



KARPAGAM ACADEMY OF HIGHER EDUCATION
(Deemed to be University Established Under Section 3 of UGC Act 1956)
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DEPARTMENT OF BIOCHEMISTRY

SYLLABUS

SUBJECT NAME: ENZYMES AND MICROBIAL TECHNOLOGY

SUB.CODE: 19BCP102

SEMESTER: I

CLASS: I M.Sc., BIOCHEMISTRY

UNIT I

Enzymes - Nomenclature and classification of Enzymes with examples; coenzymes and cofactors. Active site, the investigation of active site structure - The identification of binding sites, catalytic sites - trapping the ES complex, Use of substrate analogs, enzyme modification by treatment with proteolytic enzymes, Photooxidation and chemical modifications of amino acid side chains. Affinity labeling studies and super reactive amino acid chains. The 3D structural features of active site as revealed by X-ray crystallographic and chemical studies, Site directed mutagenesis. catalytic triad. Lock and key model, Induced fit model. Factors affecting enzyme activity. Isolation, purification and characterization of enzymes. Mechanism of enzyme action –Acid base and covalent catalysis (Chymotrypsin, lysozyme), metal activated and metalloenzymes.

UNIT II

Enzyme Kinetics : Derivation of MM equation, LB plot, Eadie Hofstee plot and Hanes plot. Bisubstrate reactions-types of bi-bi reactions, differentiating bi substrate mechanisms-diagnostic plots, isotope exchange. Enzyme inhibition-Types and differentiation of competitive, uncompetitive and non-competitive inhibition, Allosteric inhibition, feed-back inhibition and regulation. Reversible covalent modification (glycogen phosphorylase); proteolytic cleavage (Zymogen); multi enzyme complex as regulatory enzymes (PDH); isoenzymes (LDH). Mechanism based inhibitors-antibiotics as inhibitors. Mechanism of action of enzymes - chymotrypsin and lysozyme. Enzyme based diagnostic techniques.

UNIT III

Immobilization of enzymes: Methods of immobilization - adsorption, covalent binding, entrapment, membrane confinement. Effect of immobilization on enzyme. Use of enzymes in clinical diagnosis and industry. Enzyme engineering. Artificial enzymes and synzymes, Abzymes, ribozymes, enzymes in organic solvents.

Biosensors - Glucose oxidase, Cholesterol oxidase, Urease and antibiotics as biosensors

UNIT IV

Microbial Growth: Balanced and Unbalanced microbial growth; Measurement of growth; Principles of microbial growth and culture systems-batch culture, fed batch culture, semi-continuous culture and continuous culture. Isolation and screening of industrially important microbes. Important strains for better yield. Design of a fermenter. Types of bioreactor-Continuous stirred tank, Bubble column, Airlift, Fluidized bed, Packed bed and Photobioreactor.

Solid substrate fermentation and Media fermentation. Examples of bioprocess for the production of biomass. Microbial metabolic products-primary and secondary metabolites.

UNIT V

Production of fermented products and downstream processing: Production of alcohol and alcoholic beverages. Microbial production of Organic acids: Source, recovery and uses of Citric acid, Lactic acid, Acetic acid and L-ascorbic acid. Production of antibiotics: Penicillin and Tetracyclin. Bioinsecticides: Production of Bacterial and fungal polysaccharides, commercial production of Xanthan gum and pullulan. Production of edible mushroom and SCP.

Biofertilizers *Phosphobacterium* and *Rhizobium sp.*; Biopesticides, leaching of ores by microbes, Microbial treatment of waste water - aerobic and anaerobic methods.

REFERENCES

- R1. Chapline, M.F., and C. Bucke, (1990), Protein Biotechnology. Cambridge University Press, London.
- R2. Nicholas C. Price and Lewis Stevens, 2004, Fundamentals of Enzymology, 3rd Edition, Oxford Univ. Press, New York.
- R3. Stanbury P.F, A. Whitaker and S.J. Hall, 2005, Principles of Fermentation Technology, Elsevier Publishers.

TEXTBOOKS

- T1. Trevor and Palmer (2004) Enzymes, East West Press Pvt. Ltd, New Delhi.
- T2. Dixon M and Webb EC (1979) Enzymes, 3rd edition, Longman and Company Better World Books Ltd.
- T3. Nelson DL and Cox MM (2013) Lehninger - Principles of Biochemistry 6th edition, WH Freeman & Company.
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- W2. [http://what-when-how-to-me/mol-bio/affinity catalytic traid.html](http://what-when-how-to-me/mol-bio/affinity_catalytic_traid.html)
- W3. [http://www.ias.ac.in/article/fulltext/reso/009/10/0025-0033.](http://www.ias.ac.in/article/fulltext/reso/009/10/0025-0033)
- W4. http://agritech.tnau.ac.in/org_farm/orgfarm_biofertilizers.html
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LECTURE PLAN

DEPARTMENT OF BIOCHEMISTRY



STAFF NAME: Dr. S. RUBILA

SUBJECT NAME: ENZYMES AND MICROBIAL TECHNOLOGY

SUB.CODE:19BCP102

CLASS: I M.Sc., (BIOCHEMISTRY)

SEMESTER: I

S.No	Lecture Duration Period	Topics to be Covered	Support Material/Page No.
		Unit -I	
1	2	Enzymes – Nomenclature and classification of enzymes with examples.	T1: 134-137
2	1	Structure and functions of coenzymes and cofactors, Active site, the investigation of active site structure. The identification of binding sites, catalytic sites- trapping the E-S complex.	T1: 209-215
3	1	Use of substrate analogy, enzyme modification by treatment with proteolytic enzymes, photooxidation	W1
4	1	Chemical modifications of aminoacid side chains	W1
5	1	Affinity labelling studies and super reactive aminoacid chains. The 3-D structural features of active sites as revealed by x-ray chrystallographic and chemical studies.	W2
6	1	Site directed mutagenesis. Catalytic triad Lock and key model. Induced fit model.	R1:91-98
7	1	Factors affecting enzyme activity. Isolation, purification and characterization of enzymes	R1:87-88 T2:463-464
8	1	Mechanisms of enzyme action acid-base and covalent catalysis, metal activated and metalloenzymes	T2: 496-501 T3:501-504
Total No of Hours Planned For Unit 1: 09			
		Unit - II	
1	2	Derivation of MM equation, LB plot, Eadie Hofstee plot and Hanes plot.	T1: 203-207
2	1	Bisubstrate reaction types of bi-bi reactions, differentiating bi substrate mechanisms-diagnostic plots, isotope exchange	T1:487-489
3	1	Enzyme inhibition-types and differentiation of competitive, uncompetitive and non competitive inhibition, Allosteric inhibition, feed-back inhibition and regulation.	T4:505-507

4	1	Reversible covalent modification and Proteolytic cleavage	R2:256-263 T4:231-232
5	1	Multi enzyme complex as regulatory enzymes, isoenzymes.	R2:12-13 200-201
6	1	Mechanism based inhibition-antibiotics as inhibitors	T4:211-212
7	1	Mechanism of action of enzymes-chymotrypsin and lysozyme	R2:154-159
8	1	Enzyme based diagnostic techniques	R2:422-426
Total No of Hours Planned For Unit I: 09			
		Unit -III	
1	2	Immobilization of enzymes: Methods of immobilization-adsorption, covalent binding	T1:356-357
2	1	Entrapment, membrane confinement	T1:356-357
3	1	Effect of immobilization on enzyme	T1:357-358
4	1	Use of enzymes in clinical diagnosis and industries	T1:340-350
4	1	Enzyme engineering.	T1:359-360
5	1	Artificial enzymes and synzymes, Abzymes, ribozymes	T1:360-365
6	1	Enzymes in organic solvents	T1:365-369
7	1	Biosensors- glucose oxidase, cholesterol oxidase, urease and antibodies as biosensors	T4:116-128
Total No of Hours Planned For Unit III: 09			
		Unit- IV	
1	1	Balanced and Unbalanced microbial growth; Measurement of growth	T5: 119-132
2	1	Principles of microbial growth and culture systems-batch culture, fed batch culture, semi-continuous culture and continuous culture	T5:478 T6:90-91
3	1	Isolation and screening of industrially important microbes	T7: 992-997
4	1	Important strains for better yield	T7:998-1004
5	1	Design of a fermenter.	R3:167-199
6	1	Types of bioreactor-Continuous stirred tank, bubble	T8:418-419

		column, Airlift, Fluidized bed, packed bed and photobioreactor.	
7	1	Solid substrate fermentation and Media fermentation.	R3:93-116
8	1	Examples of bioprocess for the production of biomass	R3: 1-10, 221-222
9	1	Microbial metabolic products-primary and secondary metabolites.	T7:78:80
Total No of Hours Planned For Unit IV: 09			
		Unit -V	
1	1	Production of alcohol and alcoholic beverages.	T8:407-409
2	2	Microbial production of organic acids: Source, recovery and uses of citric acid, lactic acid, acetic acid and L-ascorbic acid.	T8:409-411 415-420
3	1	Production of antibiotics; penicillin and tetracyclin.	T6:107-110
4	1	Bioinsecticides; Production of bacterial and fungal polysaccharides	T7:1018-1022
5	1	Commercial production of xanthan gum and pullulan,	W3
6	1	Production of edible mushrooms and SCP.	T8:413-415
7	1	Biofertilizers phosphobacterium and rhizobium species	W4
8	1	biopesticide, leaching of ores by microbes, microbial treatment of wastewater-aerobic and anaerobic methods	W5
		Total No of Hours Planned For Unit IV: 09	
Previous year End Semester Exam- QP discussion			
1	1	Revision will be given in the End Semester question papers	-
2	1	Revision will be given in the End Semester question papers	-
3	1	Revision will be given in the End Semester question papers	-
Total No of Hours Planned for discussion: 03			
Total Planned Hours	48		

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- R1. Chapline, M.F., and C. Bucke, (1990), Protein Biotechnology. Cambridge University Press, London.
- R2. Nicholas C. Price and Lewis Stevens, 2004, Fundamentals of Enzymology, 3rd Edition, Oxford Univ. Press, New York.
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WEBSITES

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- W3. <http://www.ias.ac.in/article/fulltext/reso/009/10/0025-0033>.
- W4. http://agritech.tnau.ac.in/org_farm/orgfarm_biofertilizers.html
- W5. <http://www.krishisewa.com/articles/organic-agriculture/115-biofertilizers.html>

**UNIT I
SYLLABUS**

Enzymes - Nomenclature and classification of Enzymes with examples; coenzymes and cofactors. Active site, the investigation of active site structure - The identification of binding sites, catalytic sites - trapping the ES complex, Use of substrate analogs, enzyme modification by treatment with proteolytic enzymes, Photooxidation and chemical modifications of amino acid side chains. Affinity labeling studies and super reactive amino acid chains. The 3D structural features of active site as revealed by X-ray crystallographic and chemical studies, Site directed mutagenesis. catalytic triad. Lock and key model, Induced fit model. Factors affecting enzyme activity. Isolation, purification and characterization of enzymes. Mechanism of enzyme action – Acid base and covalent catalysis (Chymotrypsin, lysozyme), metal activated and metalloenzymes.

Enzymes - Nomenclature and classification of Enzymes with examples

All enzymes contain a protein backbone. In some enzymes this is the only component in the structure. However there are additional non-protein moieties usually present which may or may not participate in the catalytic activity of the enzyme. Covalently attached carbohydrate groups are commonly encountered structural features which often have no direct bearing on the catalytic activity, although they may well effect an enzyme's stability and solubility. Other factors often found are metal ions (**cofactors**) and low molecular weight organic molecules (**coenzymes**). These may be loosely or tightly bound by noncovalent or covalent forces. They are often important constituents contributing to both the activity and stability of the enzymes. This requirement for cofactors and coenzymes must be recognized if the enzymes are to be used efficiently and is particularly relevant in continuous processes where there may be a tendency for them to become separated from an enzyme's protein moiety.

General principles

Because of their close interdependence, it is convenient to deal with the classification and nomenclature together.

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*.

In this context it is appropriate to express disapproval of a loose and misleading practice that is found in the biological literature. It consists in designation of a natural substance (or even of an

hypothetical active principle), responsible for a physiological or biophysical phenomenon that cannot be described in terms of a definite chemical reaction, by the name of the phenomenon in conjugation with the suffix *-ase*, which implies an individual enzyme. Some examples of such *phenomenase* nomenclature, which should be discouraged even if there are reasons to suppose that the particular agent may have enzymic properties, are: *permease*, *translocase*, *reparase*, *joinase*, *replicase*, *codase*, etc..

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, cf. section 3).

To classify an enzyme according to the type of reaction catalysed, it is occasionally necessary to choose between alternative ways of regarding a given reaction. Some considerations of this type are outlined in section 3 of this chapter. In general, that alternative should be selected which fits in best with the general system of classification and reduces the number of exceptions.

One important extension of this principle is the question of the direction in which the reaction is written for the purposes of classification. To simplify the classification, the direction chosen should be the same for all enzymes in a given class, even if this direction has not been demonstrated for all. Thus the *systematic* names, on which the classification and code numbers are based, may be derived from a written reaction, even though only the reverse of this has been actually demonstrated experimentally. In the list in this volume, the reaction is written to illustrate the classification, i.e. in the direction described by the systematic name. However, the *common* name may be based on either direction of reaction, and is often based on the presumed physiological direction.

Many examples of this usage are found in section 1 of the list. The reaction for EC 1.1.1.9 is written as an oxidation of xylitol by NAD^+ , in parallel with all other oxidoreductases in subgroup EC 1.1.1, and the systematic name is accordingly, *xylitol:NAD⁺ 2-oxidoreductase (D-xylulose-forming)*. However, the common name, based on the reverse direction of reaction, is *D-xylulose reductase*.

Common and Systematic Names

The first Enzyme Commission gave much thought to the question of a systematic and logical nomenclature for enzymes, and finally recommended that there should be two nomenclatures for enzymes, one systematic, and one working or trivial. The systematic name of an enzyme, formed in accordance with definite rules, showed the action of an enzyme as exactly as possible, thus identifying the enzyme precisely. The trivial name was sufficiently short for general use, but not necessarily very systematic; in a great many cases it was a name already in current use. The introduction of (often cumbersome) systematic names was strongly criticized. In many cases the reaction catalysed is not much longer than the systematic name and can serve just as well for identification, especially in conjunction with the code number.

The Commission for Revision of Enzyme Nomenclature discussed this problem at length, and a change in emphasis was made. It was decided to give the trivial names more prominence in the

Enzyme List; they now follow immediately after the code number, and are described as Common Name. Also, in the index the common names are indicated by an asterisk. Nevertheless, it was decided to retain the systematic names as the basis for classification for the following reasons:

- (i) the code number alone is only useful for identification of an enzyme when a copy of the Enzyme List is at hand, whereas the systematic name is self-explanatory;
- (ii) the systematic name stresses the type of reaction, the reaction equation does not;
- (iii) systematic names can be formed for new enzymes by the discoverer, by application of the rules, but code numbers should **not** be assigned by individuals;
- (iv) common names for new enzymes are frequently formed as a condensed version of the systematic name; therefore, the systematic names are helpful in finding common names that are in accordance with the general pattern.

