sec (cgs) field of force.

If x represents the distance of the particles from the axis of rotation in the ultracentrifuge, then the rate at which they travel is

$$\frac{dx}{dt} = s\,\omega^2 x$$

m is the velocity of revolutions/second, m is 2 c revolutions per second or 6.2832 revolutions per second and t is the time in seconds.

To calculate s, suppose if at time ti, the boundary of the particles in the colloidal solution is xi cm from the axis of the centrifuge and at time t2, it iS X2 cm from the axis, then

$$s = \frac{l}{(t2 - t1)} \cdot \log \frac{X2}{X1}$$

For molecular weight determination, the following formula may be used:

$$M = \frac{RTs}{D\,(I - Vp)}$$

Where R is the gas constant, T is the absolute temperature, s is the sedimentation constant, D is the diffusion coefficient, V is the partial specific volume, p is the density of the solution.

By equilibrium studies

If during centrifugation in the ultracentrifuge, the protein band remains in equilibrium i.e. no change in the position of the band on prolonged centrifugation, then, molecular weight of the protein may be calculated using the following formula:

$$M = \frac{2RT\ lO\ e\ (C2/Cl)}{I\ (l - Vp)\ (i^{2} - i)}$$

Where Cz and Ci are concentrations at distances xi and x2 from the axis of the rotation.

Gel filtration chromatography method

As described above, in gel filtration chromatography, separation is on the basis of molecular size and shape. If all standard proteins and enzyme protein are globular in nature, separation will be on the basis of molecular weight. Although any of the gel filtration matrix may be used, commonly used ge1 filtration matrix is Sephadex G-200. A calibrated column of Sephadex provides a simple way of determining the molecular weight of the enzyme. Calibrated column may be used repeatedly both for molecular weight determination and for routine separation. The packed gel filtration column may be calibrated using standard markers. There are following two commonly used methods for molecular weight determination:

Whitekar method: A semi log graph paper is used. Log molecular weight is plotted versus elution volume of the standard proteins. A straight line is obtained. After determination of the elution volume of the enzyme protein, from the graph, molecular weight may be calculated.

Andrews method: Here also a semi log graph paper is used. Log molecular weight is plotted versus elution volume/ void volume for the standard proteins. A straight line is obtained. After determination of the elution volume of the enzyme protein, from the graph, molecular weight may be calculated.

4. Describe in detail different types of chromatographic techniques used in purification of enzymes

Chromatography is a separation technique based on partitioning of the proteins between moving phase and a stationary phase. The technique allows separation to be modified by changes in packing chemistry and elution buffer. Fully automatic high performance liquid chromatography equipments with more speed, resolution, sensitivity, reproducibility and recovery are available commercially.

For enzyme purification, commonly used chromatography techniques are: (i) Ion exchange chromatography; (ii) Adsorption chromatography; (iii) Gel filtration chromatography and (iv) Affinity chromatography.

In general, the procedure of carrying the work is same in all types of chromatography. First, the enzyme protein sample to be purified is applied onto the pre-equilibrated column and thereafter, the

sample from the column is eluted with buffer with a series of steps of different solute concentrations, with a gradient of solute or with a specific ligand for the desired enzyme protein. The effluent eluted out from the column is collected as a series of fractions using a fraction collector, tested for enzyme activity and protein.

Ion exchange chromatography

The basic principle involved in ion exchange chromatography is binding of charged proteins othe ion exchanger by electrostatic attraction (ionic bonds) between charged groups on the proteins and opposite charges on the exchanger. Conditions like pH are set in such a way that opposite charges be there between the proteins and ion exchanger. Unbound proteins are removed from the column by washing with the same medium used for pre-equilibrium.

Bound proteins are eluted by passing buffer of higher ionic strength (using salts like sodium or potassium chloride) or by using buffer of different pH. It is preferred to make a linear gradient of the salt or pH, instead of step-wise elution. Gradient elution is considered better since with gradient, there are more chances of removal of unwanted proteins. Fractions of the effluent are collected and analyzed for the desired enzyme activity.

Two types of ion exchangers are in common use for separation of enzymes: Anion exchangers and cation exchangers.

The most commonly used anion exchanger is diethyl amino ethyl cellulose (DEAE cellulose).

Some other are amino ethyl cellulose (AE cellulose), triethyl amino ethyl cellulose (TEAE cellulose) and guanido ethyl cellulose (GE cellulose). The most commonly used cationexchanger is carboxy methyl cellulose (CM cellulose). The other examples of cation exchangers are phospho cellulose (P cellulose) and sulfo ethyl cellulose (SE cellulose). These exchangers have cellulose matrix, which is considered to be inert. The other matrices used in exchangers are Sephadex and Sepharose.

There may be a condition when enzyme protein of interest is not bound on the exchanger and unwanted proteins are bound. Although it is not considered to be a preferred way, however, if sufficient purification and recovery is obtained, the condition may be used. Under the conditions, some times, it is called negative chromatography. On an average, we apply 2 to 5 mg proteins per ml packed bed of the exchanger. In ion exchange chromatography, amount of the protein applied on the column is more important than the volume of the sample.

Regarding the dimensions of the packed exchanger bed, it is preferred to have 1:10 diameter to length ratio.

Adsorption chromatography

The basic principle in this type of chromatography is binding of the proteins on the matrix by physical adsorption on the surface of insoluble matrix (through weaker bonds like hydrogen, van der Waals bonds). Afterwards, proteins are eluted from the column matrix by using a suitable elution buffer either having change in ionic concentration or pH. The commonly used matrices in adsorption chromatography are: (i) calcium phosphate gel; (ii) alumina gel and (iii) hydroxylapatite gel.

In this type of chromatography, gel to protein ratio is important for physical adsorption. It is preferable to carry a trial experiment in centrifuge tubes. A constant amount of the gel is put in each tube and different amounts of protein sample are added in each tube so that ratio of

0.1 to 2.0 in different tubes be obtained. After addition of sample, it is mixed with the gel and allowed to bind for few minutes. Afterwards, tubes are centrifuged and enzyme activity is determined in different tubes supernatants. If enzyme activity is present in a supernatant, it means binding of the enzyme protein (of interest) on the gel did not occur. From this trial experiment, one can determine, what will be the optimum gel to protein ratio so that enzyme protein of interest gets adsorped on the gel surface. Afterwards, accordingly, size of the packed gel in the column be decided. For elution, generally either buffer of high ionic strength or buffer with salt like NaCl or KCl is used.

The gels used in adsorption chromatography are commercially available. The gels may also be prepared in the laboratory. It is found that older gels are more effective in separation compared to newly prepared gel. In the laboratory, calcium phosphate gel is prepared by addition of sodium tri phosphate to a diluted solution of calcium chloride and pH is adjusted to 7.4. A precipitate of calcium phosphate formed is washed to remove excess ions.
Alumina gel is prepared by the addition of a hot solution of aluminum ammonium sulfate to a solution containing ammonium sulfate, ammonia and water at 60o
The solution is cooled,the precipitate of alumina formed is washed with water to remove excess ions.
Hydroxylapatite gel is prepared by addition of calcium chloride and di sodium hydrogen phosphate to a solution of one molar sodium chloride. The precipitate of hydroxylapatite formed is treated with alkali and heated to boiling for about 40 to 50 minutes. Afterwards, it is cooled and washed with water to remove excess ions.

Gel filtration (Molecular sieve) chromatography

The basic principle is based on the size and shape of the proteins. Here, gel particles have sponge like porous matrix as a structure with controlled dimension. The gel particles are swollen and equilibrated with appropriate medium and afterwards is packed in the chromatography column. Gel particles are spherical in shape. The molecules (proteins) to be separated enter in the porous matrix of the gel particles and too large molecules are not entered in the porous matrix and are eluted out from the column. Every gel is characterized by exclusion limit that means the proteins of more than that molecular weight will not enter in the matrix and eluted out as such (without separation). Void volume is considered as the space between the gel particles in the packed column. It is determined by passing bluedextran, which has very high molecular weight. Molecules with masses below the exclusion limit of the gel are eluted from the column in order of their molecular mass (weight) with the largest eluting first. Larger molecules have lesser of the interior volume of the gel available to them than the smaller molecules.

The commonly used gel filtration gels are of dextran, agarose, polyacrylamide. These gels are having registered trade names of the manufacturers. For example, dextran gels having
registered trade name' Sephadex' are in much common use. Gel filtration chromatography (with matrix having much lesser exclusion limit such as Sephadex G-25) is also used for desalting purpose. Since in Gel filtration chromatography, separation is based on molecular weight (if shape of all the molecules is same), this chromatography has been commonly used for determination of molecular weight of proteins.

Affinity chromatography

The basic principle involves bio-specific interaction of the enzyme protein of interest with an immobilized ligand, which may be substrate, analogue of the substrate, inhibitor, activator.
Inert materials like agarose, polyacrylamide, glass beads, cellulose etc have been used as supporting medium (matrix). The ligand is attached so that its enzyme interaction function is not impaired. Subsequently, elution is done by treatment resulting in dissociation of the desired enzyme ligand complex. Nowadays, affinity matrices (ligand immobilized with the matrix) are commercially available.

Immuno-affinity chromatography is also an affinity chromatography where antibody of the protein is used as ligand. The basic principle of antigen antibody interaction in this chromatography is applied. Although it is a good technique for purification of a protein, it is not in common use for enzymes since generally enzyme gets inactivated after binding with the antibody.

Besides, another affinity chromatography called Dye Affinity Chromatography is also used for enzyme purification. Specific dye bound matrices are available commercially which are used in Dye

Affinity Chromatography. One such popular dye matrix is Green A. Binding affinity of the dye is ranging from 1 to 15 mg protein per ml gel. In dye affinity chromatography too, elution is done by using higher ionic strength (presence of salt in the elution medium).

5. **Describe in the process of isolation, purification and characterization of an important** fungal enzyme **with** example

Proteases are the single class of enzymes, which occupy a pivotal position with respect to their applications in both physiological and commercial fields. They perform both degradative and synthetic functions. Proteolytic enzymes catalyze the cleavage of peptide bonds in other proteins. Advances in analytical technique have demonstrated that proteases conduct highly specific and selective modifications of proteins.

A wide range of microorganisms including bacteria, fungi, yeast and also mammalian tissues produces alkaline proteases. The Proteolytic enzymes from Fungi are so far the most important group of enzymes produced commercially.

Method for Extraction, Purification and Charecterization of Protease.

1. Screening of Neurospora for Protease Production
2. Extracellular Enzymatic activity of Microorganisms: Preparation of Crud Extract
3. Enzyme assay
4. Purification of Protease
5. Protease Assay after Purification.
6. Estimation of Standard and Purified Protease by Lowry's Method
7. Enzyme Kinetics: Effect of PH, Temperature and Substrate on activity of Protease.

6. Describe in detail **the different** steps involved in isolation, **purification** and characterization of an **important plant enzyme with example**

Along with vitamins and minerals, enzymes occur in food that is in a natural state. All raw food contains the proper types and proportion of enzymes necessary to digest itself. This occurs in our stomach when the food is eaten or in nature as the food ripens.

Four plant enzyme groups exist:

1. Proteases - break long protein chains into smaller amino acid chains and eventually into single amino acids
2. Amylases - reduce polysaccharides to disaccharides: lactose, maltose, and sucrose
3. Lipases - break triglycerides into individual fatty acids and glycerol
4. Cellulases - digest specific carbohydrate bonds found in fiber

Methods of enzyme purification

Fractionation of the proteins on the basis of solubility in aqueous solutions of salts or organic solvents
Chromatographic separation of the enzyme proteins
Ion exchange chromatography
Adsorption chromatography
Gel filtration (Molecular sieve) chromatography
Affinity chromatography
Chromatofocusing
Electrophoretic techniques
Isoelectrofocusing

Miscelleneous

Ultrafiltration
Dialysis
Crystallization

7. **Describe in detail the different** steps **involved in isolation, purification and characterization of an** animal enzyme with example

Selection of Source: Commonly animal material, plant material (or) microbial source is used. For metabolic enzymes liver is the source. For insulin pancreas is the source. For ATPase mitochondria is the source. For protein synthesis ribosomes is the source. For carbohydrate synthesis plant material is the source. For industrial and commercial enzymes microbes are the source.

Solubilities of Protein: Since enzyme is a multiple acid-base group, its solubility properties depends on the concentration of the salt pH, polarity of the solvent and temperature. This is used for precipitating enzymes. 1.Effect of salt concentration The salt concentration is expressed in terms of ionic strength I = Uz CiZi 2 where I-ionic strength C-molar concentration Z - ionic charge Usually ammonium sulphate is used in all laboratories by the process of salting-in and salting out. Salting in is the phenomenon that as the salt concentration of protein solution increases, the additional counter ions more effectively shield the protein molecules multiple ionic charges and thereby increases protein solubility. At high ionic strength the solubilities of proteins as well as those of most other substances decreases. This effect is known as salting out. In addition to ammonium sulphate , NaCl , KCl , MgSO 4, K 2 SO 4 are also used for precipitation.

Before isolation the enzyme source should be in soluble form. The material has to be break open (lysis) in a hypotonic (homogenizer) or sonication (breaking the cell through ultrasonic vibration). If the enzyme is in organelle differential centrifugation followed by the use of detergent solutions (or) Butanol to get the enzyme.

Homogenisation The particular cell from a plant or animal or a microbe has to be ground well in a mortar or in a virtishomogenicer. It has to be ground well till a mixture of homogenised solution is obtained. In all the subsequent steps the pH, ionic strength and temperature has to be maintained.

Precipitation The enzymes are charged molecules which are precipitated by charge breaking chemicals, acids, bases, salts and organic solvents. The procedure of precipitation of enzymes by the addition of concentrated solution of salts is known as "Salting out". It is a complex process and involves disruption of various physical forces involved in enzyme solubilization . The added salts alter the structure of the solvents which can lead to large changes in enzyme configuration by altering the electrostatic interaction of charged group on enzyme surfaces and solvation of polar uncharged residues exposed to the solvent. The salt may also interfere with the formation of Vanderwaal's forces between hydrophobic groups of the aminoacids . Further, it may compete with the enzyme molecules thereby lower its solvation . In other words salt dehydrates enzyme molecules which then form aggregates and precipitate out. Commonly ammonium sulphate is used for precipitation. By measuring the amount of enzyme solution various concentration ranging from 0 to 30, 30 to 60, 60 to 90 and 90 to 100 percent of ammonium sulphate is used. Out of various percentages, with the available assay procedures one can estimate in which percentage of precipitation the desired enzyme is present. Once it is established, from the susbequent precipitation directly the required percentage and amount of ammonium sulphate can be taken. Care should be taken by adding ammonium sulphate from 0 to 4 degree centigrade and high degree of purity of chemicals. While adding ammonium sulphate magnetic stirring is done to prevent air entrainment which might inactivate the enzyme.

8. Explain the various microbial, plant and animal sources of enzymes and give their uses

Biologically active enzymes may be extracted from any living organism. A very wide range of sources are used for commercial enzyme production from *Actinoplanes* to *Zymomonas,* from spinach to snake venom. Of the hundred or so enzymes being used industrially, over a half are from fungi and yeast and over a third are from bacteria with the remainder divided between animal (8%) and plant (4%) sources (Table). A very much larger number of enzymes find use in chemical analysis and clinical diagnosis. Non-microbial sources provide a larger proportion of these, at the present time. Microbes are preferred to plants and animals as sources of enzymes because:

1. they are generally cheaper to produce.

2. their enzyme contents are more predictable and controllable,
3. reliable supplies of raw material of constant composition are more easily arranged, and
4. plant and animal tissues contain more potentially harmful materials than microbes, including phenolic compounds (from plants), endogenous enzyme inhibitors and proteases.

Attempts are being made to overcome some of these difficulties by the use of animal and plant cell culture.

ENZYME	EC number	**Source**	**Intra/extra** -cellular	**Industrial** use
Animal en:::ymes				
Catalase	l.11.l.6	Liver	I	Food;milk,cheese, egg beverages, salads
Lipase	3.1.1.3	Pancreas	E	Food;cheese,fats and oil
Rennet	3.4.23.4	Abomasum	E	Cheese
Plant enzymes				
Actinidin	3.4.22.14	Kiwi fruit	E	Food
a-Amylase	3.2.1.1	Malted barley	E	Brewing
b-Amylase	3.2.1 .2	Malted barley	E	Brewing
Bromelain	3.4.22.4	Pineapple latex	E	Brewing
b-Glucanase	3.2.1.6	Malted barley	E	Brewing
Ficin	3.4.22.3	Fig latex	E	Food
Lipoxygenase	1.13.11.12	Soybeans	I	Food
Papain	3.4.22.2	Pawpaw latex	E	Meat
Bacterial enzyme				
a-Amylase	3.2.1.1	*Bacillus*	E	Starch,Fats and Oils, Cheese, Beverages, Bakery,
b-Amylase	3.2.1.2	*Bacillus*	E	Starch,Beverages
Endo-b-glucanase	3.2.1.6	Bacillus		Beverages
Glucose isomerase	5.3.1.5	*Bacillus*	I	Fructose syrup Cereal and Starch, Fruit and Vegetables, Beverages, Sugar and Honey
Hemicellulase	3.2.1.78	Bacillus		Cocoa,chocolate,coffee and tea
Protease	3.4.2l.14	*Bacillus*	E	Meat, Fish, Soups and Broths
Pullulanase	3.2.1.41	*Klebsiella*	E	Starch, Sugar and Honey, Beverages

Fungal enzymes				
a-Amylase	3.2.1.1	*Aspergillus*	E	Bakery; cereal and starch; fruit and vegetable; beverages; sugar and honey
Tannase	3.1.1.20	Aspergillus		Beverages
Glucoamylase	3.2.1.3	*Aspergillus*	E	Starch;fruit and vegetable;beverage; sugar and honey; confectionery; bakery; dietary foods
Catalase	1.11.1.6	*Aspergillus*	I	Food;mi1k;cheese; egg;salads; beverages
Cellulase	3.2.1.4	*Trichoderma*	E	Waste; fruit and vegetables; beverages;dietary foods
Dextranase	3.2.1.11	*Penicillium*	E	Food;sugar and honey
Glucose oxidase	1.1.3.4	*Aspergillus*	I	Food;egg;beverages; salads
Lactase	3.2.1.23	*Aspergillus*	E	Dairy;milk;cheese;edibleice;dietary foods
Lipase	3.1.1.3	*Rhizopus*	E	Food;cheese;fats and oils
Rennet	3.4.23.6	*Mucormiehei*	E	Cheese
Pectinase	3.2.1.15	*Aspergillus*	E	Drinks;fats and oi1s;fruit and vegetables;fish
Pectin lyase	4.2.2.10	*Aspergillus*	E	Drinks
Protease	3.4.23.6	*Aspergillus*	E	Baking
Raffinase	3.2.1.22	*Mortierella*	I	Food;beverages
Yeast enzymes				
Invertase	3.2.1.26	*Saccharomyces*	I/E	Confectionery
Lactase	3.2.1.23	*Kluyveromyces*	I/E	Dairy;milk;cheese;edibleice;dietary foods
Lipase	3.1.1.3	*Candida*	E	Food;cheese;fats and oils
Raffinase	3.2.1.22	*Saccharomyces*	I	Food;sugar and honey

9. Write short note on (4*4)

i) Cell disruption
ii) Protein precipitation
iii) Protein extraction
iv) Intracellular enzymes

v) Cell disruption

Cell disruption is the process of obtaining intracellular fluid via methods that open the cell wall. The overall goal in cell disruption is to obtain the intracellular fluid without disrupting any of its components. Though many cell disruption methods exist, certain factors must be considered in order to obtain viable cellular products.

Factors affecting cell disruption

- Sample Size

  In most cases, sample size limits the ability to obtain pure forms of the intracellular fluid. It is necessary to use precise and accurate procedures when handling samples sizes on the order of micro liters or less. Large sample sizes pose problems in reproducibility of pure product.

- **Ability to disrupt the cell and the necessary conditions**

  The ability to disrupt cells is dependent on the different components of the cell itself. The harder it is to disrupt the cell; more time and power are required to obtain the intracellular fluid.