It is recommended that for enzymes that are not the main subject of a paper or abstract, the common names should be used, but they should be identified at their first mention by their code numbers and source. Where an enzyme is the main subject of a paper or abstract, its code number, systematic name, or, alternatively, the reaction equation and source should be given at its first mention; thereafter the common name should be used. In the light of the fact that enzyme names and code numbers refer to reactions catalysed rather than to discrete proteins, it is of special importance to give also the source of the enzyme for full identification; in cases where multiple forms are known to exist, knowledge of this should be included where available.

When a paper deals with an enzyme that is not yet in the Enzyme List, the author may introduce a new name and, if desired, a new systematic name, both formed according to the recommended rules. A number should be assigned only by the Nomenclature Committee of IUBMB.

The Enzyme List contains one or more references for each enzyme. It should be stressed that no attempt has been made to provide a complete bibliography, or to refer to the first description of an enzyme. The references are intended to provide sufficient evidence for the existence of an enzyme catalysing the reaction as set out. Where there is a major paper describing the purification and specificity of an enzyme, or a major review article, this has been quoted to the exclusion of earlier and later papers. In some cases separate references are given for animal, plant and bacterial enzymes.

Scheme for the classification of enzymes and the generation of EC numbers

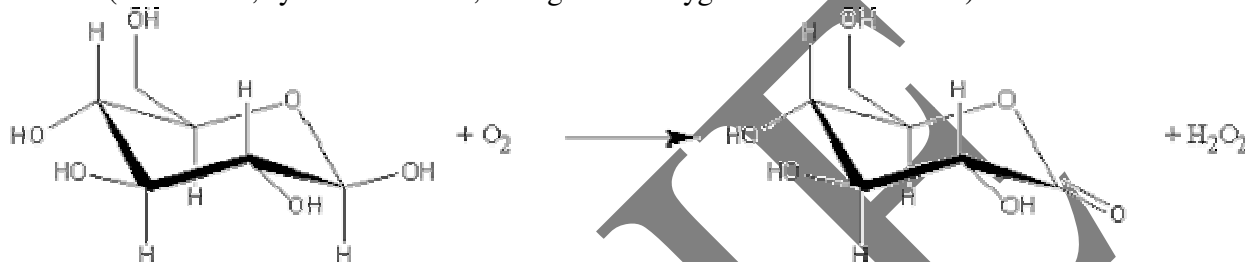
The first Enzyme Commission, in its report in 1961, devised a system for classification of enzymes that also serves as a basis for assigning code numbers to them. These code numbers, prefixed by EC, which are now widely in use, contain four elements separated by points, with the following meaning:

- (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.

Enzymes are classified according the report of a Nomenclature Committee appointed by the International Union of Biochemistry (1984). This enzyme commission assigned each enzyme a recommended name and a 4-part distinguishing number. It should be appreciated that some alternative names remain in such common usage that they will be used, where appropriate, in this

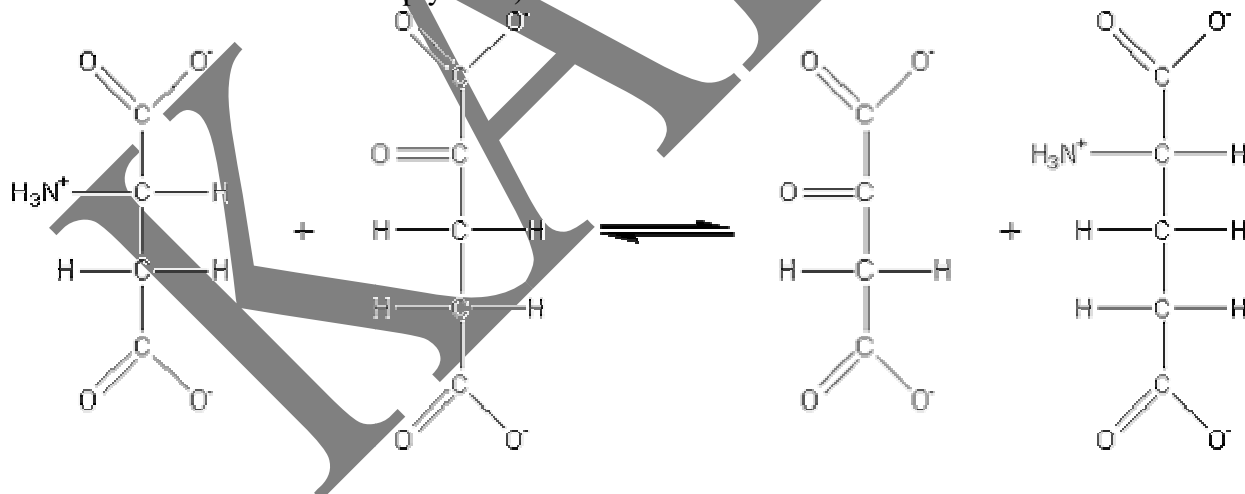
text. The enzyme commission (EC) numbers divide enzymes into six main groups according to the type of reaction catalysed:

(1) **Oxidoreductases** which involve redox reactions in which hydrogen or oxygen atoms or electrons are transferred between molecules. This extensive class includes the dehydrogenases (hydride transfer), oxidases (electron transfer to molecular oxygen), oxygenases (oxygen transfer from molecular oxygen) and peroxidases (electron transfer to peroxide). For example: glucose oxidase (EC 1.1.3.4, systematic name, b-D-glucose:oxygen 1-oxidoreductase).



b-D-glucose + oxygen \longrightarrow D-glucono-1,5-lactone + hydrogen peroxide

(2) **Transferases** which catalyse the transfer of an atom or group of atoms (e.g. acyl-, alkyl- and glycosyl-), between two molecules, but excluding such transfers as are classified in the other groups (e.g. oxidoreductases and hydrolases). For example: aspartate aminotransferase (EC 2.6.1.1, systematic name, L-aspartate:2-oxoglutarate aminotransferase; also called glutamic-oxaloacetic transaminase or simply GOT).



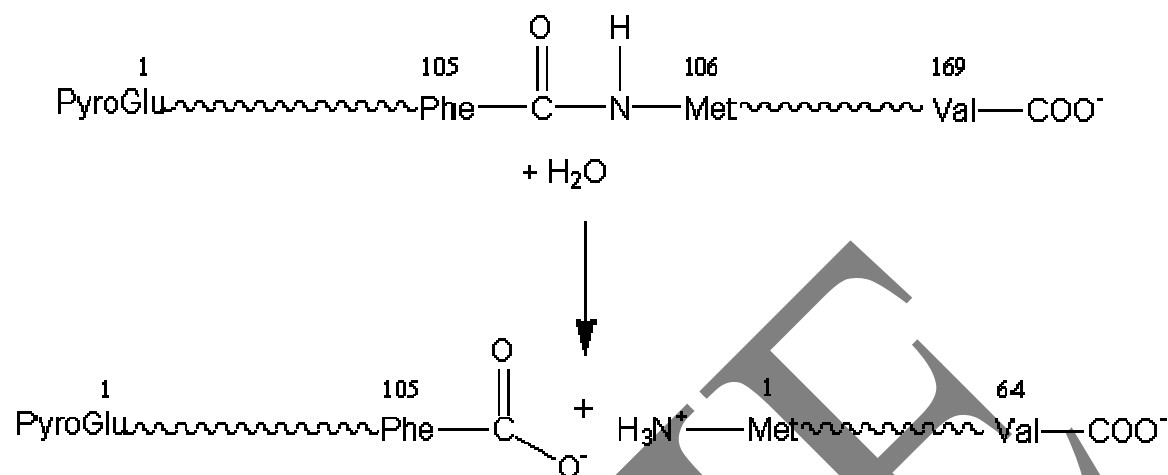
L-aspartate + 2-oxoglutarate \rightleftharpoons oxaloacetate + L-glutamate

(3) **Hydrolases** which involve hydrolytic reactions and their reversal. This is presently the most commonly encountered class of enzymes within the field of enzyme technology and includes the esterases, glycosidases, lipases and proteases. For example: chymosin (EC 3.4.23.4, no systematic name declared; also called rennin).

CLASS: I M.Sc., BIOCHEMISTRY COURSE NAME: ENZYMES AND MICROBIAL TECHNOLOGY

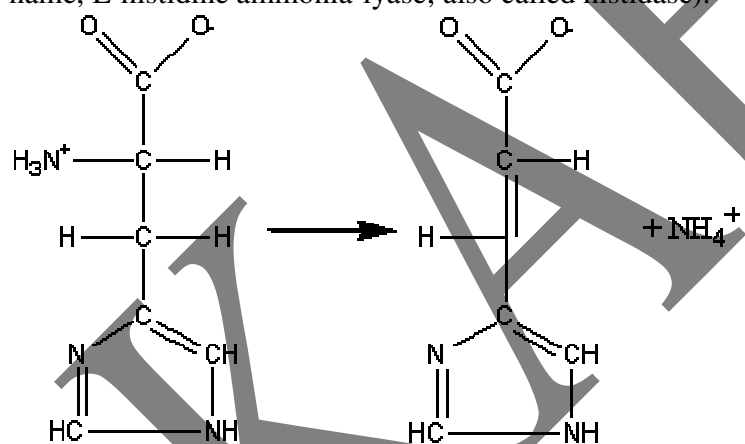
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UNIT-I: ENZYMES (BATCH-2019-2021)



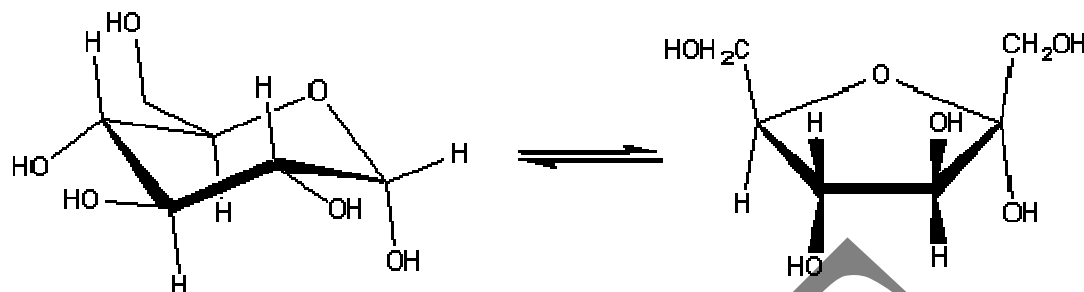
k-casein + water \longrightarrow para-k-casein + caseino macropeptide

(4) **Lyases** which involve elimination reactions in which a group of atoms is removed from the substrate. This includes the aldolases, decarboxylases, dehydratases and some pectinases but does not include hydrolases. For example: histidine ammonia-lyase (EC 4.3.1.3, systematic name, L-histidine ammonia-lyase; also called histidase).



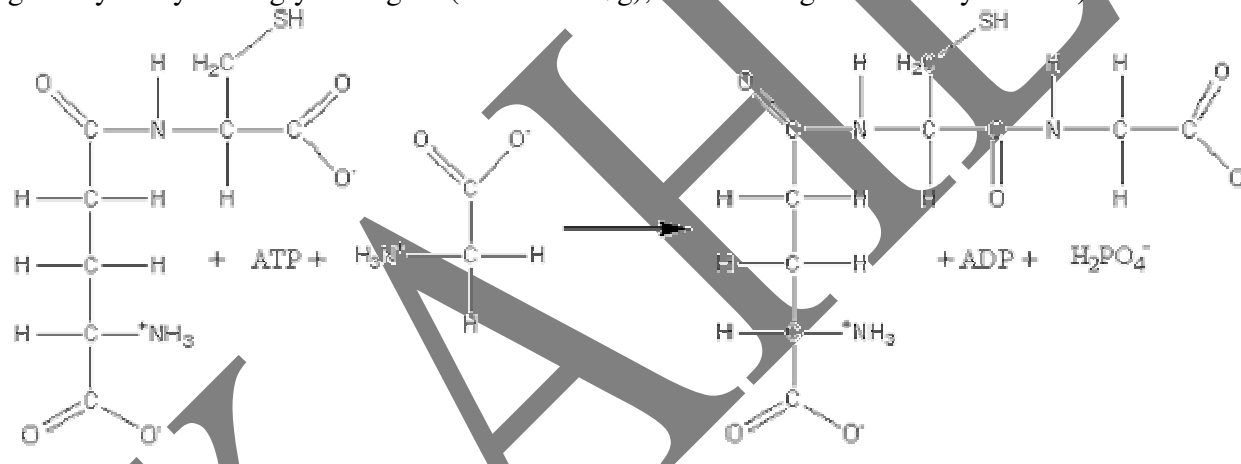
L-histidine → urocanate + ammonia

(5) **Isomerases** which catalyse molecular isomerisations and includes the epimerases, racemases and intramolecular transferases. For example: xylose isomerase (EC 5.3.1.5, systematic name, D-xylose ketol-isomerase; commonly called glucose isomerase).



α -D-glucopyranose \rightleftharpoons α -D-fructofuranose

(6) **Ligases**, also known as synthetases, form a relatively small group of enzymes which involve the formation of a covalent bond joining two molecules together, coupled with the hydrolysis of a nucleoside triphosphate. For example: glutathione synthase (EC 6.3.2.3, systematic name, g-L-glutamyl-L-cysteine:glycine ligase (ADP-forming); also called glutathione synthetase).

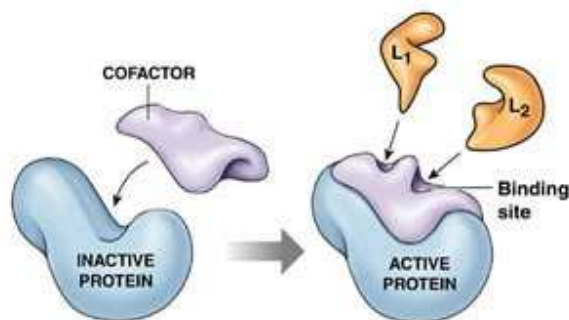


ATP + g-L-glutamyl-L-cysteine + glycine \longrightarrow ADP + phosphate + glutathione

Coenzymes and cofactors

Coenzymes and cofactors are molecules or ions that are used by enzymes to help catalyse reactions. Coenzymes are typically organic molecules that contain functionalities not found in proteins, while

Key difference: Both, cofactor and coenzymes play an extremely important role in the metabolic functions of the body. A coenzyme is technically a type of cofactor, wherein coenzymes are defined as molecules that are bound loosely to an enzyme, and cofactors are those chemical compounds that bind to proteins.



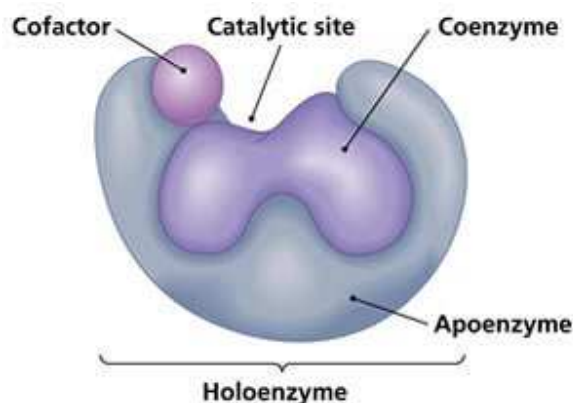
The ability of an organism to carry out hundreds of chemical reactions, within their cells, is what separates the organism from an inanimate object. These reactions are extremely important for survival, and carried out by the enzymes, coenzymes, cells and units present in the body. Out of these, enzymes are the essential proteins which help in controlling the metabolic and chemical reactions of a body.

Other compounds influencing these reactions are the coenzymes and cofactors, which are vital for helping the enzymes to carry out the reactions. Though, both have different functions and properties in a reaction, coenzyme is a derivative of cofactor. This can be very confusing, which is why, it is important to understand the differences between the two chemical compounds.

A cofactor is a non-protein chemical compounds that are bound tightly or loosely to an enzyme (protein), in order to increase the biological activity of the compound. It is extremely vital and used as a catalyst in a reaction. It is also termed as 'helper molecule' because of its biochemical transformations in the reactions. There are two types of cofactors:

- Coenzymes
- Prosthetic groups

On the other hand, coenzymes are defined as small, organic, non-protein molecules, such as vitamins, that carry chemical groups between enzymes. Although, it is not considered a part of an enzyme's structure, coenzymes are acted upon by the enzymes for a reaction. It is also known as co-substrates.



Although, coenzyme is a type of cofactor, it is a chemical molecule and a cofactor is a chemical compound. Also, coenzyme is a loosely bound cofactor to an enzyme, while cofactor is tightly bound to proteins in a reaction. During a

reaction, the coenzymes function as intermediate carriers, wherein they make sure that specific atoms are carried out to the specific group, such that the overall reaction is carried out and finalized easily.

Cofactors, on the other hand, are needed and required to increase and analyze the speed of the reaction i.e. how fast the catalyst would act to complete the reaction. Many a times, cofactors and coenzymes have similar functions such as regulating, controlling, and adjusting the pace of these chemical reactions, and their effect on the body. Though, the differences between the two are given in the table below.

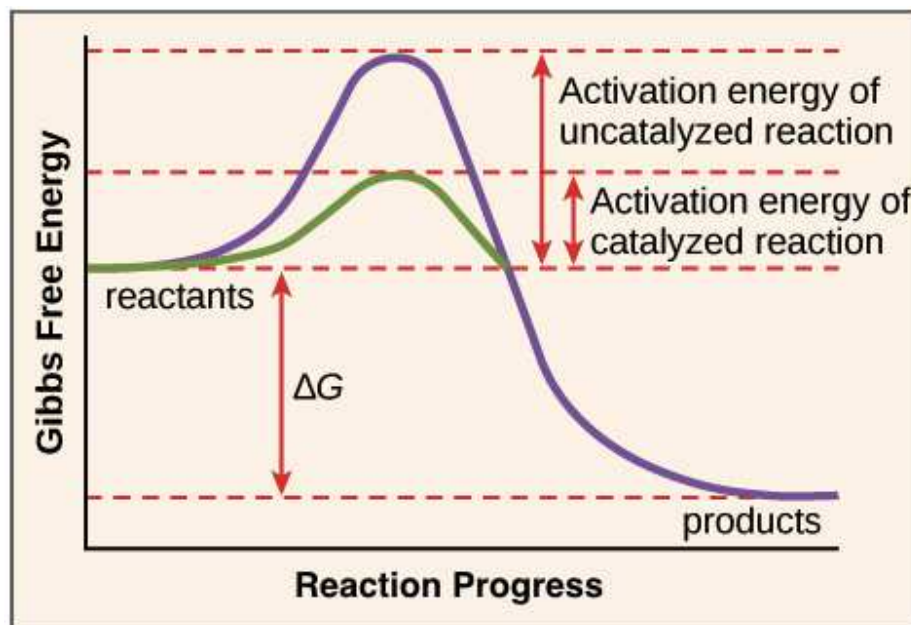
Comparison between Cofactor and Coenzyme:

	Cofactor	Coenzyme
Definition	It is a non-protein chemical compounds that are bound tightly or loosely to an enzyme (protein).	It is defined as small, organic, non-protein molecules, which carry chemical groups between enzymes.
Characteristics	These are inorganic substances.	These are organic substances.
Function	It assists in biological transformations.	It aids or helps the function of an enzyme.
Type	They are chemical compounds.	They are chemical molecules.
Bound	It is tightly bound to an enzyme.	It is loosely bound to an enzyme.
Action	They act on catalyst to increase the speed of the reaction.	They act as carries to the enzymes.
Example	Metal Ions like Zn^{++} , K^{+} and Mg^{++} , etc.	Vitamins, Biotin, Coenzyme A, etc

Enzymes and activation energy

A substance that speeds up a chemical reaction—without being a reactant—is called a **catalyst**. The catalysts for biochemical reactions that happen in living organisms are called **enzymes**. Enzymes are usually proteins, though some ribonucleic acid (RNA) molecules act as enzymes too.

Enzymes perform the critical task of lowering a reaction's activation energy—that is, the amount of energy that must be put in for the reaction to begin. Enzymes work by binding to reactant molecules and holding them in such a way that the chemical bond-breaking and bond-forming processes take place more readily.



Reaction coordinate diagram showing the course of a reaction with and without a catalyst. With the catalyst, the activation energy is lower than without. However, the catalyst does not change the ΔG for the reaction.

To clarify one important point, enzymes don't change a reaction's ΔG value. That is, they don't change whether a reaction is energy-releasing or energy-absorbing overall. That's because enzymes don't affect the free energy of the reactants or products.

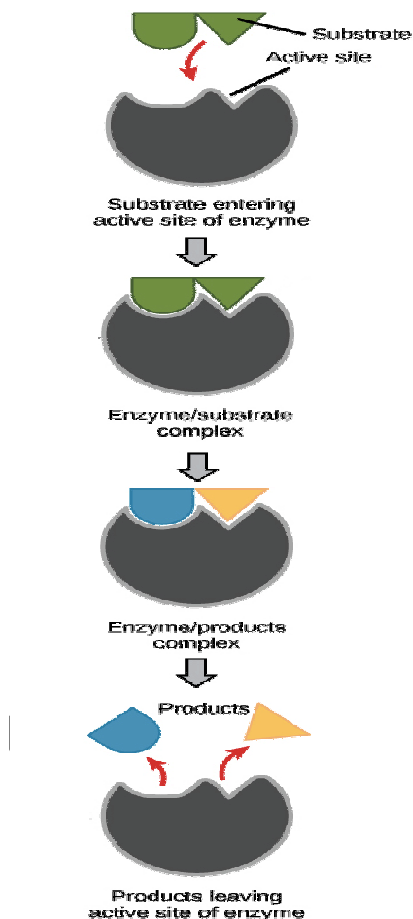
Instead, enzymes lower the energy of the **transition state**, an unstable state that products must pass through in order to become reactants. The transition state is at the top of the energy "hill" in the diagram above.

Active site

To catalyze a reaction, an enzyme will grab on (bind) to one or more reactant molecules. These molecules are the enzyme's **substrates**.

In some reactions, one substrate is broken down into multiple products. In others, two substrates come together to create one larger molecule or to swap pieces. In fact, whatever type of biological reaction you can think of, there is probably an enzyme to speed it up!

The part of the enzyme where the substrate binds is called the **active site** (since that's where the catalytic "action" happens).



A substrate enters the active site of the enzyme. This forms the enzyme-substrate complex. The reaction then occurs, converting the substrate into products and forming an enzyme products complex. The products then leave the active site of the enzyme.

Proteins are made of units called amino acids, and in enzymes that are proteins, the active site gets its properties from the amino acids it's built out of. These amino acids may have side chains that are large or small, acidic or basic, hydrophilic or hydrophobic.

The set of amino acids found in the active site, along with their positions in 3D space, give the active site a very specific size, shape, and chemical behavior. Thanks to these amino acids, an enzyme's active site is uniquely suited to bind to a particular target—the enzyme's substrate or substrates—and help them undergo a chemical reaction.

Types of Active Site

Because enzymes, like all proteins, are made of amino acids, they can create active sites with a wide variety of properties that can bind specifically to different substrates. Properties of amino acids which enzymes can use to bind to substrates include:

- **Size and shape of active site** – Can be created specifically to fit around a substrate.

- **Polarity or non-polarity** – Polar molecules are attracted to other polar molecules, while non-polar molecules prefer other non-polar molecules. In this way, parts of the active site can attract or repel different parts of the substrate to create a better fit.
- **Positive or negative charge** – When it comes to ions, opposites really do attract! Positive charges are attracted to negative charges, and vice versa. Similar charges – two positive charges, for example – will actively repel each other instead of attracting.
This is another way in which an enzyme active site can attract certain parts of substrates, while repelling others to create the right fit.
- **Hydrophobicity or hydrophilicity** – Just like with polarity, in this case “like attracts like.” Hydrophobic amino acids attract other hydrophobic molecules, and hydrophilic amino acids attract hydrophilic substrates.
- **Special properties of co-factors** – Some vitamins and minerals are important because they are used as co-factors that help enzymes bind to their substrates.
Several B vitamins, for example, are used as co-factors by enzymes involved in producing energy. That’s why many energy “shots” and supplements contain a collection of B vitamins.

The investigation of active site structure

The identification of binding sites

In biochemistry, a binding site is a region on a protein or piece of DNA or RNA to which ligands (specific molecules and/or ions) may form a chemical bond. Characteristics of binding sites are chemical specificity, a measure of the types of ligands that will bond, and affinity, which is a measure of the strength of the chemical bond.

An equilibrium exists between unbound ligands and bound ligands. *Saturation* is the fraction of total binding sites that are occupied at any given time. When more than one type of ligand can bind to a binding site, the ligands can compete with each other.

Binding sites are often an important component of the functional characterization of biomolecules. For example, the characterization of the active site of a substrate to an enzyme is essential to model the reaction mechanism responsible for the chemical change from substrate to product.

Binding sites on proteins can sometimes recognize other proteins. When a binding site of one protein identifies with another protein's surface, a non-covalent bond is formed between the two polypeptide (peptide) chains and a combined new protein is formed.

A more specific type of binding site is the transcription factor binding site present on DNA. Short, recurring patterns in DNA often indicate sequence-specific binding sites for proteins such as nucleases and transcription factors; ribosome binding, mRNA processing, and transcription termination are also signaled by these sequence motifs. Prediction of protein (esp. transcription factors) binding sites on DNA has recently become an area of active research and different tools have been produced for it. With the advent of deep learning, newer and more accurate methods have been produced these methods often benefit from the large volume of available data which is generated from high-throughput technologies, such as the protein binding microarrays and use

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deep learning modules such as the convolutional neural networks (CNNs) and the recurrent neural nets (RNNs).

Binding sites also exist on antibodies as specifically coded regions that bind antigens based upon their structure. Several supervised machine learning models and applications were suggested to identify the binding sites, including techniques involving 3D convolutional neural networks.

Catalytic sites-trapping ES complex

The identification of substrates of protein tyrosine phosphatases (PTPs) is an essential step toward a complete understanding of the physiological function of members of this enzyme family. PTPs are defined by a conserved catalytic domain harboring 27 invariant residues. From a mutagenesis study of these invariant residues that was guided by our knowledge of the crystal structure of PTP1B, we have discovered a mutation of the invariant catalytic acid (Asp-181 in PTP1B) that converts an extremely active enzyme into a “substrate trap.” Expression of this D181A mutant of PTP1B in COS and 293 cells results in an enzyme that competes with endogenous PTP1B for substrates and promotes the accumulation of phosphotyrosine primarily on the epidermal growth factor (EGF) receptor as well as on proteins of 120, 80, and 70 kDa. The association between the D181A mutant of PTP1B and these substrates was sufficiently stable to allow isolation of the complex by immunoprecipitation. As predicted for an interaction between the substrate-binding site of PTP1B and its substrates, the complex is disrupted by vanadate and, for the EGF receptor, the interaction absolutely requires receptor autophosphorylation. Furthermore, from immunofluorescence studies, the D181A mutant of PTP1B appeared to retain the endogenous EGF receptor in an intracellular complex. These results suggest that the EGF receptor is a bona fide substrate for PTP1B *in vivo* and that one important function of PTP1B is to prevent the inappropriate, ligand-independent, activation of newly synthesized EGF receptor in the endoplasmic reticulum. This essential catalytic aspartate residue is present in all PTPs and has structurally equivalent counterparts in the dual-specificity phosphatases and the low molecular weight PTPs. Therefore we anticipate that this method may be widely applicable to facilitate the identification of substrates of other members of this enzyme family.

Use of substrate analogs

Substrate analogs (substrate state analogues), are chemical compounds with a chemical structure that resemble the substrate molecule in an enzyme-catalyzed chemical reaction. Substrate analogs can act as competitive inhibitors of an enzymatic reaction. An example is phosphoramidate to the *Tetrahymena* group I ribozyme

As a competitive inhibitor, substrate analogs occupy the same binding site as its analog, and decrease the intended substrate's efficiency. The V_{max} remains the same while the intended substrate's affinity is decreased. This means that less of the intended substrate will bind to the enzyme, resulting in less product being formed. In addition, the substrate analog may also be missing chemical components that allow the enzyme to go through with its reaction. This also causes the amount of product created to decrease.

Substrate analogs bind to the binding site reversibly. This means that the binding of the substrate analog to the enzyme's binding site is non-permanent. The effect of the substrate analog can be nullified by increasing the concentration of the originally intended substrate.

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Other examples of substrate analogs include: 5'-adenylyl-imidodiphosphate: substrate analog of ATP 3-acetylpyridine adenine dinucleotide: substrate analog of NADH

Some substrate analogs can still allow the enzyme to synthesize a product despite the enzyme's inability to metabolize the substrate analog.¹ These substrate analogs are known as gratuitous inducers.

Example of a substrate analog that is also a gratuitous inducer: IPTG (isopropyl β -thiogalactoside: substrate analog and gratuitous inducer of β -galactosidase activity

There are even substrate analogs that bind to the binding site of an enzyme irreversibly. If this is the case, the substrate analog is called an inhibitory substrate analog, a suicide substrate, or even a Trojan horse substrate.

Example of a substrate analog that is also a suicide substrate/Trojan horse substrate: Penicillin: substrate analog and suicide substrate/Trojan horse substrate of peptidoglycan.

Enzyme modification by treatment with proteolytic enzymes

Enzymatic hydrolysis led to improve functional properties and biological activity of protein by-products, which can be further used as protein ingredients for food and feed applications. The effects of proteolytic enzyme modification of egg-yolk protein preparation (YP) and white protein preparation (WP), obtained as the by-products left during the course of lecithin, lysozyme, and cystatin isolation on their biological and functional properties, were evaluated by treating a commercial Neutrase. The antihypertensive and antioxidative properties of YP and WP hydrolysates were evaluated based on their angiotensin-converting enzyme (ACE)-inhibitory activity and radical scavenging (DPPH) capacity, ferric reducing power, and chelating of iron activity. The functionality of obtained hydrolysates was also determined. Neutrase caused a degree of hydrolysis (DH) of YP and WP by-products: 27.6% and 20.9%, respectively. In each of them, mixture of peptides with different molecular masses was also observed. YP hydrolysate showed high levels of antioxidant activity.

Photo oxidation and chemical modification of amino acid side chains

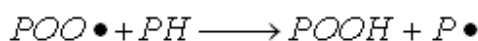
Photo-oxidation is the degradation of a polymer surface in the presence of oxygen or ozone. The effect is facilitated by radiant energy such as UV or artificial light. This process is the most significant factor in weathering of polymers. Photo-oxidation is a chemical change that reduces the polymer's molecular weight. As a consequence of this change the material becomes more brittle, with a reduction in its tensile, impact and elongation strength. Discoloration and loss of surface smoothness accompany photo-oxidation. High temperature and localized stress concentrations are factors that significantly increase the effect of photo-oxidation.