- **Efficiency of disruption**

  Disruption efficiency must be sacrificed for experiments that require pure and intact forms of the product under scrutiny. Not only that, the cost (explicitly and implicit) of processing cells is a major factor in laboratory experiments as well.

- **Stability of the component needed to be isolated**

  It is important to combine materials in cell disruption with the conditions required to keep the component intact and pure. Different cell disruption methods have been created and enhanced to ensure the safety of the component under investigation.

- **Problems with Cell Disruption methods**

  Though cell disruption is necessary for obtaining intracellular fluid, the process of doing so could pose problems in purification of certain biomolecules. Some adverse effects of cell disruption include, but not limited to:

  - Heat generation
  - Release of proteases
  - Contamination (Nucleic acids, heavy metal, etc.)
  - Foaming

vi) Protein precipitation

Protein Precipitation is widely used in downstream processing of biological products in order to concentrate proteins and purify them from various contaminants. For example, in the biotechnology industry protein precipitation is used to eliminate contaminants commonly contained in blood. The underlying mechanism of precipitation is to alter the solvation potential of the solvent, more specifically, by lowering the solubility of the solute by addition of a reagent.

General Principles

Repulsive electrostatic force
Attractive electrostatic force
Precipitate formation
Methods
Salting out
Energetics involved in salting out
Hofmeister series
Salting out in practice
Isoelectric precipitation

Precipitation with miscible solvents
Non-ionic hydrophilic polymers
Flocculation by polyelectrolytes
Polyvalent metallic ions

vii) Protein extraction

Protein purification is a series of processes intended to isolate one or a few proteins from a complex mixture, usually cells, tissues or whole organisms. Protein purification is vital for the characterization of the function, structure and interactions of the protein of interest. The purification process may separate the protein and non-protein parts of the mixture, and finally separate the desired protein from all other proteins. Separation of one protein from all others is typically the most laborious aspect of protein purification. Separation steps usually exploit differences in protein size, physico-chemical properties, binding affinity and biological activity. The pure result may be termed protein isolate.

Preliminary steps

- Extraction
- Precipitation and differential solubilization
- Ultracentrifugation
- Purification strategies
- Size exclusion chromatography

Separation based on charge or hydrophobicity

- Hydrophobic interaction chromatography
- Ion exchange chromatography
- Free-flow-electrophoresis

Affinity chromatography

- Metal binding
- Immunoaffinity chromatography
- Purification of a tagged protein

HPLC

viii) Intracellular enzymes

Enzymes that act inside cells are responsible for catalysing the millions of reactions that occur in metabolic pathways such as glycolysis in the mitochondria and in the **photosynthetic pathway** in the chloroplast. The lysosome contains many enzymes that are mainly responsible for destroying old cells.

10. Explain in detail about Development of enzymatic assays

When embarking on an assay development project the researcher should ask a number of questions in order to define the exact requirements of the assay.

Question to ask	Points to consider
What is the exact molecule to be assayed?	Isoform/splice variant Total or modified (e.g. phosphorylated / acetylated / methylated)? Soluble or membrane-bound?
Parameter to assay?	Amount of molecule present? Biological function?
Source of molecule?	Sample availability Volume of sample Likely concentration of molecule Stability of molecule
Quantitative or semi-quantitative?	Is semi-quantitative measurement of the molecule sufficient, or does the study require rigorous quantitation?
Number of assay points to be run?	10s, 100s, 1000s? Sample-to-data streamlining Automation

Having considered these questions regarding the molecule to be assayed, careful attention must be paid to a number of fundamental technical/practical issues that apply regardless of the particular molecule of interest or the specific assay format adopted.

Assay **parameter**	**Key considerations**
Specificity	Will the assay detect only the desired molecule?
Sensitivity	Will the assay detect the levels of the molecule in the samples of interest?
Dynamic range	Will the levels of the molecule fall within the dynamic range of the assay?
Interference	Will components in the assay sample interfere with the assay?
Robustness	Can the assay cope with small changes in the assay sample/equipment/operator?
Reproducibility	Does the assay display low inter and intra assay variability?
Accuracy (precision)	Is the assay capable of accurately determining the absolute amount/concentration of the molecule?
Analysis of assay performance	Is it appropriate/desirable to statistically analyse assay performance? Does the assay have sufficient discriminating power?

11. Explain the steps involved in the purification of bacterial enzyme.(May 2015,Nov 2015)

Preliminary steps

- Extraction
- Precipitation and differential solubilization
- Ultracentrifugation
- Purification strategies
- Size exclusion chromatography

Separation based on charge or hydrophobicity

- Hydrophobic interaction chromatography
- Ion exchange chromatography
- Free-flow-electrophoresis

Affinity chromatography

- Metal binding
- Immunoaffinity chromatography
- Purification of a tagged protein

HPLC

12. Describe the method of enzyme characterizations and its importance in detail.(May 2015)

Protein characterization:

i) molecular weight
   i. electrophoresis (SDS-PAGE --> *individual polypeptide chain molecular weights)*
   ii. gel filtration (calibrated column --> approx. *native molecular weight* if column run under nondenaturing conditions)
   iii. ultracentrifugation (depends on *size* and *shape*, but can give very accurate *molecular weight)*

ii) isoelectric point (charge properties)
   i. isoelectric focusing (often used as the first dimension in 2-D gel separations to look at ALL the proteins in a complex mixture)

iii) spectroscopic properties (give various kinds of structural and functional information)
   i. UV-visible spectroscopy
      1. absorbance spectroscopy
      2. fluorescence spectroscopy
      3. circular dichroism spectroscopy
   ii. NMR spectroscopy

iv) determination of primary structure
   i. inference from sequence of nucleotides in the gene, and/or
   ii. chemical methods
      1. amino acid composition
      2. amino terminal residue determination
      3. Edman degradation
      4. fragmentation and determination of overlapping fragment sequences
      5. mass spectrometry (useful in many other ways, too, e.g. for identifying proteins, even in complex mixtures)

v) complete 3-dimensional structure determination
   i. X-ray diffraction from crystals of protein
   ii. NMR of protein in solution (only for small proteins, with current technology)

13. **Describe in detail the different steps involved in isolation, purification and characterization** of **an important intracellular enzyme. How do you characterize the purity of the** enzymes

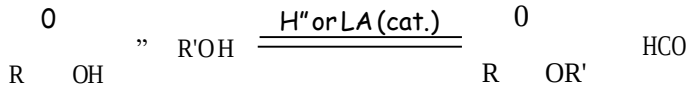
Module 5
APPLICATIONS

What are **catalytic antibodies?**

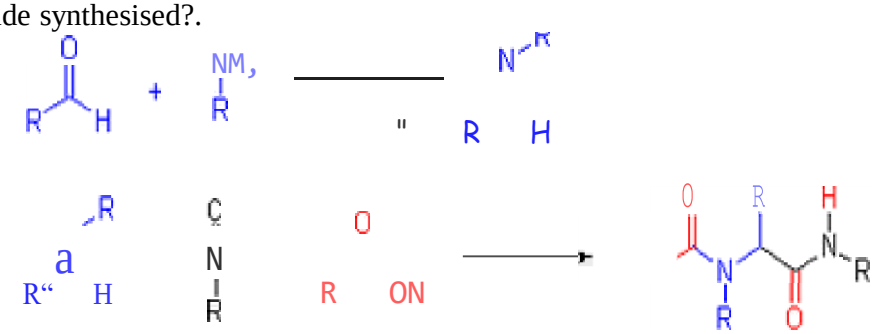
An abzyme (from antibody and enzyme), also called catmab (from catalytic monoclonal antibody), and most often called catalytic antibody, is a monoclonal antibody with catalytic activity. Abzymes are usually raised in lab animals immunized against synthetic haptans, but some natural abzymes can be found in normal humans (anti-vasoactive intestinal peptide autoantibodies) and in patients with autoimmune diseases such as systemic lupus erythematosus, where they can bind to and hydrolyze DNA.

1. Give the advantages and disadvantages **for the utilization of catalytic antibody?**
   - Catalyse reactions cannot be done by natural enzymes
   - Construction of catalytic antibodies having an opposite stereochemical preference is possible
   - Efficiency is low than the naturel enzyme
   - Production is tedious
   - Product inhibition limits the overall performance
2. What are **artificial** enzymes ?
   An **artificial** enzyme is a synthetic, organic molecule prepared to recreate the active site of an enzyme.
3. **How are modified enzymes prepared to make them soluble in lipophilic solvents?**
   By covalent attachment of the amphipathic polymer PEG to the surface of enzymes
4. **What is lipid coating?**
   Attachment of lipids such as simple long chain fattyacids or amphiphilic compounds to the enzyme surface by simple adsorption
5. **What is bio imprinting?**
   The induction of a new catalytic specificity in an enzyme in an organic solvent. An enzyme and a weakly binding non-substrate/non-product (e.g. chymotrypsin and N-acetyl-D-tryptophan) are suddenly precipitated together from an aqueous solution by addition of a miscible organic solvent. The complex reverts to the native conformation and behaviour when redissolved in water, but if redissolved in a suitable organic solvent it will be found to possess a new enzymic activity, e.g. for synthesis of N-acetyl-D-tryptophan ethyl ester from N-acetyl-D-tryptophan and ethanol.
6. **Biocatalytic transformations performed in organic** media is advantageous - **explain?**
   Over all yield is better in organic medium, nonpolar substrates are transformed at better rates due to their increased solubility, microbial contamination is negligible.
7. **What is partition coefficient?**
   Partition-coefficient (logP) or distribution-coefficient (logD) is the ratio of concentrations of a compound in a mixture of two immiscible phases at equilibrium. These coefficients are a measure of the difference in solubility of the compound in these two phases.
8. **List down the parameters that facilitate a biocatalytic reaction in a monophasic organic** solvent system?
   pH, enzyme state, choice of solvent, water content, effect of additives, super critical gases.

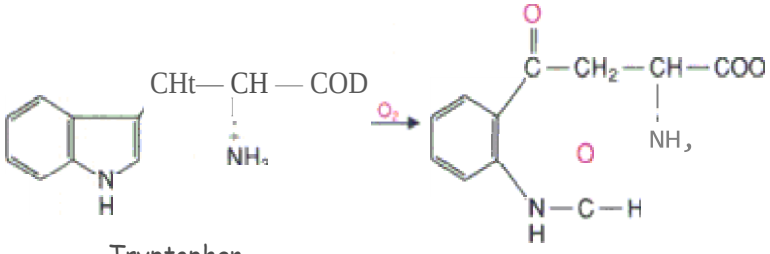
9. What are super critical gases?
A supercritical fluid is a state where matter is compressible and behaves like a gas (i.e. it fills and takes the shape of its container), which is not the case when it is in a liquid state (an incompressible fluid that occupies the bottom of its container). However, a supercritical fluid has the typical density of a liquid and hence its characteristics dissolving power. That is why we cannot define the supercritical fluid as a liquid or as a gas. This is a new state of matter in principle. Eg. Carbon di oxide, freons (CHFS), hydrocarbons (ethane, ethane, propane), inorganic compounds (SF6, NzO).
10. What are the draw backs of oxidation reaction by traditional method ?
Many oxidants are based on toxic metal ions such as chromium which are environmentally incompatible, undesired side reactions are common due to lack of specificity, Molecular oxygen cannot be used efficiently , extremely difficult toperform oxidation in a regio and stereo selective fashion.
11. **What is esterification?**



12. What are the principles of enzymatic peptide synthesis ?.
Reversal of hydro1ysis,transpeptidation, aminolysis of esters.
13. How is amide synthesised?.



14. What are oxygenases? Give any two examples
Oxygenasesare enzymes that incorporate oxygen atoms from Oz into the oxidized products. Dioxygenases are uncommon enzymes that incorporate both atoms of O2 into one substrate. An example is tryptophan 2,3-dioxygenase, which catalyzes the reaction below:



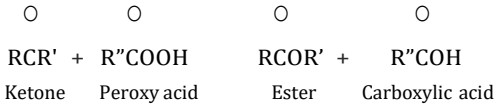
Tryptophan

MForMylkynurenine

Monooxygenases are much more common than dioxygenases. They incorporate one atom from O2 into a product and reduce the other atom to water. A monooxygenase has one substrate that accepts oxygen and another that furnishes the two H atoms that reduce the other oxygen to water. Because two substrates are oxidized, enzymes of this type are also called mixed-function oxidases. The general reaction catalyzed bymonooxygenases is the following:

$$AH + BH_Z + O2 <-> A\text{-}OH + B + H2O$$

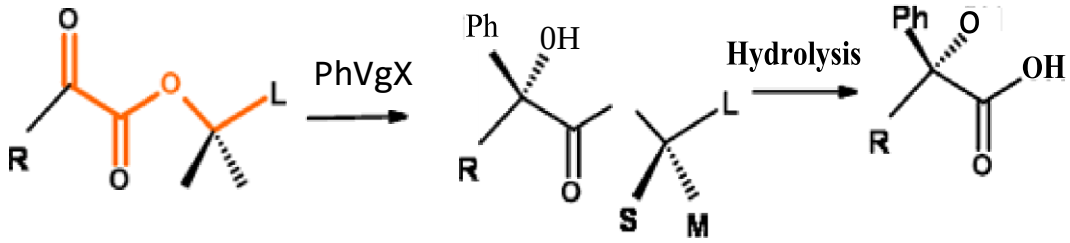
The substrate AH usually becomes hydroxylated by this class of ezymes, so they are also called hydroxylases. For example, this type of enzyme is used to hydroxylate steroids.
15. What is Baeyer-VilligerOxidation ?
An oxygen from the peroxy acid is inserted between the carbonyl group of a ketone and one of the attached carbons of the ketone to give an *ester*. Reactions of this type are known as Baeyer—Villiger oxidations.



16. **Name the organisms that asymmetrically oxidizethio ethers?**
Bacteria: *corynebacteriumequi, Rhodococcusequi,*
Fungi: *Helminthosporium sp., Mortierellaisabellina*

17. **Classify the enzymes employed in redox reactions?**
Dehydrogenases, Oxygenases, Oxidases

18. Define **prelog's rule?**
The rule has been applied for asymmetric synthesis of on-hydroxyacids and for assigning the configuration of secondary and tertiary alcohols. The *anti* configurational arrangement of the two on-carbonyl moieties could be rationalized. The negative end of these dipoles would prefer to be as far removed as possible. The two lone pairs would sit on ether oxygen like the 'Rabit Ears'. The keto-carbonyl would orient between the two ears. This will place the bonds shown in red in the same plane as thrketo-carbonyl group. The attack from the side of the small (S) group is an extension of Cram's Rules. The asymmetric induction could be at times poor due to the large distance between the reaction center and the asymmetric center inducing asymmetry at the developing chiral center.



19. Name few **naturally occurring epoxides?**

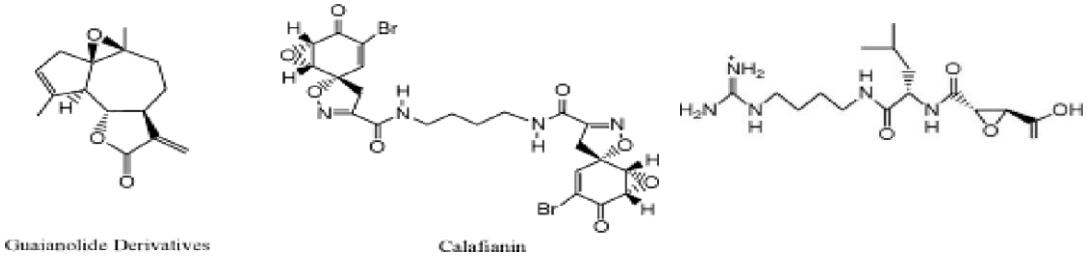


Figure 1. Naturally occurring epoxides

20. What is kazlauskasrule ?

A rule to predict which enantiomer of a secondary alcohol reacts faster in reactions catalyzed by cholesterol esterase, lipase from *Pseudomonas cepacia*, and lipase from *Candida rugosa.*

21. **Write a short note on epoxide.**

An epoxide is cyclic ether with a three-atom ring. This ring approximates an equilateral triangle, which makes it strained and hence highly reactive, moreso than other ethers. They are produced on a large scale for many applications. In general, low molecular weight epoxides are colourless and nonpolar, and often volatile.

22. **What are** enzyme **mimics?**

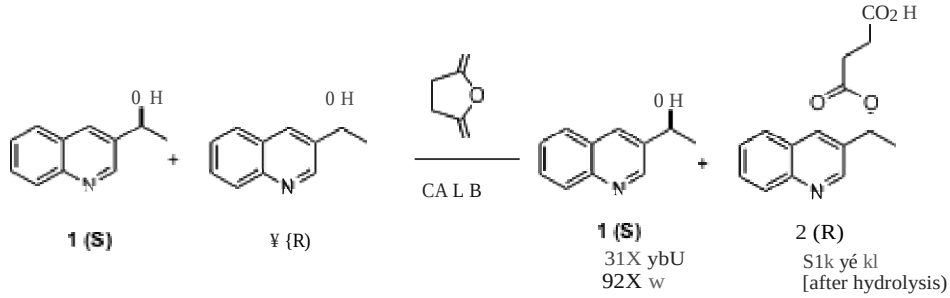
Enzyme mimic (or Artificial enzyme) is a branch of biomimetic chemistry, which aims at imitating the function of natural enzymes. An enzyme mimic is a small molecule complex that models the molecular structure, spectroscopic properties, or reactivity of an enzyme, sometimes called bioinspired complexes.

23. What **are abzymes?** Give an example

An abzyme (from antibody and enzyme), also called catmab (from catalytic monoclonal antibody), and most often called catalytic antibody, is a monoclonal antibody with catalytic activity. Abzymes are usually raised in lab animals immunized against synthetic haptans, but some natural abzymes can be found in normal humans (anti-vasoactive intestinal peptide autoantibodies) and in patients with autoimmune diseases such as systemic lupus erythematosus, where they can bind to and hydrolyze DNA. Ex. Cyclic phosphonate ester is the structural analog of the cyclic intermediate.

24. Outline the application of enzymes in organic synthesis with an example

Enzymes have many applications in organic synthesis. One of the most common is the resolution of a secondary alcohol. A shortcoming of this approach is that the separation of the residual alcohol from the product ester has required column chromatography, adding to the expense. Louisa Aribi-Zouioueche of the University of Annaba, Algeria, and Jean-CaludeFiaud of the University Paris-Sud, Orsay, have been exploring the use of succinic anhydride as an alternative to the more typical vinyl acetate or isopropenyl acetate acylating agents. Using this procedure, the residual alcohol and the product ester can be separated by simple acid-base extraction.

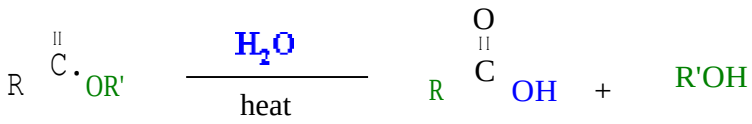


25. Ilustrate the mechanism used for the hydrolysis of epoxides in prokaryotes and eukaryotes. (Nov 2016)

Part-B

1. Explain in detail about the hydrolytic reaction involving ester and amide bond?

Hydrolysis of Esters



Reaction type: Nucleophilic Acyl Substitution

Summary

- Carboxylic esters hydrolyze to the parent carboxylic acid and an alcohol.
- Reagents : aqueous acid *(e.g.* H2SO4) / heat, or aqueous NaOH / heat (known as *"saponification").*
- These mechanisms are among some of the most studied in organic chemistry.
- Both are based on the formation of a tetrahedral intermediate which then dissociates.
- In both cases it is the C-O bond between the acyl group and the oxygen that is cleaved.

Hydrolytic reaction of amide bond

Step 1:

An acid/base reaction. Since we only have a weak nucleophile and a poor electrophile we need to activate the ester. Protonation of the amide carbonyl makes it more electrophilic.

Step 2:

The water O functions as the nucleophile attacking the electrophilic C in the C=O, with the electrons moving towards the oxonium ion, creating the tetrahedral intermediate.

Step 3:

An acid/base reaction. Deprotonate the oxygen that came from the water molecule.

Step 4:

An acid/base reaction. Need to make the -NH2 leave, but need to convert it into a good leaving group first by protonation.