Aldehydes, ketones and carboxylic acids along or at the end of polymer chains are generated by oxygenated species in photolysis of photo-oxidation. The initiation of photo-oxidation reactions is due to the existence of chromophoric groups in the macromolecules. Photo-oxidation can occur simultaneously with thermal degradation and each of these effects can accelerate the other. The photo-oxidation reactions include chain scission, cross linking and secondary oxidative reactions. The following process steps can be considered: Initial step: Free radicals are formed by photon absorption.

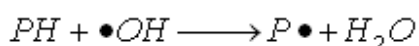
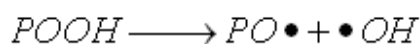
1. Chain Propagation step: A free radical reacts with oxygen to produce a polymer peroxy radical (POO•). This reacts with a polymer molecule to generate polymer hydroperoxide (POOH) and a new polymer alkyl radical (P•).
2. Chain Branching: Polymer oxy radicals (PO•) and hydroxy radicals (HO•) are formed by photolysis.
3. Termination step: Cross linking is a result of the reaction of different free radicals with each other.



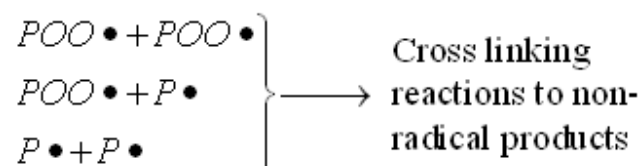
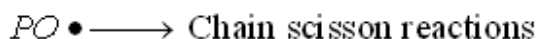
Initial step



Chain Propagation



Chain branching



Termination

where^[1] PH = Polymer

P• = Polymer alkyl radical

PO• = Polymer oxy radical (Polymer alkoxy radical)

POO• = Polymer peroxy radical (Polymer alkylperoxy radical)

POOH = Polymer hydroperoxide

HO• = hydroxy radical

Effects of dyes/pigments

Adding pigment light absorbers and photostabilizers (UV absorbers) is one way to minimise photo-oxidation in polymers. Antioxidants are used to inhibit the formation of hydroperoxides in the photo-oxidation process.

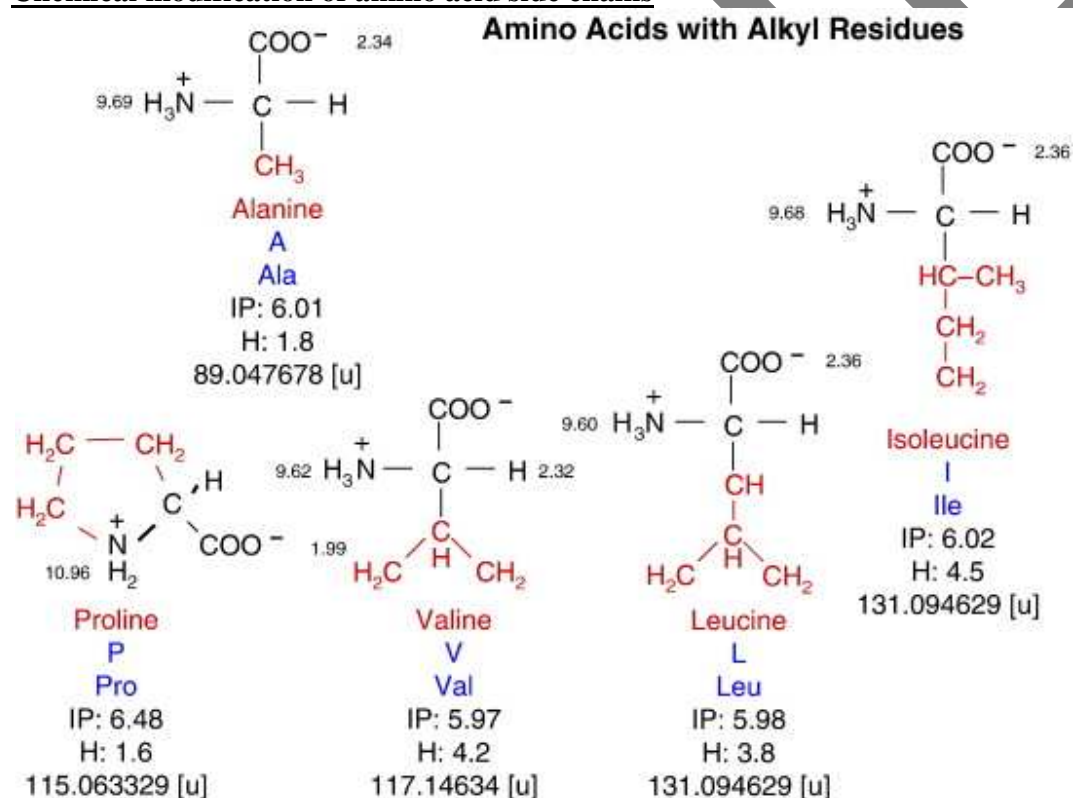
Dyes and pigments are used in polymer materials to provide color changing properties. These additives can reduce the rate of polymer degradation. Cu-phthalocyanine dye can help stabilize against degradation, but in other situations such as photochemical aging can actually accelerate degradation. The excited Cu-phthalocyanine may abstract hydrogen atoms from methyl groups in the PC, which increase the formation of free radicals. This acts as the starting points for the sequential photo-oxidation reactions leading to the degradation of the PC.

Electron transfer sensitization is a mechanism where the excited Cu-phthalocyanine abstracts electrons from PC to form Cu-Ph radical anion and PC radical cations. These species in the presence of oxygen can cause oxidation of the aromatic ring.

Photo oxidation Production

Poly (ethylene-naphthalate) (PEN) can be protected by applying a zinc oxide coating, which acts as protective film reducing the diffusion of oxygen.^[5] Zinc oxide can also be used on polycarbonate (PC) to decrease the oxidation and photo-yellowing rate caused by solar radiation.

Chemical modification of amino acid side chains



General scheme of an enzyme-catalyzed reaction by which the side chain residue of an amino acid in a protein is modified can be described as the covalent addition of an electrophilic chemical group to the nucleophilic electron-rich side chain of the amino acid.

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Affinity labeling studies

Affinity labels are molecules similar in structure to a particular substrate for a specific enzyme and are considered to be a class of enzyme inhibitors. These molecules covalently modify active site residues in order to reveal the active site and thus elucidate its structure. The label binds covalently to the enzyme so that the substrates can no longer bind, causing a permanent and irreversible change. Affinity labels are used in affinity labeling, a technique for the validation of substrate-specific binding of compounds.

These types of compounds are highly reactive and usually alkylate nucleophilic amino acids present in the enzyme. Their usefulness is limited as they are often indiscriminate in their action, and therefore non-selective and inherently toxic

Super reactive amino acid chains

-amino acid side chains can function as nucleophiles

-better nucleophiles=less electronegative (lone pairs more willing to react)

RNH₂ is better nuc than ROH (N is less electronegative)

OH⁻ is better than H₂O

RSH is better than H₂O

S is bigger and its electron cloud is more polarizable-more reactive

order of side chains from most nuc to least Cys (RSH, pK_a 8.5-9.5), His (pK_a 6-7), Lys (pK_a 10.5) and Ser (ROH, pK_a 13).

Ser is a potent nucleophile in a certain class of proteins (proteases, for example) when it is *deprotonated*.

Reactions of lysine

Lys: (or N-terminal RNH₂) This Is a Potent Nu: Only When Deprotonated

phosphorylation

Phosphorylation usually occurs on serine, threonine, tyrosine and histidine residues in eukaryotic proteins.

Histidine phosphorylation of eukaryotic proteins appears to be much more frequent than tyrosine phosphorylation.

In prokaryotic proteins phosphorylation occurs on the serine, threonine, tyrosine, histidine or arginine or lysine residues. The addition of a phosphate (PO₄) molecule to a polar R group of an amino acid residue can turn a hydrophobic portion of a protein into a polar and extremely hydrophilic portion of molecule. In this way protein dynamics can induce a conformational change in the structure of the protein via long-range allostery with other hydrophobic and hydrophilic residues in the protein.

phosphorylase

phosphorylate with phosphate from inorganic phosphate

kinase

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phosphorylate with Phosphate from ATP
phosphatase
remove phosphate group
methylation
Only cytosine is methylated on DNA
Amino acids on histones
modifications at certain amino acids on histones

Lysine:
acetylation
ubiquitination

arginine: methylation

threonine and serine: phosphorylation

The 3D structural features of active sites revealed by X-Ray crystallography

X-ray crystallography can reveal the detailed three-dimensional structures of thousands of proteins. The three components in an X-ray crystallographic analysis are a protein crystal, a source of x-rays, and a detector.

X-ray crystallography is used to investigate molecular structures through the growth of solid crystals of the molecules they study. Crystallographers aim high-powered X-rays at a tiny crystal containing trillions of identical molecules. The crystal scatters the X-rays onto an electronic detector. The electronic detector is the same type used to capture images in a digital camera. After each blast of X-rays, lasting from a few seconds to several hours, the researchers precisely rotate the crystal by entering its desired orientation into the computer that controls the X-ray apparatus. This enables the scientists to capture in three dimensions how the crystal scatters, or diffracts, X-rays. The intensity of each diffracted ray is fed into a computer, which uses a mathematical equation to calculate the position of every atom in the crystallized molecule. The result is a three-dimensional digital image of the molecule.

Crystallographers measure the distances between atoms in angstroms. The perfect “rulers” to measure angstrom distances are X-rays. The X-rays used by crystallographers are approximately 0.5 to 1.5 angstroms long, which are just the right size to measure the distance between atoms in a molecule. That is why X-rays are used.

Protein X-ray crystallography is a technique used to obtain the three-dimensional structure of a particular protein by x-ray diffraction of its crystallized form. This three dimensional structure is crucial to determining a protein's functionality. Making crystals creates a lattice in which this technique aligns millions of proteins molecules together to make the data collection more sensitive. It's like getting a stack of papers, measuring the width with a ruler, and dividing that length with the number of pages to determine the width of one piece of paper. By this averaging technique, the noise level gets reduced and the signal to noise ratio increases.^[2] The specificity of the protein's active sites and binding sites is completely dependent on the protein's precise

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conformation. X-ray crystallography can reveal the precise three-dimensional positions of most atoms in a protein molecule because x-rays and covalent bonds have similar wavelength, and therefore currently provides the best visualization of protein structure. It was the X-ray crystallography by Rosalind E. Franklin, that made it possible for J.D. Watson and F.H.C. Crick to figure out the double-helix structure of DNA.

Steps 1

The process begins by crystallizing a protein of interest. Crystallization of protein causes all the protein atoms to be orientated in a fixed way with respect to one another while still maintaining their biologically active conformations - a requirement for X-ray diffraction. A protein must be precipitated out or extracted from a solution. The rule of thumb here is to get as pure a protein as possible to grow lots of crystals (this allows for the crystals to have charged properties, and surface charged distribution for better scattering results). 4 critical steps are taken to achieve protein crystallization, they are:

1. Purify the protein. Determine the purity of the protein and if not pure (usually >99%), then must undergo further purification.
2. Must precipitate protein. Usually done so by dissolving the protein in an appropriate solvent (water-buffer soln. w/ organic salt such as 2-methyl-2,4-pentanediol). If protein is insoluble in water-buffer or water-organic buffer then a detergent such as sodium lauryl sulfate must be added.
3. The solution has to be brought to supersaturation (condensing the protein from the rest of the solvent forming condensation nuclei). This is done by adding a salt to the concentrated solution of the protein, reducing its solubility and allowing the protein to form a highly organized crystal (this process is referred to as salting out). Other methods include batch crystallization, liquid-liquid crystallization, vapor diffusion, and dialysis.
4. Let the actual crystals grow. Since nuclei crystals are formed this will lead to obtaining actual crystal growth.

NOTES ON RECRYSTALLIZATION TECHNIQUE TO ACHIEVE A MORE PURE PROTEIN:

Recrystallization is an incredibly important technique used for the purification of substances. Understanding the solubility of the solid in a certain solvent is the key to recrystallization. One of the applications of this technique can be seen in pharmaceuticals and in many other fields. For example, crystallographers use methods of nuclear magnetic resonance and x-ray diffraction to gain insight into different compounds. X-ray diffraction requires the formation of pure crystals in order to acquire accurate results. Crystallographers can gain insight into protein structure by using x-ray diffraction, but in order to be able to use x-rays to examine their crystals, they must first spend time forming pure protein crystals. It is very difficult to form protein crystals. It may even take years and incredibly specific conditions. Temperature, pH, and concentration have to be very specific to form larger crystals with a pure structure. Recrystallization in this process is vital to get rid of impurities in the crystal lattice. Scientists today use crystallography and recrystallization techniques to understand protein structure and help understand how a single abnormality in the protein's primary structure can cause diseases. All in all, purification techniques are vital in order to use x-ray diffraction to understand structure. In this experiment

we explore the differences in micro and macro recrystallization. The techniques employed in recrystallization include finding a good solvent to work in, gravity filtration, slow cooling, and vacuum filtration. The key to a successful recrystallization is a good solvent. We need a solvent that will not dissolve the sample at cool temperatures but will dissolve it at high temperatures. This allows the precipitation of the solute after the solution is dissolved in warm temperatures. Since the solute is only soluble in the warm solute, upon cooling, a precipitate forms. Gravity Filtration is used to remove insoluble impurities remaining in the solution before recrystallization and it is used to filter out the charcoal used to remove the color impurities. Gravity filtration is effective, but we must avoid crystallization during this process as to avoid losing pure crystals in the filter paper. Slow cooling is also essential to ensure the purity and size of the crystals. When the solution is allowed to cool slowly, the dissolved impurities have time to interact with the solvent instead of remaining trapped in the crystal lattice. During fast cooling, impurities may remain trapped in the crystal lattice because crystallization occurs too quickly and impurities do not have time to return to the solvent. After the crystals are put in an ice bath to ensure maximum recrystallization, the solution is filtered using a vacuum filtration to extract the pure crystals from the solution with the impurities. After it is vacuumed, the pure crystals are collected and weighed. Micro recrystallization differs from macro recrystallization in the instruments and techniques used for filtration of the pure crystals. Micro recrystallization involves using a Craig tube and centrifugation instead of vacuum filtration. It is used for a recrystallization of less than 300mg of solid.