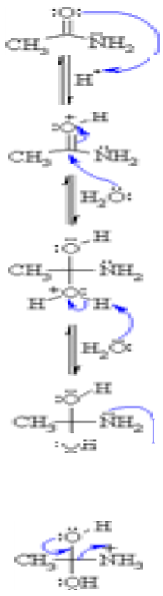
Step 5:

Use the electrons of an adjacent oxygen to help "push out" the leaving group, a neutral ammonia molecule.

Step 6:

An acid/base reaction. Deprotonation of the oxonium ion reveals the

carbonyl in the carboxylic acid product and regenerates the acid catalyst.

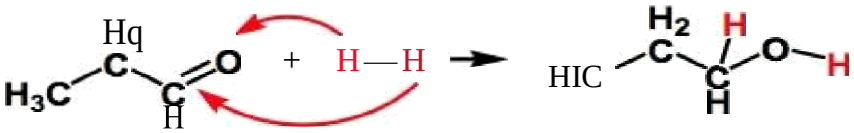


2. Explain in detail about the hydrolytic reaction involving Epoxides and nitriles?
3. Explain in detail about the reduction reaction involving aldehydes and ketones? (Nov 2016)

General equation: reduction of aldehydes and ketones

aldehyde + H2 -- > primary alcohol
ketone + H2z - -> secondary alcohol

Reduction of an Aldehyde:

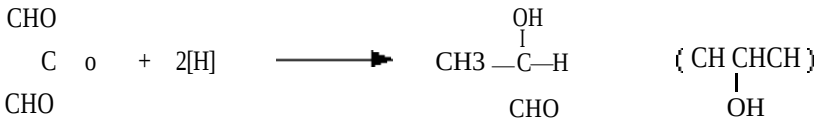


Example:

1) One hydrogen adds to the carbon with the double bond oxygen.
2) One hydrogen adds to the double bond oxygen.
3) The two electrons which were in the double bond are used in the bonding of both hydrogens.
4) The double bond is converted to a single bond.
5) The final product is a primary alcohol.

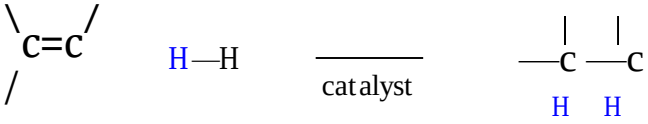
The reduction of a ketone

For example, with propanone you get propan-2-ol:



Reduction of a ketone leads to a secondary alcohol. A secondary alcohol is one which has two alkyl groups attached to the carbon with the -OH group on it. They all contain the grouping -CHOH.

4. **Explain in** detail about **the reduction reaction involving** C=C?



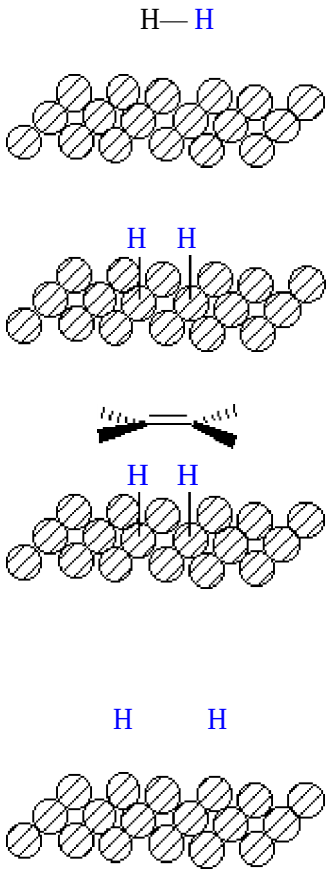
Reaction Type: Electrophilic Addition

- Alkenes can be reduced to alkanes with H2 in the presence of metal catalysts such as Pt, Pd, Ni or Rh.
- The two new C-H o bonds are formed simultaneously from H atoms absorbed into the metal surface.
- The reaction is stereospecific giving only the *syn* addition product.
- This reaction forms the basis of experimental *"heats **of hydrogenation**"* which can be used to establish the stability of isomeric alkenes.

CATALYTIC HYDROGENATION

Step 1:
Hydrogen gets absorbed onto the metal surface.

Step 2:
Alkene approaches the H atoms absorbed on the metal surface.



Step 3:
C=C reacts with the H atoms on the surface forming the two new C-H o bonds.

5. **Explain in detail about the oxidation reaction involving alkanes and aromatic compounds?**
Alkanes can be oxidized to carbon dioxide and water via a free-radical mechanism. The energy released when an alkane is completely oxidized is called the **heat** of **combustion.** For example, when propane is oxidized, the heat of combustion is 688 kilocalories per mole.

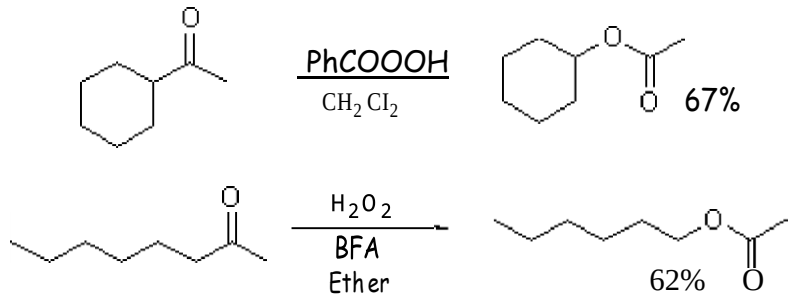
—

In a homologous series like the straight-chain alkanes, the energy liberated during oxidation increases by approximately 157 kilocalories for each additional methylene (CH z) unit.

Heat of combustion data is often used to assess the relative stability of isomeric hydrocarbons. Because the heat of combustion of a compound is the same as the enthalpy of that compound in its standard state, and because potential energy is comparable to enthalpy, the differences in heats of combustion between two alkanes translate directly to differences in their potential energies. The lower the potential energy of a compound, the more stable it is. In the alkanes, the more highly branched isomers are usually more stable than those that are less branched.

6. **Explain in detail about the Baeyer-Villiger reactions?**

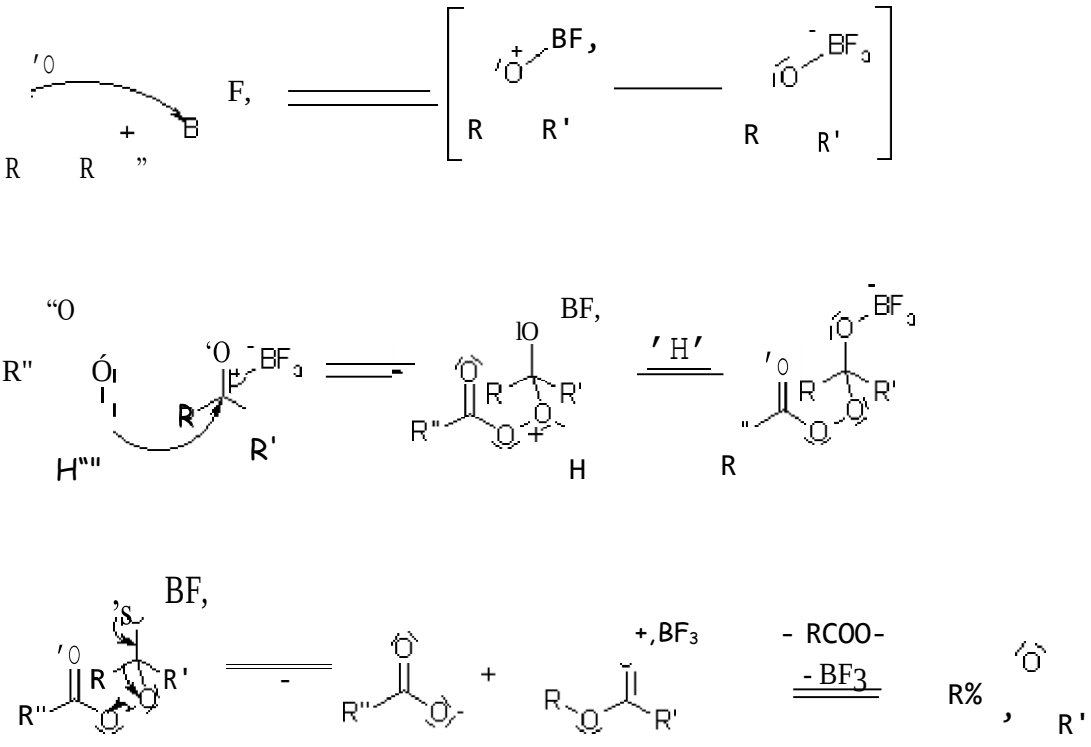
Baeyer-Villiger ***Oxidation***



The Baeyer-Villiger Oxidation is the oxidative cleavage of a carbon-carbon bond adjacent to a carbonyl, which converts ketones to esters and cyclic ketones to lactones. The Baeyer-Villiger can be carried out with peracids, such as MCBPA, or with hydrogen peroxide and a Lewis acid.

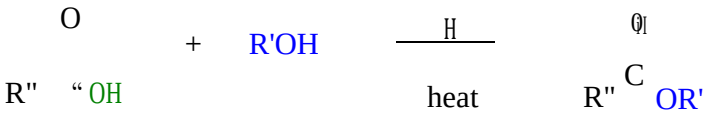
The regiospecificity of the reaction depends on the relative migratory ability of the substituents attached to the carbonyl. Substituents which are able to stabilize a positive charge migrate more readily, so that the order of preference is: *tert.* alkyl>cyclohexyl> sec. alkyl > phenyl > prim. a1kyl> CH3. In some cases, stereoelectronic or ring strain factors also affect the regiochemical outcome.

Mechanism of the Baeyer-Villiger Oxidation



7. Discuss in detail **about** the synthesis of esters?

Synthesis of Esters

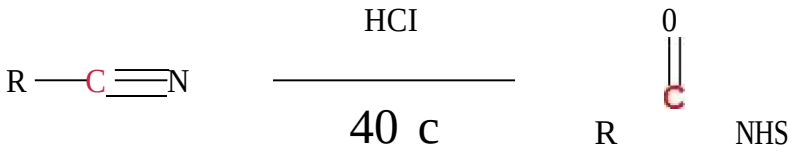


Reaction type: Nucleophilic Acy1 Substitution

8.Discuss in detail about **the synthesis** of amides?

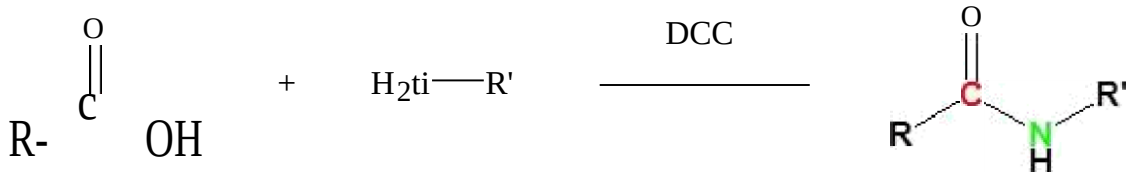
Synthesis of Amides

Nitriles can be converted to amides. This reaction can be acid or base catalyzed



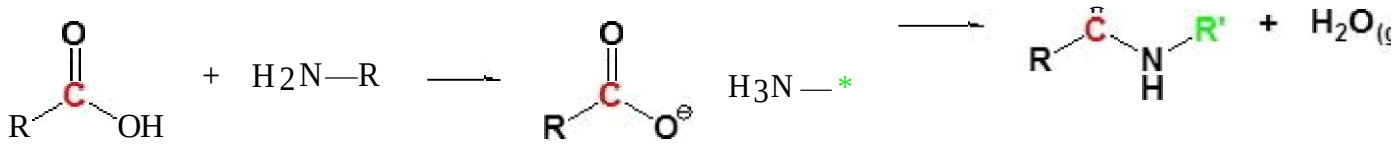
Nitrile Amide

Carboxylic acid can be converted to amides by using DCC as an activating agent



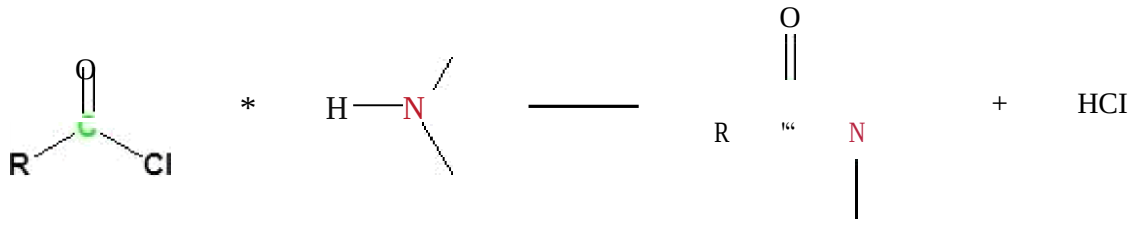
Carboxylic Acid Alcohol Amide

Direct conversion of a carboxylic acid to an amide by reaction with an amine.



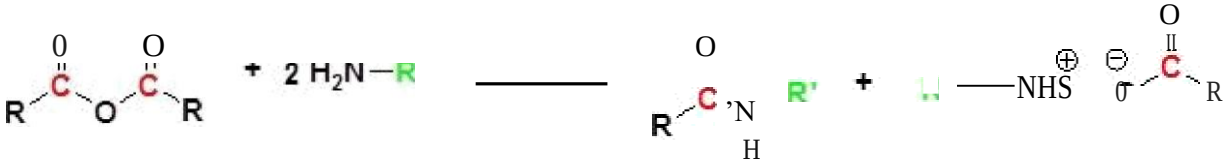
Amm onium Carbo xylate Salt

Acid chlorides react with ammonia, 1° amines and 2° amines to form amides



Acid Chl oride A m ine A m ide

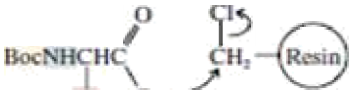
Acid Anhydrides react with ammonia, 1° amines and 2° amines to form amides



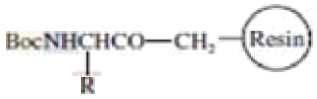
Acid A n **hydri de** **A** m in e A m i de

9.Discuss in detail about the synthesis of peptides?

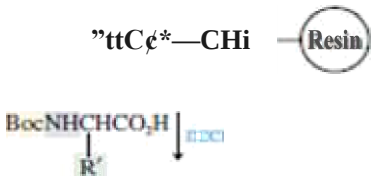
Step 1: The Boc-protected amino acid is anchored to the resin. Nucleophilic substitution of the benzylic chloride by



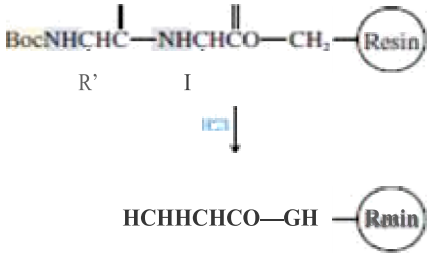
ren+owed @ rroam<at •'ah hydrcclf/ortc acid in dikite acm c a A Mter ihe resin has been washed. the C-terminal amino



Step1 Tbc rcein-t«xind E-lermirul amir acid is coupled to an N-protected amino acid by using *N,N'*-dicyclohexylcarbodiimide. Excess reagent and *N,N'*-dicyclohexylurea are waxf›ed a•'ay from Mr resin aflcs coupling is



Step 4: The Boc protecting group is removed as in step 2. If desired, steps 3 and 4 may be



in trifluoroacetic acid.

10. Discuss in detail about the modified and artificial enzymes?

An **artificial enzyme** is a synthetic, organic molecule prepared to recreate the active site of an enzyme.

Enzyme catalysis of chemical reactions occur with high selectivity and rate in a small part of the enzyme macromolecule known as the active site. There, the binding of a substrate close to functional groups in the enzyme causes catalysis by so-called proximity effects. It is therefore possible to create similar catalysts from small molecule mimics of enzyme active sites by combining, in a small molecule, the ability to bind substrate with catalytic functional groups. Since the artificial enzymes need to bind molecules, they are made based on a host-molecule such as a cyclodextrin, crown ethers or calixarene etc.

A number of artificial enzymes have been reported catalysing various reactions with rate increases up to 103; this is nevertheless substantially lower than natural enzymes that typically causes rate increases above 10^6. One of the pioneers in artificial enzyme research is chemist Ronald Breslow. He has published a book on the subject entitled

Artificial Enzymes.

New approaches based on amino acids or peptides as characteristic molecular moieties have led to a significant expansion of the field of artificial enzymes or enzyme mimics. For instance, recent results by the group of Rob Liskamp have shown that scaffoldedhistidine residues can be used as mimics of certain metalloproteins and -enzymes. Especially the structural mimicry of

certain copper proteins (e.g. hemocyanin,tyrosinase and catechol oxidase), containing so-called type-3 copper binding sites, has been shown. This is a significant improvement since the use of scaffoldedhistidine residues is one step closer to the mimicry of enzymes by biological relevant species such as amino acids and peptides.

Recently, a new kind of artificial enzymes, **nanozymes** (or **nanozyme),** has been reported. Nanozymes are nanomaterials with enzyme-like characteristics. The term of nanozymes was coined by FlavioManea, Florence BodarHouillon, Lucia Pasquato, PaoloScrimin in 2004. (Note: **nanozyme** (or **nanozymes)** has also been used in other cases.) They have been widely used in various areas, such as biosensing, bioimaging, tumor diagnosis and therapy, antibiofouling, etc. Several conferences (meetings) has been focused on the nanozymes. In 2015, a workshop for nanozyme has been held in 9th Asian Biophysics Associatation (ABA) Symposium.InPittcon 2016, a Networking entitled "Nanozymes in Analytical Chemistry and Beyond" is devoted to nanozymes.A book chapter on nanozymes has been written by Xiyun Yan and coworkers.An integrated nanozymes have been developed for real time monitoring the dynamic changes of cerebral glucose in living brains

11. Describe in detail about the synthesis of catalytic antibodies with an example?

Antibodies molecules are produced by the immune system to bind and neutralize foreign substances called antigens. Foreign proteins of bacteria and viruses, as well as some small chemical molecules called haptens, act as antigens, and elicit the production of antibodies to protect the host from harm. In fact, the human body is capable of producing antibodies to virtually any encountered antigen. Each antibody binds its own unique target similar to a key fitting in a lock.

Structurally, antibodies (immunoglobulin G-type) are "Y-shaped" molecules (see animation l). They consist of two identical heterodimers joined together by disulfide bonds. Each heterodimer consists of a short peptide called the light chain and a longer peptide called the heavy chain. The heavy and light chains are also joined together by disulfide bonds. One end of the antibody contains conserved regions, called constant domains (Fc, CH 1-3), that are formed by the interface of two of the two heavy chains. CH domains have similar amino acid compositions in most IgG's, despite the antigen to which the antibody binds. The opposite end of the molecule (Fv) is variable in structure and amino acid sequence, however. These variable domains are responsible for specifically binding antigen. The interface of two of the heavy and light chain variable regions (VL and VH) forms a single deep pocket (antigen binding site) that molds to the shape of the antigen. Notice that each IgG molecule contains two identical antigen binding sites because of the "Y-like" shape. "Hot spots" within the variable domains are called complementarity determining regions (CDRs). Amino acids within the CDRs specifically contact the antigen via non-covalent interactions to mediate binding to the foreign particles.

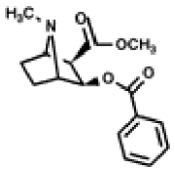
In most cases, antibodies tightly bind the antigen, but do not specifically alter its chemical nature. Natural enzymes within the body, on the other hand, bind biomolecules and subsequently catalyze their conversion to new products. According to "transition state theory," enzymes catalyze a reaction by stabilizing the chemical intermediate, or transition state, between substrates and products <1ink to enzyme unit>. Formation of this transition state geometry is energetically unfavorable in the absence of enzyme. However, enzymes provide the chemical momentum (activation energy) to push a reaction through its transition state. The net result of enzyme catalysis is the acceleration of the reaction rate.

Theoretically, if an antibody binds to a transition-state molecule, it may be expected to catalyze a corresponding chemical reaction by forcing substrates into transition-state geometry. But how can an

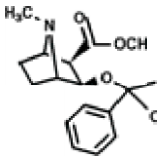
antibody be raised against such a fleetingly unstable chemical? The answer lies in the synthesis of"look-alikes" called transition-state analogs. These molecules are more stable than the transition state itself, but mimic its three-dimensional structure. If injected into the bloodstream of an animal, transition state analogs act as haptens, and elicit antibody production. Antibodies are isolated from the serum of the animal, and then screened by experimental assays to determine which catalyze the selected reaction.