Steps 2

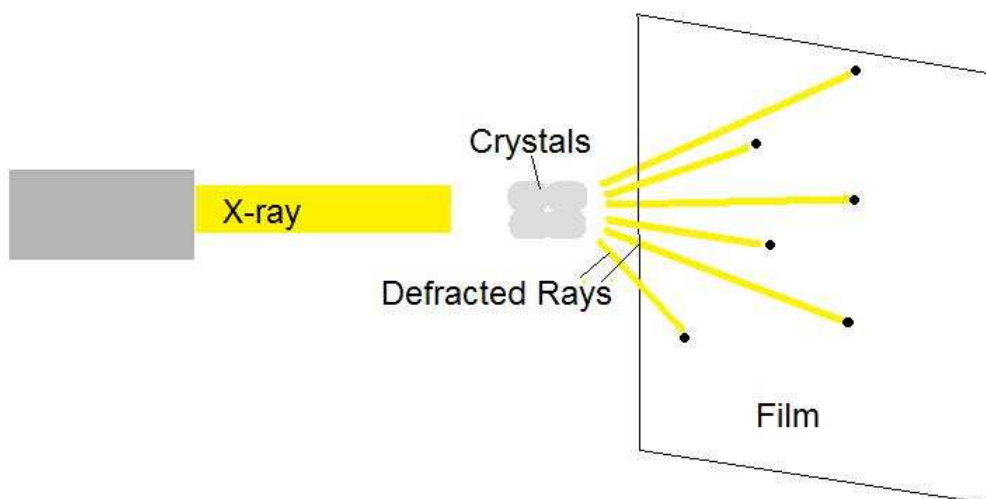
For the next step, x-rays are generated and directed toward the crystallized protein. X-rays can be generated in four different ways,

1. by bombarding a metal source with a beam of high-energy electrons,
2. by exposing a substance to a primary beam of X-rays to create a secondary beam of X-ray fluorescence,
3. from a radioactive decay process that generates X-rays (Gamma rays are indistinguishable from X-rays), and
4. from a synchrotron (a cyclotron with an electric field at constant frequency) radiation source.

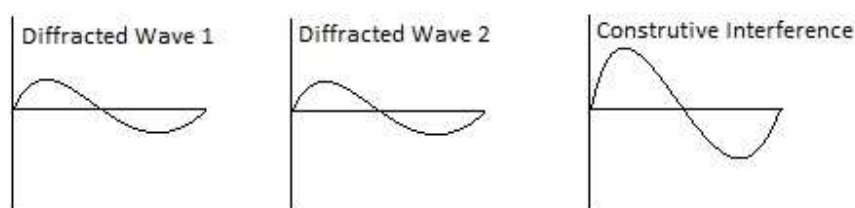
The first and last method utilizes the phenomenon of bremsstrahlung, which states that an accelerating charge will give off radiation.

Then, the x-rays are shot at the protein crystal resulting in some of the x-rays going through the crystal and the rest being scattered in various directions. The scattering of x-rays is also known as "x-ray diffraction". Such scattering results from the interaction of electric and magnetic fields of the radiation with the electrons in the atoms of the crystal.

The patterns are a result of interference between the diffracted x-rays governed by Bragg's Law: where d is the distance between two regions of electron density, θ is the angle of diffraction, λ is the wavelength of the diffracted x-ray and n is an integer. If the angle of reflection satisfies the following condition: the diffracted x-rays will interfere constructively. Otherwise, destructive interference occurs.



Here is an example of constructive interference:



Here is an example of destructive interference:

Constructive interference indicates that the diffracted x-rays are in phase or lined up with each other, while destructive interference indicates that the x-rays are not exactly in phase with each other. The result is that the measured intensity of the x-rays increases and decreases as a function of angle and distance between the detector and the crystal.

The x-rays that have been scattered in various directions are then caught on x-ray film, which show a blackening of the emulsion in proportion to the intensity of the scattered x-rays hitting the film, or by a solid-state detector, like those found in digital cameras. The crystal is rotated so that the x-rays are able to hit the protein from all sides and angles. The pattern on the emulsion reveals much information about the structure of the protein in question. The three basic physical principles underlying this technique are:

1. Atoms scatter x-rays. The amplitude of the diffracted x-ray is directly proportional to the number of electrons in the atom.
2. Scattered waves recombine. The beams reinforce one another at the film if they are in phase or cancel one another out if they are out of phase. Every atom contributes to a scattered beam.
3. Three-dimensional atomic arrangement determines how the beams recombine.

The intensities of the spots and their positions are thus the basic experimental data of the analysis.

Final Steps

The final step involves creating an electron density map based on the measured intensities of the diffraction pattern on the film. A Fourier Transform can be applied to the intensities on the film to reconstruct the electron density distribution of the crystal. In this case, the Fourier Transform takes the spatial arrangement of the electron density and gives out the spatial frequency (how closely spaced the atoms are) in the form of the diffraction pattern on the x-ray film. An everyday example of the Fourier Transform is the music equalizer on a music player. Instead of displaying the actual music waveform, which is difficult to visualize, the equalizer displays the intensity of various bands of frequencies. Through the Fourier Transform, the electron density distribution is illustrated as a series of parallel shapes and lines stacked on top of each other (contour lines), like a terrain map. The mapping gives a three-dimensional representation of the electron densities observed through the x-ray crystallography. When interpreting the electron density map, resolution needs to be taken into account. A resolution of 5\AA - 10\AA can reveal the structure of polypeptide chains, 3\AA - 4\AA of groups of atoms, and 1\AA - 1.5\AA of individual atoms. The resolution is limited by the structure of the crystal and for proteins is about 2\AA .

Types of X rays device

Protein molecules are very large, thus their crystals diffract x-ray beams much less than crystals from smaller molecules. Because larger molecules have fewer crystals, diffraction scattering and hence intensity emitted is very weak. Proteins contain carbon, nitrogen, and oxygen, and so are lighter elements (that is they have fewer electrons/atom); this is important since electrons are responsible for the diffraction and intensity, and therefore they scatter x-rays weaker than heavy elements. Knowing this, protein crystallographers use high intensity x-ray sources such as a rotating anode tube or a strong synchrotron x-ray source for analyzing the protein crystals.

1. The number of electrons in an atom is proportional to the wave's amplitude. An example would be comparing a carbon atom and hydrogen atom; you would see that the carbon atom would scatter six times as strongly as the hydrogen atom.
2. If in phase the waves combine with one another at the film but if the waves are out of phase then they cancel out one another at the film.
3. The only thing that matters when looking at how scattered waves recombine is the atomic arrangement.

Energy of X-ray:

Where f is frequency and λ is wavelength. The SI unit of energy is the joule (J).

X-rays have higher energy than visible light due to its small wavelength.

Phase problem

The interaction of X-rays with the electrons in a crystal gives rise to a diffraction pattern, which mathematically is the Fourier transform of the electron density distribution. The detectors used to measure the X-rays, however, can only measure the amplitude of the diffracted x-rays; the phase shifts, which are required to use the Fourier Transform and find the electron density distribution, are not measurable directly using this method. This is known in the physics community as the

"Phase Problem". In simpler terms the phases cannot be found from the measured amplitudes of the X-rays. Other extrapolations must be made and additional experiments must be done in order to get an electron density map. Many times, the existing data on the compound's physical and chemical properties can help aid when there is a poor density map. Another method known as Patterson Synthesis is very useful to find out an initial estimate of phases and it is very useful for the initial stages to determine the structure of proteins when the phases are not known. The problem can be simplified by finding an atom, usually a heavy metal, using Patterson Synthesis and then using that atom's position to estimate the initial phases and calculate an initial electron density map that can further help in the modeling of the position of other atoms and improve the phase estimate even more. Another method is called Molecular Replacement; it locates the location of the protein structure in the cell. In addition to the molecular replacement method, the phase problem can also be solved by the isomorphous replacement method, the multiple wavelength anomalous diffraction method, the single-wavelength anomalous diffraction method, and direct methods.

Molecular Replacement

Phase problem can be solved by having an atomic model that can compute phases. A model can be obtained if the related protein structure is known. However, in order to build this atomic model, the orientation and position of the model in the new unit cell needs to be determined. This is when the technique, molecular replacement (or MR) comes in.

Molecular Replacement, also known as MR, is a method to solve phase problems in x-ray crystallography. MR locates the orientation and position of a protein structure with its unit cell, whose protein structure is homologous to the unknown protein structure that needs to be determined. The obtained phases can help generate electron density maps and help produce calculated intensities of the position of the protein structure model to the observed structures from the x-ray crystallography experiment.

MR method is also effective for solving macromolecular crystal structures. This method requires less time and effort for structural determination, since heavy atom derivatives and collecting data do not need to be prepared. The method is straight forward and model building is simplified because it needs no chain tracing.

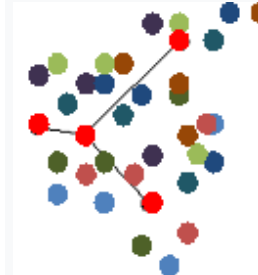
This method consists of two steps:

1. a rotational search to orient the homologous model in the unit cell or target
2. a translational target where the new oriented model is positioned in the unit cell

Patterson-based (Molecular Replacement)

Patterson maps are interatomic vector maps that contain peaks for each related atom in the unit cell. If the Patterson maps were generated based on the data derived from the electron density maps, the two Patterson maps should be closely related to each other only if the model is correctly oriented and placed in the correct position. This will allow us to infer information about the location of the unknown protein structure with its cell. However, there is a problem with molecular replacement; it has six dimensions, three parameters to specify orientation and position. With the Patterson maps, it can be divided into subsets of the parameters to look at each part separately.

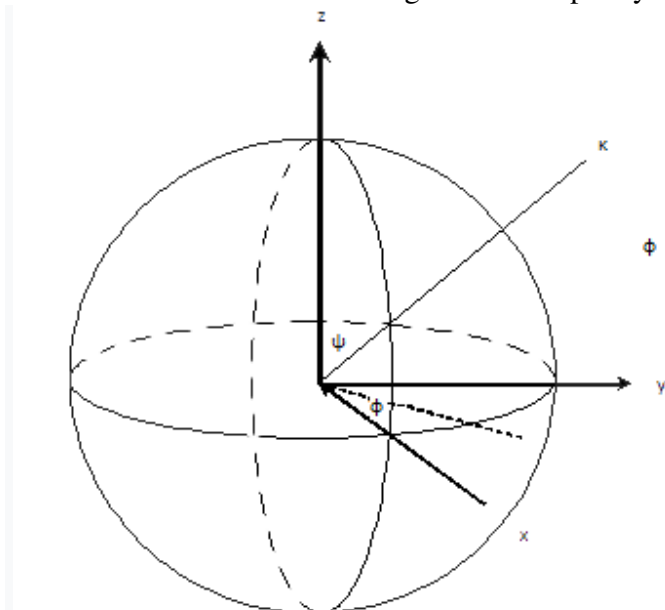
The figure shows the molecule in a random orientation (left) and together with the rest of the intramolecular vectors (right).



This is a Patterson map of the above structure. The intramolecular vectors are shown in red.

Classic Rotation Function

To find the orientation, determine the rotation axis and rotation angle about that axis. Two parameters will be needed to define an axis (a vector from the center of the sphere to a point on the sphere surface). The rotation axis starts off parallel to the z-axis and is rotated around the y-axis with angle ψ , then the object rotates around the z-axis with angle ϕ , and finally it rotates around the rotation axis with angle κ . These specify a point on the surface of a unit sphere.



The $\kappa/\phi/\psi$ description is useful if looking for rotations with a particular rotation angle (κ). For instance, a 2-fold rotations will have $\kappa=180^\circ$, while a 6-fold rotations will have $\kappa=60^\circ$

Fast Rotation Function

The rotation function can be computed by comparing two Patterson maps or the peaks in those Pattersons. Rotation function can be computed much faster with Fourier transforms only if the Pattersons were expressed in terms of spherical harmonics.

Direct Rotation Function

In direct rotation function, the protein structure can be placed in the unit cell of the unknown structure and the Patterson for the oriented molecule is compared with the entire unknown structure Patterson.

Translation Function

Once the orientation of the known structure is known its model (electron density map) can be oriented to compute structure factors where a correlation function is used to determine the vector to translate the model on top of the homologous one within an asymmetric unit.

With the correct oriented and translated phasing models of the protein structure, it is accurate enough to derive the electron density maps from the derived phases. The electron density maps can be used to build and refine the model of the unknown structure.

Multiwavelength Anomalous Diffraction

X-Rays are generated in large machines called synchrotrons. Synchrotrons accelerate electrons to nearly the speed of light and travel them through a large, hollow metal polygon-ring. At each corner, magnets bend the electron stream, causing the emission of energy in the form of electromagnetic radiation. Since the electrons are moving at the speed of light, they emit high energy X-rays.

The benefits of using synchrotrons is that researches do not have to grow multiple versions of every crystallized molecule, but instead only grow one type of crystal that contains selenium. They then have the ability to tune the wavelength to match the chemical properties of selenium. This technique is known as Multiwavelength Anomalous Diffraction. The crystals are then bombarded several times with wavelengths of different lengths, and eventually a diffraction pattern emerges which enables researchers to determine the location of the selenium atoms. This position can be used as a reference, or marker to determine the rest of the structure. The benefits of this allow researchers to collect their data much more quickly.

Isomorphous Replacement Method

This method compares the x-ray diffraction patterns between the original protein crystal and the same type of crystal with an addition of at least one atom with high atomic number. The method was used to determine the structure of small molecules and eventually that of hemoglobin by Max Ferdinand Perutz (1914–2002). A perfect isomorphism is when the original crystal and its derivative have exactly the same conformation of protein, the position and orientation of the molecules, and the unit cell parameters. The only difference that the crystal and its derivative have in a perfect isomorphism is the intensity differences due to the addition of heavy atoms on the derivative. These differences can be identified manually or by an automatic Patterson search procedure, such as SIR 2002, SHELXD, nB, and ACORN, and such information is important as to determine the protein phase angles. However, perfect isomorphism hardly occurs because of the change in cell dimensions. For the protein with heavy atom, its tolerable change in cell dimension is $d_{\min}/4$, for d_{\min} is the resolution limit. Other factors, such as rotation, also contribute to nonisomorphism.

Procedures

1. Prepare a few derivatives of the protein in crystalline structure. Then, measure the cell dimension to check for isomorphism.

2. Collect x-ray intensity data of the original protein and its derivative.
3. Apply the Patterson function to determine the coordinates of the heavy atom.
4. Refine the heavy atom parameters and calculate the phase angle of the protein.
5. Calculate the electron density of the protein.

The derivatives are made through two different methods. The preferred method is to soak the protein crystal in a solution that is composed identically to the mother liquor, but with a slight increase of precipitant concentration. Another method is co-crystallization, but it is not commonly used because the crystal will not grow or grow nonisomorphously. The soaking procedure depends on how wide the crystal pores are. The pores should be wide enough for the reagent to diffuse into the crystal and to reach the reactive sites on the surface of all protein molecules in the crystal.

Multiple Wavelength Anomalous Diffraction Method

Multiple Wavelength Anomalous Diffraction (abbreviated MAD) is a method utilized in X-ray crystallography that allows us to determine the structures of biological macromolecules, such as proteins and DNA, in order to solve the phase problem. Requirements for the structure include atoms that cause significant scattering from X-rays; notably sulfur or metal ions from metalloproteins. Since selenium can replace natural sulfur, it is more commonly used. The use of this technique greatly facilitates the crystallographer from using the Multiple Isomorphous Replacement (MIR) method as preparation of heavy compounds is superfluous.

this method is used to solve phase problems, when there is no available data regarding scattered diffraction besides amplitudes. Moreover, it is used when a heavy metal atom is already bound inside the protein or when the protein crystals are not isomorphous which is unsuitable for MIR method. The method has been mostly used for heavy metallo solution, these metallo enzyme normally comes from the 1st transition series and their neighbors. it is important to have a source for a powerful magnetic field to carry out this experiment, environment such as underground should be considered. A particle accelerator called a synchrotron is also required for the method.

Single-Wavelength Anomalous Diffraction Method

In comparison to multi-wavelength anomalous diffraction (MAD), single-wavelength anomalous diffraction (SAD) uses a single set of data from a single wavelength. The main beneficial difference between MAD and SAD is that the crystal spends less time in the x-ray beam with SAD, which reduces potential radiation damage to the molecule. Also, since SAD uses only one wavelength, it is more time-efficient than MAD.

The electron density maps derived from single-wavelength anomalous diffraction data do need to undergo modifications to resolve phase ambiguities. A common modification technique is solvent flattening, and when SAD is combined with solvent flattening, the electron density maps that result are of comparable quality to those that are derived from full MAD phasing. Solvent flattening involves adjusting the electron density of the interstitial regions between protein molecules occupied by the solvent. The solvent region is assumed to be relatively disordered and featureless compared to the protein. Smoothing the electron density in the solvent regions will enhance the electron density of the protein to an interpretable degree. This method is called ISAS, iterative single-wavelength anomalous scattering.

Direct Methods

The direct method can help recover the phases using the data it obtains. Direct Method estimates the initial and expanding phases using a triple relation. Triple (trio) relation is the relation of the intensity and phase of one reflection with two other intensities and phases. When using this method, the size of the protein structure matters since the phase probability distribution is inversely proportionate to the square root of the number of atoms. Direct method is the most useful technique to solve phase problems.

Site directed mutagenesis

Mutation is an alteration in any of the base of a DNA sequence sometime's leading to a defective protein or prematurely terminated non-functional protein. Mutations are spontaneous in nature although rare (*e.g* sickle cell haemoglobin). In the technique of site-directed mutagenesis a Biotechnologist is able to create mutation selectively, rather than that which occurs randomly in nature. Using this technique amino acids can be substituted in the expressed proteins making them more stable or functionally better. Furthermore the role of specific amino acids in proteins has led to a better understanding of protein structure and function.

The principle of site-directed mutagenesis as schematically explained in Fig. 17 involves cloning the target gene into an M13 vector wherein it is presented as a single stranded part of the phage genome. A small oligonucleotide is added containing a complementary sequence to the gene but with one or more altered nucleotides. This allows the oligonucleotide to bind to a complementary portion in the target gene. This then acts like a primer *in vitro* to synthesise a double stranded replicative form. Note that the duplex RF form has one strand with the original target gene sequence, wild type and the other strand with the altered nucleotide(s). The duplex DNA molecule is then introduced into bacterial cells by transformation. Subsequent replication inside bacterial cells will produce either wild type or mutant gene containing plasmids. If appropriate expression signals are present altered protein can be expressed and studied.

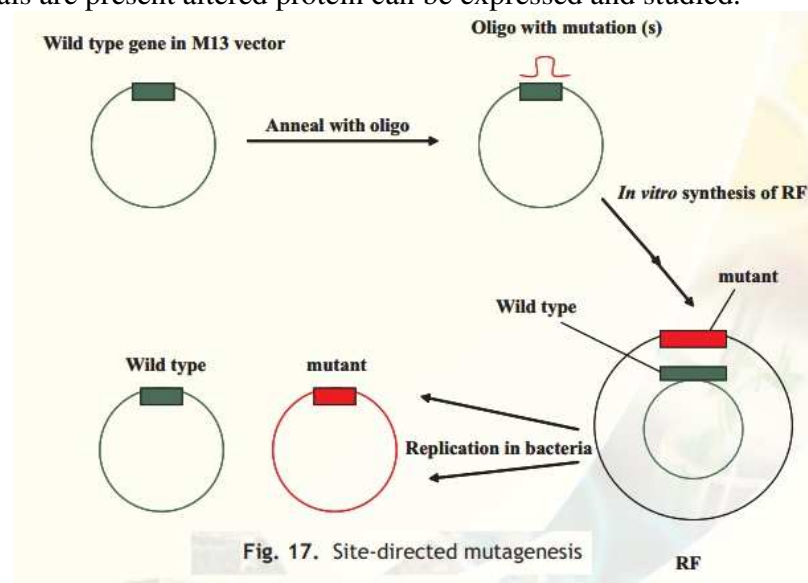


Fig. 17. Site-directed mutagenesis

Catalytic triad

A **catalytic triad** is a group of three amino acids that are found in the active sites of some proteases involved in catalysis.

Three different proteases that have catalytic triads are:

chymotrypsin, trypsin and elastase. In chymotrypsin, the catalytic triad is made from *serine 195*, *histidine 57*, and *aspartate 102*. The side chain of serine is bonded to the imidazole ring of the histidine residue which accepts a proton from serine to form a strong alkoxide nucleophile in the presence of a substrate for attack. The aspartate residue orients histidine to make it a better proton acceptor via hydrogen bonding and electrostatic reactions.

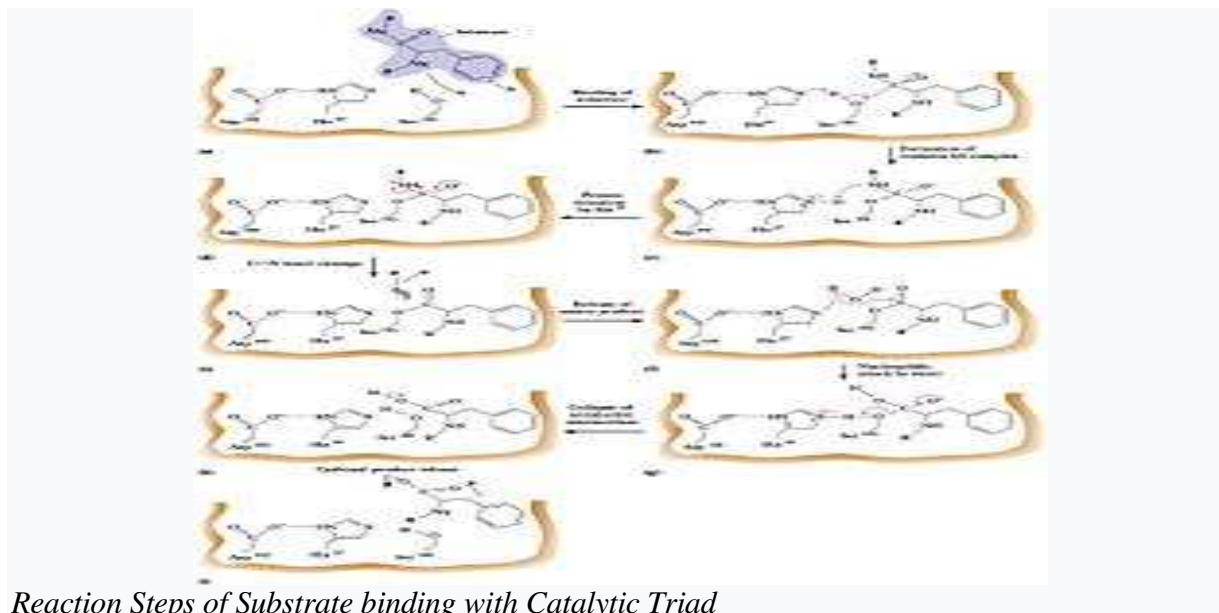
The active site of chymotrypsin is marked by serine 195. Serine lies in a small pocket on the surface of the enzyme. Serine is bonded to histidine 57 which is then bound to aspartate 102. All three of these residues are hydrogen bonded at this pocket. These three residues participate in concerted mechanisms that allows chymotrypsin and other proteases to be activated by incoming substrates. This is called the catalytic triad.

We know that serine is the final reactive site but serine actually depends on the histidine and aspartate residue to make it a good nucleophile. The histidine residue forces serine into a position that facilitates nucleophilic attack later on through the process of catalysis by approximation. In the presence of a substrate, a chain reaction occurs. First since aspartate is acidic, it will be deprotonated first by bases. Aspartate that flanks the histidine residue also provides it with favorable electrostatic effects and makes it a better proton acceptor. So after aspartate is deprotonated, proton transfer from histidine goes to aspartate. Now that histidine is deprotonated, it grabs the proton from serine's hydroxyl group. This creates a much more reactive alkoxide group on serine.

Now that the serine is activated we can proceed onto peptide hydrolysis. The alkoxide can attack an incoming substrate to form a tetrahedral intermediate. In this stage we form a resonating oxyanion hole which is a common motif in these kind of reactions. The oxyanion hole stabilizes the tetrahedral intermediate by distributing the negative charge around. Next comes the acyl-enzyme and eventually we see the release of the amine component and water binding.

The catalytic triad actually reveals a deep hydrophobic pocket where serine is sticking out in the center. This pocket positions incoming side chains of a substrate. There is a lot of specificity involved as chymotrypsin has a specific pocket with serine while other enzymes such as trypsin and elastase have different composition of pockets. Therefore we can now know that chymotrypsin likes large aromatic or long, nonpolar side chain.

Catalytic triads also exist in trypsin and elastase. Instead of serine, in trypsin, the center amino acid at the pocket is aspartate. Therefore, its pocket is specific to positively charged species of side chains. Elastase has a pocket that contains two residues of valine, which makes it very hard for big bulky side chains to enter the pocket; therefore, it favors small side chains. Trypsin and elastase are obviously homologs of chymotrypsin. They have 40% similarity in composition and have similar structures.



Reaction Steps of Substrate binding with Catalytic Triad

Site-Directed Mutagenesis helps us understand the Catalytic Triad

Site-directed mutagenesis can be used to test the involvement of individual amino acid residues to the catalytic influence of a protease. Each of the triad's residues has been converted to alanine. The cleaving ability of each mutant enzyme is examined. The conversion of active-site serine 221, aspartate 32, and histidine 64 into alanine reduces catalytic power. These results strongly support the fact that the catalytic triad, especially the serine-histidine pair, act together to generate a nucleophile that attacks the carbonyl carbon atom of a peptide bond. Site-directed mutagenesis can also tell us the importance of the oxyanion hole for catalysis. The mutation of asparagine 155 to glycine removes the side chain NH group reduced by the oxyanion hole. This shows that the NH group of asparagine residue helps to stabilize the tetrahedral intermediate and the following transition state.

Theories about Active Sites

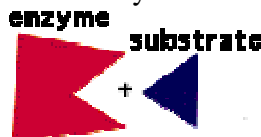
The enzyme's active site is the site at which the enzyme binds to the substrates and increases their chances of reacting.

There are two theories about how exactly an enzyme active site binds to substrates. These are:

The Lock-and-Key Model

- The lock-and-key model of enzyme active sites postulates that enzyme active sites are perfectly shaped to receive substrates and "pop" them into their new forms.
- In the picture below, the active site of the enzyme and the substrate have complementary shapes. This is so illustrated to indicate that the enzyme can recognize the substrate based, at least in part, on its shape.
- Secondly, it is meant to illustrate that the enzyme and substrate form a very close interaction.

- Thirdly, it shows that the substrate will preferentially bind to the active site and not to other sites on the enzyme.



Induced fit model

The matching between an enzyme's active site and the substrate isn't just like two puzzle pieces fitting together (though scientists once thought it was, in an old model called the "lock-and-key" model).

Instead, an enzyme changes shape slightly when it binds its substrate, resulting in an even tighter fit. This adjustment of the enzyme to snugly fit the substrate is called **induced fit**.

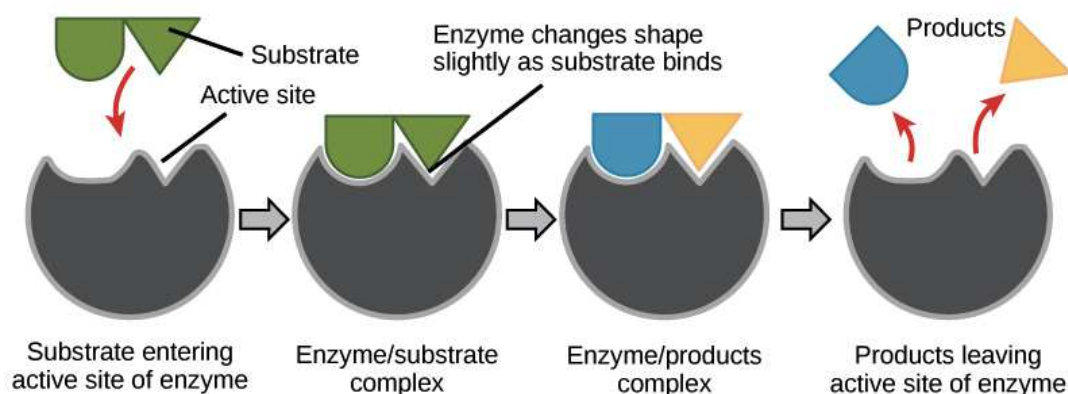


Illustration of the induced fit model of enzyme catalysis. As a substrate binds to the active site, the active site changes shape a little, grasping the substrate more tightly and preparing to catalyze the reaction. After the reaction takes place, the products are released from the active site and diffuse away.

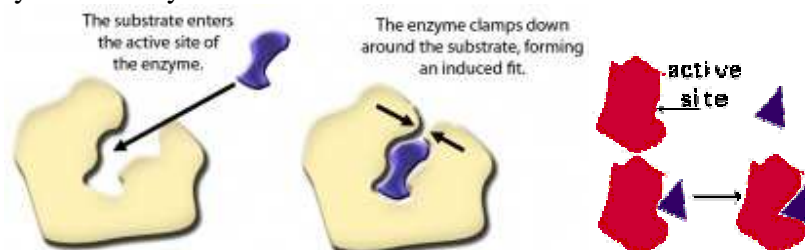
When an enzyme binds to its substrate, we know it lowers the activation energy of the reaction, allowing it to happen more quickly. But, you may wonder, what does the enzyme actually do to the substrate to make the activation energy lower?

The answer depends on the enzyme. Some enzymes speed up chemical reactions by bringing two substrates together in the right orientation. Others create an environment inside the active site that's favorable to the reaction (for instance, one that's slightly acidic or non-polar). The enzyme-substrate complex can also lower activation energy by bending substrate molecules in a way that facilitates bond-breaking, helping to reach the transition state.

Finally, some enzymes lower activation energies by taking part in the chemical reaction themselves. That is, active site residues may form temporary covalent bonds with substrate molecules as part of the reaction process.

An important word here is "temporary." In all cases, the enzyme will return to its original state at the end of the reaction—it won't stay bound to the reacting molecules. In fact, a hallmark

property of enzymes is that they aren't altered by the reactions they catalyze. When an enzyme is done catalyzing a reaction, it just releases the product (or products) and is ready for the next cycle of catalysis.



Induced Fit Enzyme Catalyst

A competing theory, the induced fit model, states that the active site and the substrate are not necessarily an ideal fit for each other in their resting states. Instead, as the substrate draws near to the enzyme, one or both undergo shape changes as a result of interacting with each other.

In this model, it is the continuing interaction of the binding site and the substrate that drive the substrate into its new formation. After the reaction has completed and new products are formed, the product and enzyme are no longer compatible and they separate.

Because active sites are finely tuned to help a chemical reaction happen, they can be very sensitive to changes in the enzyme's environment. Factors that may affect the active site and enzyme function include:

- **Temperature.** A higher temperature generally makes for higher rates of reaction, enzyme-catalyzed or otherwise. However, either increasing or decreasing the temperature outside of a tolerable range can affect chemical bonds in the active site, making them less well-suited to bind substrates. Very high temperatures (for animal enzymes, above 40°C or 104°F) may cause an enzyme to denature, losing its shape and activity.
- **pH.** pH can also affect enzyme function. Active site amino acid residues often have acidic or basic properties that are important for catalysis. Changes in pH can affect these residues and make it hard for substrates to bind. Enzymes work best within a certain pH range, and, as with temperature, extreme pH values (acidic or basic) can make enzymes denature.