In 1986, Peter Schultz and Richard Lerner demonstrated the feasibility of this proposal by generating abzymes that catalyze ester hydrolysis, the breakage of an ester bond through the addition of water. Animation 2 illustrates the hydrolysis reaction of p-nitrobenzoate. The mechanism involves the nucleophilic attack of the oxygen atom of water on the carbonyl atom in p-nitrobenzoate. This interaction produces a transition state with a tetrahedral geometry. Organic chemists have synthesized an analog of the proposed intermediate, also shown in Figure 2. By replacing the carbon at the center of the tetrahedron with phosphate, the chemical is stable for synthesis and injection into laboratory animals. The rates of reactions catalyzed with abzymes, as measured by kinetic parameters such as *Kg* and max, are up to a million-fold greater than the corresponding uncatalyzed reactions; however, in many cases, catalytic antibodies have not yet approached the rates of reactions catalyzed by natural enzymes. Since the experiments of Schultz and Lerner, however, over 100 different abzymes have been generated that catalyze a wide variety of reactions.

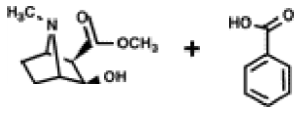
Through the use of protein engineering, abzyme catalysis can be improved even further, perhaps even to surpass the activity of natural enzymes. Molecular biologists have developed methods to clone the array of genes that encode IgG molecules. The millions of gene products from an immunized animal are screened for the production of antibodies with desirable catalytic activites. Once candidates are isolated, these so-called "recombinant antibodies" can be produced in bacteria in large amounts. In this way, an antibody gene can be "immortalized" for unlimited study.



Cocaine



Trans tion-state
(approximation}



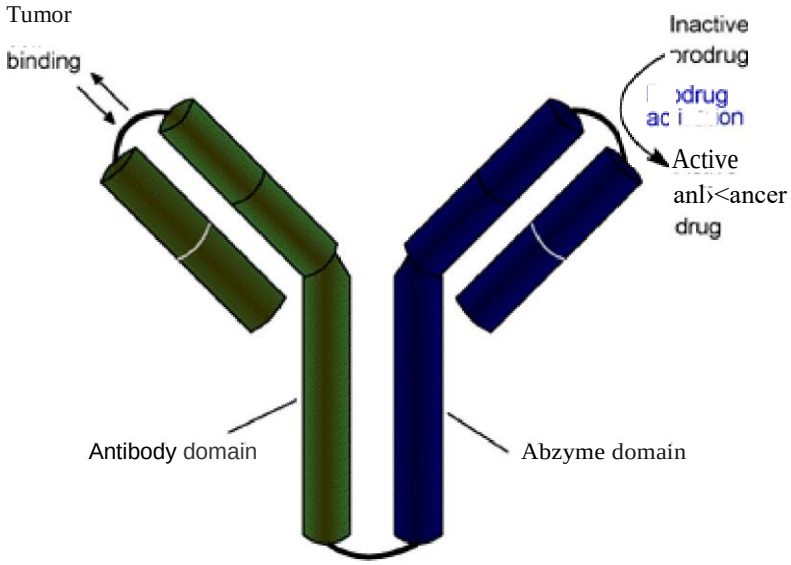
Breakdown to harmless products

cocaine detoxification

The gene encoding a selected recombinant antibody can be subsequently altered (mutated), selectively or randomly, in attempts to improve

the activity of the original catalytic antibody. Scientists often target the CDR's for mutational analysis since these regions contain amino acids that directly contact the antigen. The resulting protein mutants are once again screened for improvement in function. In this way, a starting abzyme with moderate catalytic activity can be vastly improved.

Catalytic antibodies have great potential in the pharmaceutical industries. Abzymes have been implicated for use in the detoxification of cocaine. Catalytic antibodies have been generated that cleave the cocaine molecule at specific bonds (Figure 3), thereby eliminating the toxic effect of the drug. Notice the similarity between the hydrolysis of cocaine and the hydrolysis of p-nitrobenzoate in Figure 2. Both reactions proceed through tetrahedral intermediates and the transition-state analogs mimic this geometry. As a pharmaceutical reagent, anti-cocaine abzymes could treat patients who are addicted to cocaine, or reverse the lethal effects of a cocaine overdose.

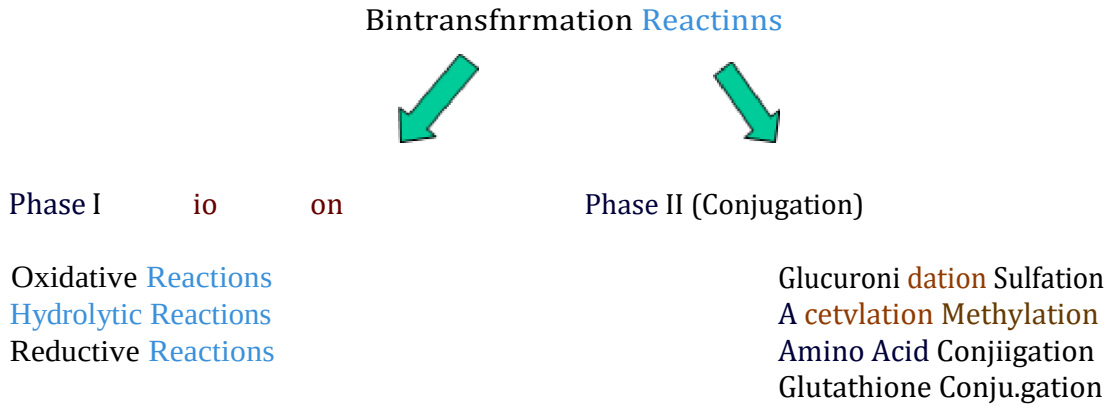


ftader and Lis1, 200ß

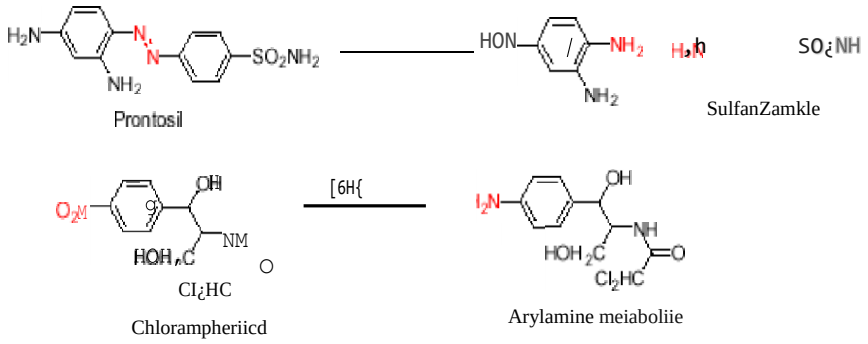
Perhaps the most exciting application of abyzme technology is the specific targeting of cancer cells for destruction. Cancer cells contain unique determinants, called tumor cell antigens, on their surface that are lacking in normal cells. By utilizing antibodies that specifically bind these tumor cell antigens, cancer drugs can be delivered directly to the tumor. In the case of abzymes, scientists have envisioned antibodies with two distinct antigen binding sites (Figure 4): one site binds with high affinity to a tumor cell antigen, while the second site catalyzes the cleavage of a prodrug. The prodrug is a non-toxic precursor of a cytotoxic drug. First, the antibody is administered to patients, and it binds the tumor cells with high affinity. Secondly, the prodrug is introduced into the bloodstream, but only becomes activated in the vicinity of the targeted antibody. By this technique, tumors are selectively destroyed while healthy cells are spared from the toxic affect of cancer drugs.

While still in the early stages, other reports have indicated possible uses of abzymes to inactivate viruses. For instance, abzymes have been isolated that cleave viral coat proteins of human immunodeficiency virus (HIV). Researchers have also developed abzymes that catalyze the specific destruction of viral genes. Perhaps in the future, we will have the tools to treat a wide variety of diseases through the use of catalytic antibody technology.

12. Explain in detail the enzyme in organic synthesis.



13. Explain in detail about the reduction reaction involved in biotransformation of enzymes.



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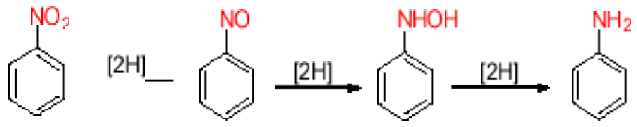


14. Describe the following:

(i) Biotransformation of steroids

(ii) Synzymes

Biotransformation of steroids

Biotransformation (regiospecific and steriospecific bioconversion) is a biological process whereby an organic compound is modified into reversible product. These involves simple, chemically defined reactions catalyzed by enzymes present in the cell. OR Microbial transformation • When the transformation of the organic compounds is carried out by microorganism then the process is called as microbial transformation.

• Naturally occurring steroids possess remarkable hormonal properties which are of therapeutic importance to human well-being, such as hormones of adrenal cortex (cortisone, cortisol, corticosterone), the progestational hormone (progesterone), the androgens or male sex hormones(testosterone, dihydrotestosterone) and the estrogens or female sex hormones (estradiol, estrone, etc.)

• The pharmaceutical industry has great interest in the biotransformation of steroids for the production of steroid hormones. • Steroid hormones and their derivatives have been used for a wide range of therapeutic purposes. • Beside the established utilization as immunosuppressive, anti-inflammatory, anti-rheumatic, progestational, diuretic, sedative, anabolic and contraceptive agents, recent applications of steroid compounds include the treatment of some forms of cancer, osteoporosis, HIV infections and treatment of declared AIDS

• Nowadays steroids represent one of the largest sectors in pharmaceutical industry with world markets in the region of US$ 10 billion and the production exceeding 1,000 000 tons per year

TYPES OF STEROIDAL TRANSFORMATION • Oxidation — Hydroxylation — Dehydrogenation. — Epoxidations — Oxidation to ketone through hydroxylation — Ring A Aromatization — Degradation of steroid nucleus

— Oxidation of alcohols to ketone: 3b-OH to 3-keto — Side chain cleavage of steroids — Decarboxylation of acids • Reduction — Double bond — aldehyde and ketone to alcohol • Hydrolysis • Isomerization • Resolution of racemic mixture • Other reactions — Aminations — Enolization of carbonyl compounds — Esterification. Hydroxylation • Hydroxylation involves the substitution of hydroxyl group directly for the hydrogen at the position, be it o or §, in the steroid with a retention of configuration. The oxygen atom in the hydroxyl

group is derived form molecular oxygen (gaseous), not from water, and the hydroxyl group thus formed always retains the stereochemical configuration of the hydrogen atom that has been replaced. Example : Certain microorganisms can introduce hydroxyl groups at any of several of the carbon atoms of the steroid molecule. • .

Fungi are the most active hydroxylating microorganisms, but some bacteria particularly the Bacilli, Nocardia and Streptomyces show fair good activity. The hydroxylation at the 11-position of progesterone was one of the first hydroxylation described

Dehydrogenation • Dehydrogenation with the concomitant introduction of a double bond has been reported for all four rings of the steroid nucleus, although the introduction of unsaturated bonds in Ring A is the only reactions of commercial importance. Example : • In 1955, Charney and co-worker observed that they could greatly enhance the anti-inflammatory properties of cortisol by causing the compound to be dehydrogenated at 1st position by Corynebacterium simplex. The resultant product, prednisolone, was 3-5 times more active than the parent compound and produced fewer side effects. cortisol prednisolone Corynebacterium simplex

Epoxidation The epoxidation of steroidal double bonds is a rare example of biological epoxidation. The 9,11-epoxidation of 9(11)-dehydro-compounds , and the 14, 15-epoxidation of 14(15)-dehydrocompounds

, using Curvalaria lunata. CH3 CH3 OCurvalaria lunata

Ring A Aromatization • The microbial aromatization of suitable steroid substrates can lead to ring A aromatic compounds, particularly the estrogens which constitutes an important ingredient in oral contraceptives drugs and play important role in replacement therapy for menopause treatment • Cell free extracts of Pseudomonas testosteroni could transform 19-nor-testosterone into estrone with small quantities of estradiol-17β. 19-nortestosterone Estrone Estradoil-17β

Degradation of steroid nucleus • Side chain degradation of steroids Selectively removal of the aliphatic side chain with out further breakdown of the steroidal nucleus. The breakdown of the side chain to yield C-17 keto steroids can be done by several organisms as given below. (Nocardia species) COOH + CH3-CH2-COOH COOH + CH3-COOH O C27 C24 C22 C17 + CH3-CH2-COOH

Reduction • Reduction of aldehydes and ketones to alcohols OH Estradiol Streptimyces

16. Hydrolysis • Hydrolysis of esters- Flavobacterium dehydrogenans contain a specific enzyme acetolase which hydrolyses the steroidal acetates OAc OH Estradiol Flavobacterium dehydrogenans

Esterification • Usually involve acetylation O O Androstenedione OAc O Testosteron acetate Sacromyces fragilis

• Steroid Ring Degradation

COMMERCIAL DEVELOPMENT THE CULTURE IN FERMENTATION TANK (AERATION & AGITATION) THE STEROID IS DISSOLVED IN SUITABLE SOLVENT ADDED AT DIFFERENT GROWTH STAGES RXN COMPLETE IN REASONABLE TIME

Fermentation condition of some steroids M/O Steroid substrate Steroid product Length of incubation , temperature, aeration Alcaligenes faecalis Cholic acid Ketocholic acids (90-100%) 2 days (monoketo acid) 4 days (diketo acid) 6 days (triketo acid) 37-39 ,surface culturę Fusarium solani Progesterone 1,4-androstadiene-3, 17-dione(85%) 4 days , 25 C , rotarў shaker (100 rpm) Corynebacterium mediolanum 21-acetoxy -3 β- hydroxy -5-pregnen- 20-one 21-hydroxy-4- pregnene-3, 20- dione (30%) 6 days , 36-37 C , purę oxygen with agitation

ADVANTAGES • The ability of microorganisms, e.g., bacteria, to produce large amounts of biomass and a great variety of different enzymes in a short time. • The chemo-, regio-, and enantioselectivity of enzymes, because of their small size bacteria have by far the largest surface- to-volume ratio in the living world, which allows them to maximize their metabolic rates because of a high exchange of molecules and metabolites through their surface.

• Microorganisms have great potential for inducing new or novel enzyme systems capable of converting foreign substrates. • Microorganisms are capable of producing unique enzymes which are stable toward heat, alkali and acid. • A combination of microbial transformation and chemical transformations (chemo-enzymatic synthesis) can be exploited for partial, as well as the total synthesis of the organic compounds

DISADVANTAGES • If the substrate is toxic, it can kill the microorganisms. Hence no transformation will be observed. • Alternatively, if the micro-organisms use the substrate as an energy source (carbon source food), no transformed or untransformed material will be recovered. • Very low chemical yields are obtained due to the involvement of a complex biological system

• Many of the ground rules for applying biotransformations are not yet well understood or well-defined. • Many chemical reactions have no equivalent biotransformations and vice- versa

Synzymes

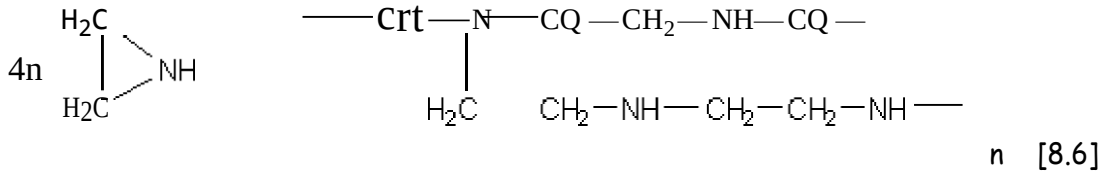
A number of possibilities now exist for the construction of artificial enzymes. These are generally synthetic polymers or oligomers with enzyme-like activities, often called synzymes. They must possess two structural entities, a substrate-binding site and a catalytically effective site. It has been found that producing the facility for substrate binding is relatively straightforward but catalytic sites are somewhat more difficult. Both sites may be designed separately but it appears that, if the synzyme has a binding site for the reaction transition state, this often achieves both functions. Synzymes generally obey the saturation Michaelis-Menten kinetics.. For a one-substrate reaction the reaction sequence is given by

synzyme + S ⟶ (synzyme-S complex) ⟶ synzyme * P

Some synzymes are simply derivatised proteins, although covalently immobilised enzymes are not considered here. An example is the derivatisation of myoglobin, the oxygen carrier in muscle, by attaching $(Ru(NH3)5)^{3}$“ to three surface histidine residues. This converts it from an oxygen carrier to an oxidase, oxidising ascorbic acid whilst reducing molecular oxygen. The synzyme is almost as effective as natural ascorbate oxidases.

It is impossible to design protein synzymes from scratch with any probability of success, as their conformations are not presently predictable from their primary structure. Such proteins will also show the drawbacks of natural enzymes, being sensitive to denaturation, oxidation and hydrolysis. For example, polylysine binds anionic dyes but only 10% as strongly as the natural binding protein, serum albumin, in spite of the many charges and apolar side-chains. Polyglutamic acid, however, shows synzymic properties. It acts as an esterase in much the same fashion as the acid proteases, showing a bell-shaped pH-activity relationship, with optimum activity at about pH 5.3, and Michaelis-Menten kinetics with a K> of 2 mm and Vmax of 10^{4} to 10^{5} s^{1} for the hydrolysis of 4-nitrophenyl acetate. Cyclodextrins (Schardinger dextrins) are naturally occurring toroidal molecules consisting of six, seven, eight, nine or ten a-1, 4-linked D-glucose units joined head-to-tail in a ring (a-, b-, g-, d- and e-cyclodextrins, respectively: they may be synthesised from starch by the cyclomaltodextrin glucanotransferase (EC 2.4.1.19) from *Bacillus macerans).* They differ in the diameter of their cavities (about 0.5-1 nm) but all are about 0.7 nm deep. These form hydrophobic pockets due to the glycosidic oxygen atoms and inwards-facing C-H groups. All the C-6 hydroxyl groups project to one end and all the C-2 and C-3 hydroxyl groups to the other. Their overall characteristic is hydrophilic, being water soluble, but the presence of their hydrophobic pocket enables them to bind hydrophobic molecules of the appropriate size. Synzymic cyclodextrins are usually derivatised in order to introduce catalytically relevant groups. Many such derivatives have been examined. For example, a C-6 hydroxyl group of b-cyclodextrin was covalently derivatised by an activated pyridoxal coenzyme. The resulting synzyme not only acted a transaminase but also showed stereoselectivity for the L-amino acids. It was not as active as natural transaminases, however.

Polyethyleneimine is formed by polymerising ethyleneimine to give a highly branched hydrophilic three-dimensional matrix. About 25% of the resultant amines are primary, 50% secondary and 25% tertiary:



[8.6]

Ethyleneimine polyethyleneimine

The primary amines may be alkylated to form a number of derivatives. If 40% of them are alkylated with 1-iodododecane to give hydrophobic binding sites and the remainder alkylated with 4(5)-chloromethylimidazole to give general acid-base catalytic sites, the resultant synzyme has 27% of the activity of a-chymotrypsin against 4-nitropheny1 esters. As might be expected from its apparently random structure, it has very low esterase specificity. Other synzymes may be created in a similar manner.

Antibodies to transition state analogues of the required reaction may act as synzymes. For example, phosphonate esters of general formula (R-PO z-OR') are stable analogues of the transition state occurring in carboxylic ester hydrolysis. Monoclonal antibodies raised to immunising protein conjugates covalently attached to these phosphonate esters act as esterases. The specificities of these catalytic antibodies (also called abzymes) depends on the structure of the side-chains (i.e. R and R' in (R-PO2-OR')) of the antigens. The K, values may be quite low, often in the micromolar region, whereas the Vmax values are low (below 1 s '), although still 1000-fold higher than hydrolysis by background hydroxyl ions. A similar strategy may be used to produce synzymes by molecular 'imprinting' of polymers, using the presence of transition state analogues to shape polymerising resins or inactive non-enzymic protein during heat denaturation.