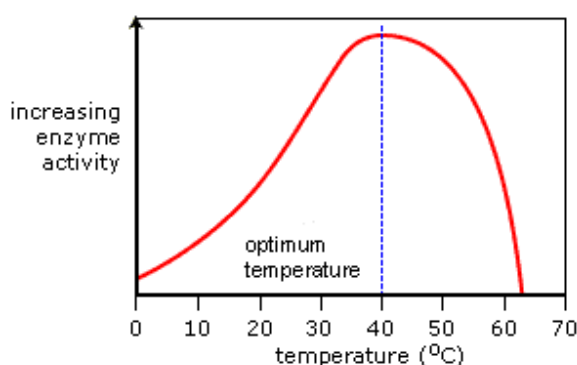
Factors affecting enzyme activity

- 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 molecules possess. In a fluid, this means that there are more random collisions between 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 Energy that molecules have, specifically in this case enzyme molecules, which puts strain on the bonds that hold them together.
- As temperature increases, more bonds, especially the weaker Hydrogen and 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, and more enzymes will be Denatured. This will decrease the rate of reaction.
- In summary, as temperature increases, initially the rate of reaction will increase, because of increased Kinetic Energy. However, the effect of bond breaking will become greater and greater, and the rate of reaction will begin to decrease.

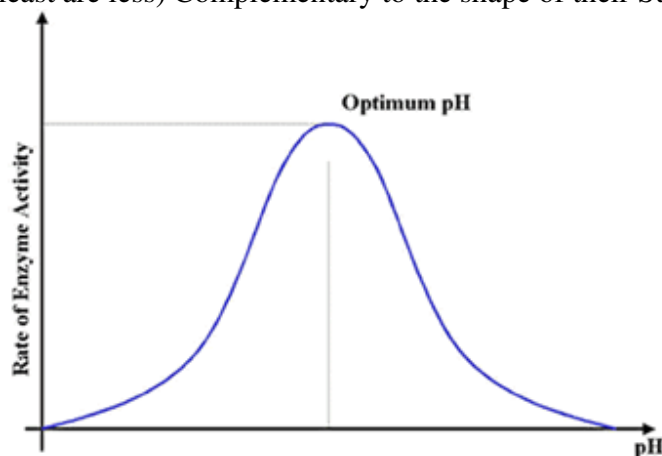


- The temperature at which the maximum rate of reaction occurs is called the enzyme's Optimum Temperature. This is different for different enzymes. *Most enzymes in the human body have an Optimum Temperature of around 37.0 °C.*

pH - Acidity and Basicity

- pH measures the Acidity and Basicity of a solution. It is a measure of the Hydrogen Ion (H^+) concentration, and therefore a good indicator of the Hydroxide Ion (OH^-) concentration. It ranges from pH1 to pH14. Lower pH values 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 and Ionic bonds that hold together an enzyme, since they will be attracted or repelled by the charges created by the bonds. This interference causes a change 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 the shape 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 a decrease 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 a permanent change to the enzyme, since the bonds can be reformed. However, extreme changes in pH can cause enzymes to Denature and permanently 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).*

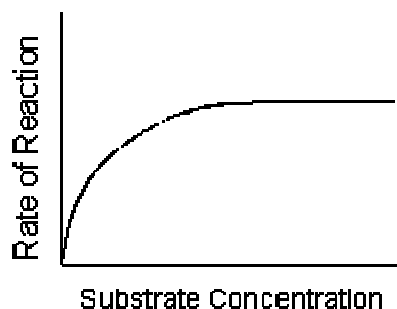
Concentration

- Changing the Enzyme and Substrate concentrations affect the rate of reaction of an enzyme-catalysed reaction. Controlling these factors in a cell is one way that an organism regulates its enzyme activity and so its Metabolism.
- 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 proceeding at a higher rate.
- If it is the limiting factor, increasing concentration will increase the rate of 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 and another factor will be limiting the maximum rate of reaction.
- As a reaction proceeds, the rate of reaction will decrease, since the Substrate will get used up. The highest rate of reaction, known as the Initial Reaction Rate is the maximum reaction rate for an enzyme in an experimental situation.

Substrate Concentration

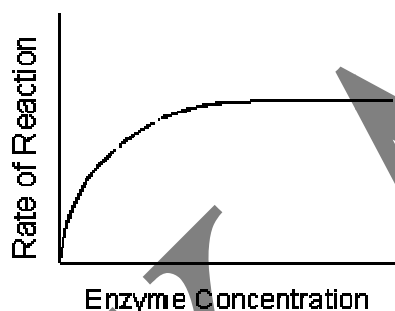
- Increasing Substrate Concentration increases the rate of reaction. This is because more substrate molecules will be colliding with enzyme molecules, so more product will be formed.

- However, after a certain concentration, any increase will have no effect on 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, as more 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.



Mechanism of enzyme action - Acid base catalysis (Chymotrypsin)

- very often-used mechanism in enzyme reactions, e.g., hydrolysis of ester/ peptide bonds, phosphate group reactions, addition to carbonyl groups, etc.
- Enzyme *avoids unstable charged intermediates in reaction* (which would have high free energies) by having groups appropriately located to
 - donate a proton (act as a *general acid*), or
 - accept a proton (abstract a proton, act as a *general base*)
- If a group *donates* a proton (acts as a general acid) in chemical mechanism, it has to *get a proton (a different one!) back* (act as a general base) by end of catalytic cycle, and vice versa.
- Protein functional groups that can function as general acid/base catalysts:
 - His imidazole
 - -amino group
 - -carboxyl group
 - thiol of Cys

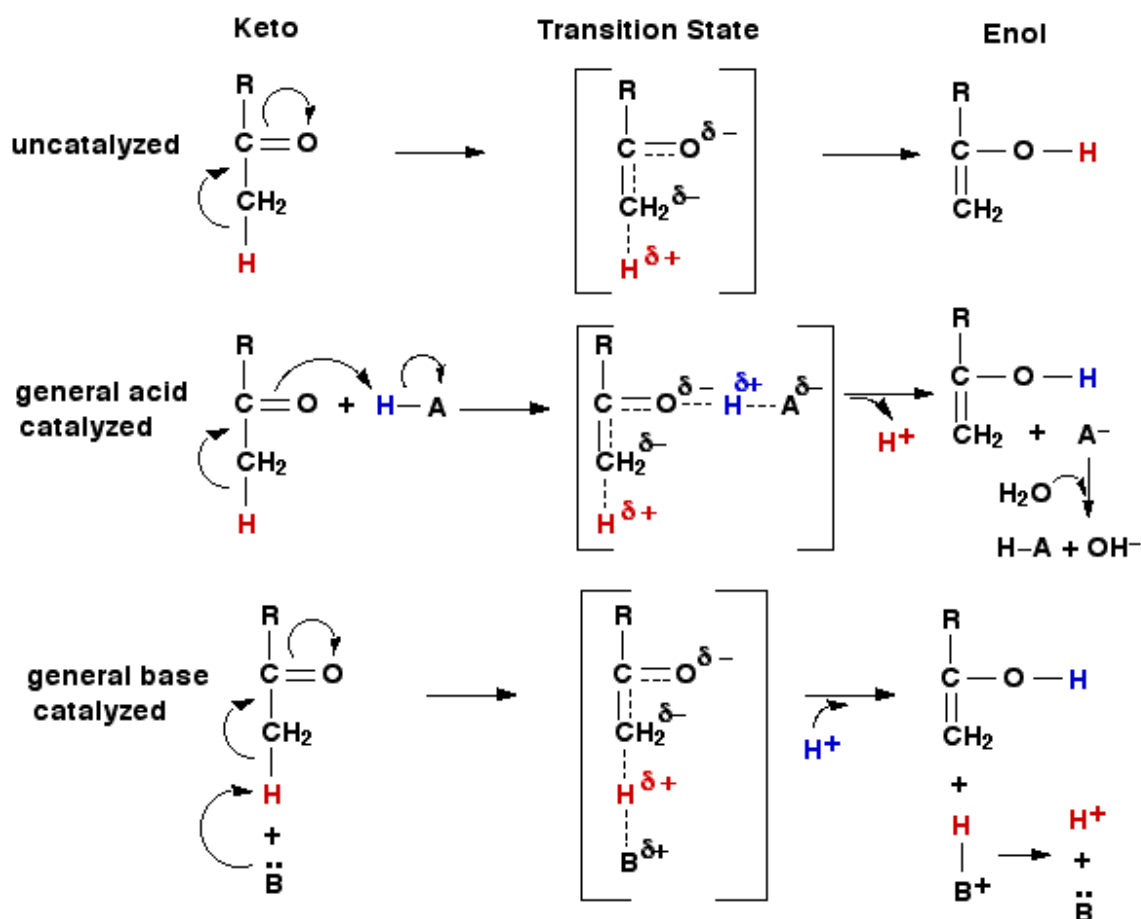
R group carboxyls of Glu, Asp

□-amino group of Lys

aromatic OH of Tyr

guanidino group of Arg

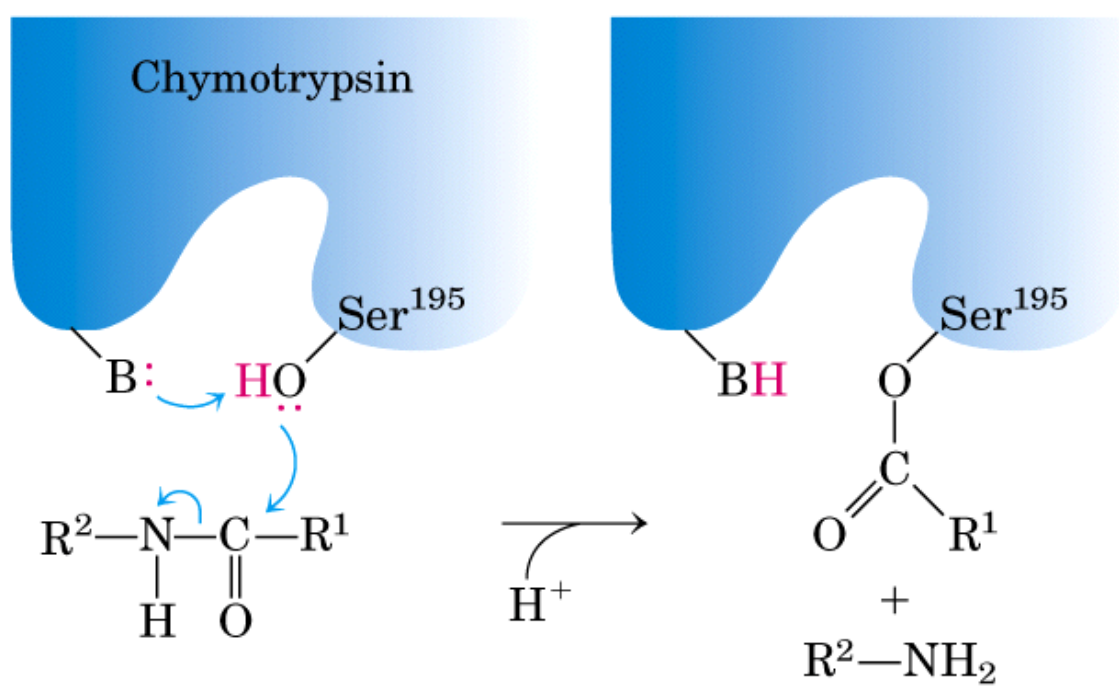
- Obviously, pH influences state of protonation of enzyme functional groups, so catalytic activity of enzymes using general acid-base catalysis is *sensitive to pH*.
- Example: Mechanisms of uncatalyzed, acid-catalyzed, and base-catalyzed keto-enol tautomerization (redrawn from Fig. 11-6, Voet, Voet & Pratt, *Fundamentals of Biochemistry*, 1999)
- General acid catalysis: partial proton transfer from an acid lowers the free energy of the high-free energy carbanion like transition state of the keto-enol tautomerization.
- Alternatively, the rate can be increased by partial proton abstraction by a base.
- *Concerted acid-base catalyzed reactions* involve both processes occurring simultaneously.



6. Covalent catalysis (also sometimes called nucleophilic catalysis):

- very often used in enzyme mechanisms

- rate enhancement by the transient formation of a catalyst-substrate covalent bond
- side chains of His, Cys, Asp, Lys and Ser can participate in covalent catalysis by acting as nucleophiles
- coenzymes pyridoxal phosphate and thiamine pyrophosphate function mainly by covalent catalysis
- Example of covalent catalysis coupled to general acid-base catalysis:
Fig: First step in chymotrypsin-catalyzed peptide bond hydrolysis -- *general base catalyzed formation of covalent acyl-enzyme intermediate*



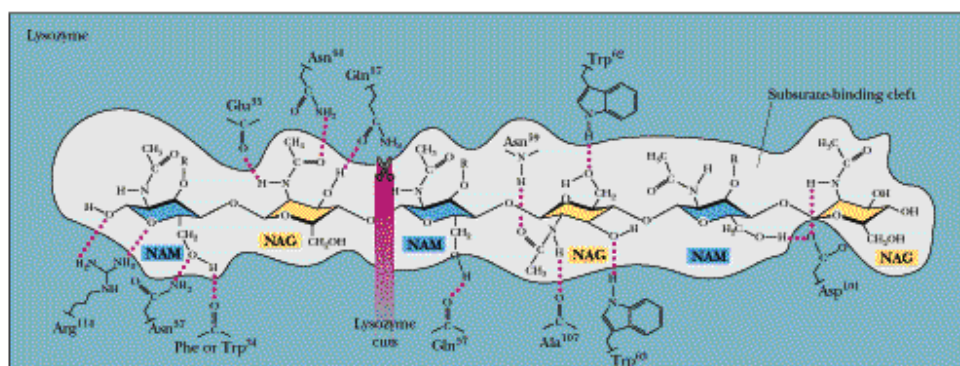
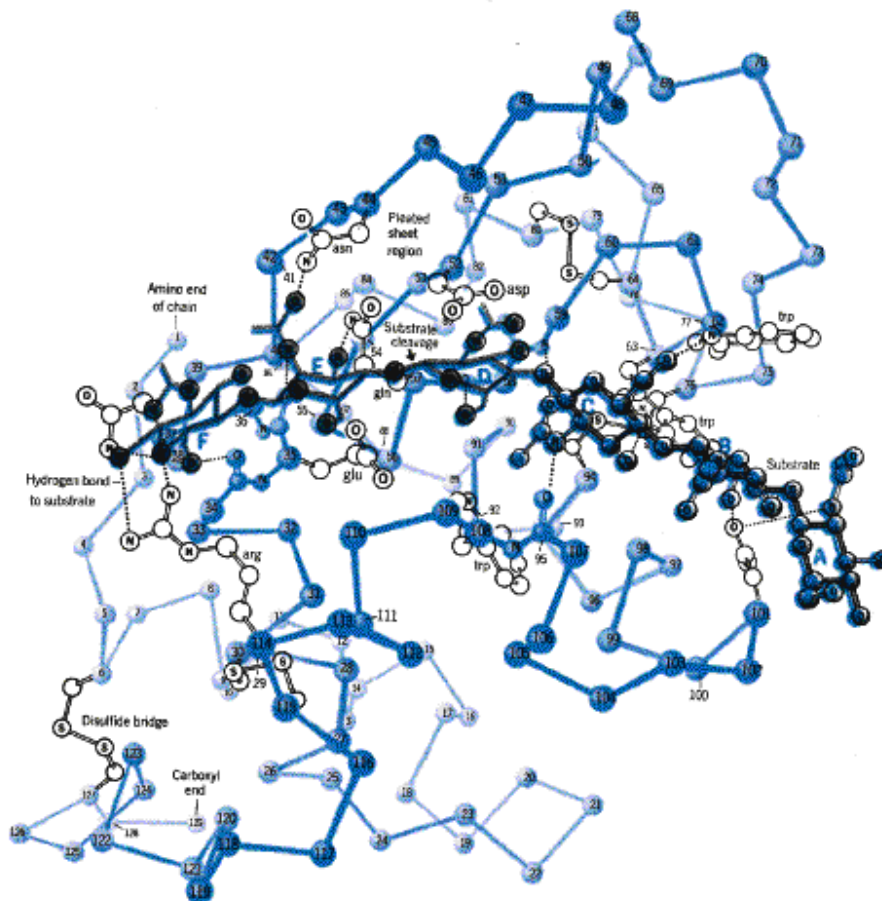
Covalent catalysis (lysozyme)

Lysozyme catalyzes the hydrolysis of the $\beta(1 \rightarrow 3)$ linkage between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) in bacterial cell wall polysaccharides and also the $\beta(1 \rightarrow 4)$ -linked poly NAG in chitin.

- 6 sugar residues of the polysaccharide substrate fit into the active site as shown in the figure below (structure of lysozyme and of its active site) -- cleavage is between the 4th and 5th residues *from the right* in active site diagram at bottom of figure.
- 4th sugar residue (adjacent to bond to be cleaved) has to be distorted out of its preferred chair conformation to fit into active site, and the inference is that the distorted structure is closer to the structure of the transition state for hydrolysis -- enzyme uses some of the favorable binding

energy from binding the *rest* of the substrate structure to raise the substrate closer to the transition state, *stabilizing the transition state relative to the ES complex*.

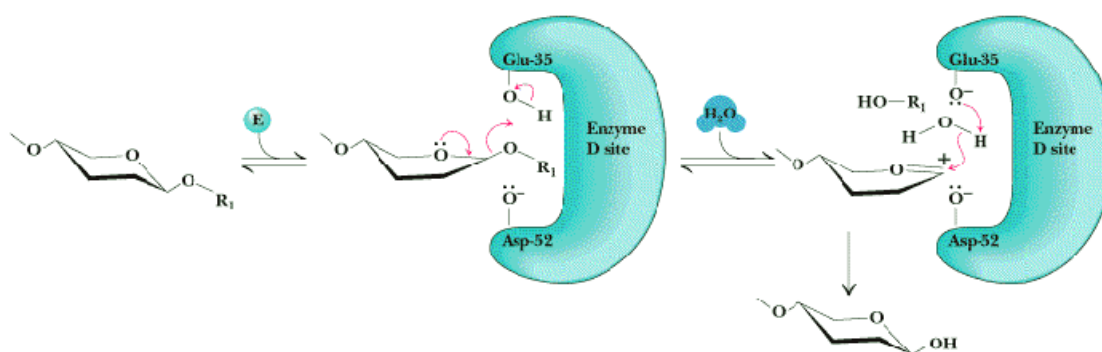
- Fig: Structure and active site of lysozyme



- Hydrolysis reaction is accelerated also by participation of Glu³⁵ and Asp⁵².

- Bell-shaped pH-activity profile for lysozyme suggests involvement of ionizable groups with pK_a values of about 5.9 and 4.5.
- Glu³⁵ is in a nonpolar environment and has a higher than expected pK_a , and has to be *protonated* at start of reaction because it first must *donate* a proton to the oxygen of the leaving group from hydrolysis (an alcohol), and subsequently accepts a proton from H₂O, the 2nd substrate (see figure below).
- Asp⁵² has a lower pK_a , 4.5, close to expected value for an R group carboxyl, and it must be *unprotonated* for activity because it's required to stabilize carbonium ion intermediate (+) formed when the alcohol leaves, until carbonium ion can react with H₂O, completing hydrolysis reaction:

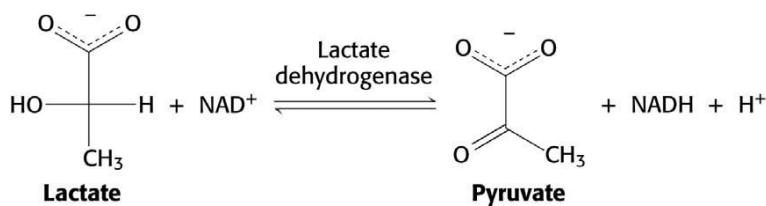
Fig. Lysozyme mechanism



- The lysozyme mechanism illustrates
 - Transition state stabilization
 - Acid-base catalysis
 - Electrostatic catalysis
 - Several residues in the protein participate in substrate binding.
 - The binding of NAM4 in the chair conformation is unfavorable.
 - But the binding of residue 4 in the half-chair conformation is favorable (preferential transition state binding).
- Hydrolysis involves acid-base catalysis:
 - Glu35 serves as a proton donor to the oxygen of the leaving alcohol.
 - The resulting carbonium ion (+) is stabilized by the ionized side chain of Asp52 (electrostatic catalysis) until it can react with water.
 - (lysozyme)
 - Many glycosidases utilize a covalent intermediate in their mechanism. Here is a good example of transition state stabilization using a bacterial glycosidase.
 - Another excellent example that clearly shows how transition state stabilization works is found in the structure of this transferase.

Isolation, purification and Characterization of Enzyme

Assay: a biochemical or biological means used to monitor the presence of the target protein. For example, enzyme activity, specific label, functional properties such as binding.



Homogenization: First step, disrupting cells.

Fractionation: Crude separation of homogenate into fractions.

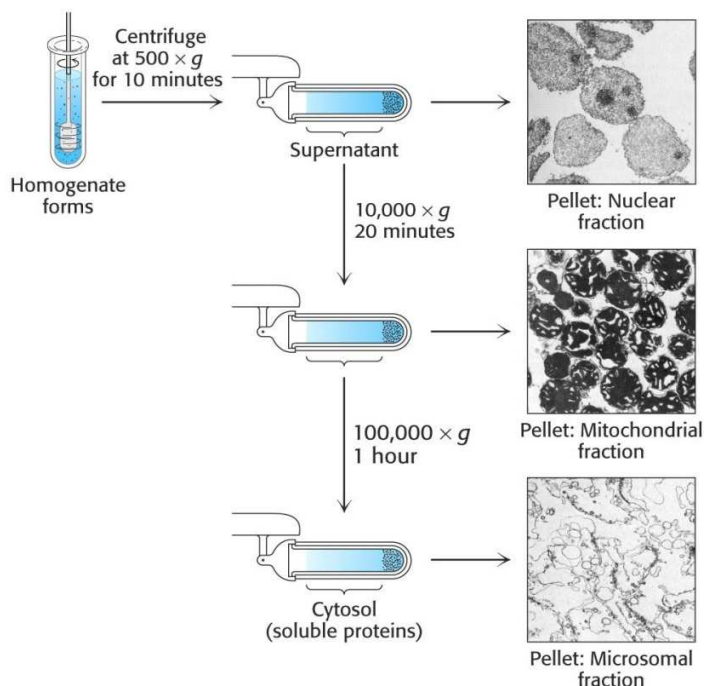
Homogenization

Dounce Homogenizer Sonication
Osmotic shock French Press

Fractionation

Fractional precipitation (pH, heat) Ammonium Sulfate Precipitation Differential Centrifugation
Soluble, membrane bound, vesicle bound, insoluble Enzymes

1 Bacteria, mammalian, plant, yeast cells 2 detergent and/enzymatic lysis
3 protease and phosphatase inhibitors Cell lysis,



The methods used in protein purification, can roughly be divided into analytical and preparative methods.

The distinction is not exact, but the deciding factor is the amount of protein, that can practically be purified with that method. Analytical methods aim to detect and identify a protein in a mixture, whereas preparative methods aim to produce large quantities of the protein for other purposes, such as structural biology or industrial use. In general, the preparative methods can be used in analytical applications, but not the other way around.

Method # 1. Extraction:

Depending on the source, the protein has to be brought into solution by breaking the tissue or cells containing it. There are several methods to achieve this; Repeated freezing and thawing, sonication, homogenization by high pressure or permeabilization by organic solvents. The method of choice depends on how fragile the protein is and how sturdy the cells are.

After this extraction process soluble protein will be in the solvent, and can be separated from cell membranes, DNA, etc. by centrifugation. The extraction process also extracts proteases, which will start digesting the proteins in the solution. If the protein is sensitive to proteolysis, it is usually desirable to proceed quickly, and keep the extract cooled, to slow down proteolysis.

Method # 2. Precipitation and Differential Solubilisation:

In bulk protein purification, a common first step to isolate proteins is precipitation with ammonium sulphate $(\text{NH}_4)_2\text{SO}_4$. This is performed by adding increasing amounts of ammonium sulphate and collecting the different fractions of precipitate protein. One advantage of this method is that it can be performed inexpensively with very large volumes.

The first proteins to be purified are water-soluble proteins. Purification of integral membrane proteins requires disruption of the cell membrane in order to isolate any one particular protein from others that are in the same membrane compartment. Sometimes a particular membrane fraction can be isolated first, such as isolating mitochondria from cells before purifying a protein located in a mitochondrial membrane.

A detergent such as sodium dodecyl sulphate (SDS) can be used to dissolve cell membranes and keep membrane proteins in solution during purification; however, because SDS causes denaturation, milder detergents such as Triton X-100 or CHAPS can be used to retain the protein's native conformation during purification.

Method # 3. Ultracentrifugation:

Centrifugation is a process that uses centrifugal force to separate mixtures of particles of varying masses or densities suspended in a liquid. When a vessel (typically a tube or bottle) containing a mixture of proteins or other particulate matter, such as bacterial cells, is rotated at high speeds, the angular momentum yields an outward force to each particle that is proportional to its mass.

The tendency of a given particle to move through the liquid because of this force is offset by the resistance the liquid exerts on the particle. The net effect of "spinning" the sample in a centrifuge is that massive, small, and dense particles move outward faster than less massive particles or particles with more "drag" in the liquid.

When suspensions of particles are "spun" in a centrifuge, a "pellet" may form at the bottom of the vessel that is enriched for the most massive particles with low drag in the liquid. The remaining, non-compacted particles still remaining mostly in the liquid are called the "supernatant" and can be removed from the vessel to separate the supernatant from the pellet.

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The rate of centrifugation is specified by the angular acceleration applied to the sample, typically measured in comparison to the g. If samples are centrifuged long enough, the particles in the vessel will reach equilibrium wherein the particles accumulate specifically at a point in the vessel where their buoyant density is balanced with centrifugal force. Such an “equilibrium” centrifugation can allow extensive purification of a given particle.

Sucrose gradient centrifugation:

A linear concentration gradient of sugar (typically sucrose glycerol, or Percoll) is generated in a tube such that the highest concentration is on the bottom and lowest on top. A protein sample is then layered on top of the gradient and spun at high speeds in an ultracentrifuge. This causes heavy macromolecules to migrate towards the bottom of the tube faster than lighter material.

During centrifugation in the absence of sucrose, as particles move farther and farther from the centre of rotation, they experience more and more centrifugal force (the further they move, the faster they move). The problem with this is that the useful separation range within the vessel is restricted to a small observable window.

Spinning a sample twice as long does not mean the particle of interest will go twice as far; in fact, it will go significantly farther. However when the proteins are moving through a sucrose gradient, they encounter liquid of increasing density and viscosity.

A properly designed sucrose gradient will counteract the increasing centrifugal force, so the particles move in close proportion to the time they have been in the centrifugal field. Samples separated by these gradients are referred to as “rate zonal” centrifugations. After separating the protein/particles, the gradient is then fractionated and collected.

Method # 4. Chromatographic Methods:

Usually a protein purification protocol contains one or more chromatographic steps. The basic procedure in chromatography is to flow the solution containing the protein through a column packed with various materials. Different proteins interact differently with the column material, and can thus be separated by the time required to pass the column, or the conditions required to elute the protein from the column. Usually proteins are detected as they are coming off the column by their absorbance at 280 nm.

Many different chromatographic methods exist:

1. Size Exclusion Chromatography:

Chromatography can be used to separate protein in solution or denaturing conditions by using porous gels. This technique is known as size exclusion chromatography. The principle is that smaller molecules have to traverse a larger volume in a porous matrix. Consequentially, proteins of a certain range in size will require a variable volume of eluant (solvent) before being collected at the other end of the column of gel.

In the context of protein purification, the eluant is usually pooled in different test tubes. All test tubes containing no measurable trace of the protein to purify are discarded. The remaining solution is thus made of the protein to purify and any other similarly-sized proteins.

2. Ion Exchange Chromatography:

Ion exchange chromatography separates compounds according to the nature and degree of their ionic charge. The column to be used is selected according to its type and strength of charge. Anion exchange resins have a positive charge and are used to retain and separate negatively

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charged compounds, while cation exchange resins have a negative charge and are used to separate positively charged molecules.

Before the separation begins a buffer is pumped through the column to equilibrate the opposing charged ions. Upon injection of the sample, solute molecules will exchange with the buffer ions as each competes for the binding sites on the resin. The length of retention for each solute depends upon the strength of its charge.

The most weakly charged compounds will elute first, followed by those with successively stronger charges. Because of the nature of the separating mechanism, pH, buffer type, buffer concentration, and temperature all play important roles in controlling the separation. Ion exchange chromatography is a very powerful tool for use in protein purification and is frequently used in both analytical and preparative separations.

3. Affinity Chromatography:

Affinity Chromatography is a separation technique based upon molecular conformation, which frequently utilizes application specific resins. These resins have ligands attached to their surfaces which are specific for the compounds to be separated. Most frequently, these ligands function in a fashion similar to that of antibody-antigen interactions. This “lock and key” fit between the ligand and its target compound makes it highly specific, frequently generating a single peak, while all else in the sample is un-retained.

Many membrane proteins are glycoproteins and can be purified by lectin affinity chromatography. Detergent solubilized proteins can be allowed to bind to a chromatography resin that has been modified to have a covalently attached lectin.

Proteins that do not bind to the lectin are washed away and then specifically bound glycoproteins can be eluted by adding a high concentration of a sugar that competes with the bound glycoproteins at the lectin binding site. Some lectins have high affinity binding to oligosaccharides of glycoproteins that is hard to compete with sugars, and bound glycoproteins need to be released by denaturing the lectin.

4. Metal Binding:

A common technique involves engineering a sequence of 6 to 8 histidines into the C-terminal of the protein. The polyhistidine binds strongly to divalent metal ions such as nickel and cobalt. The protein can be passed through a column containing immobilized nickel ions, which binds the polyhistidine tag. All untagged proteins pass through the column.

The protein can be eluted with imidazole, which competes with the polyhistidine tag for binding to the column, or by a decrease in pH (typically to 4.5), which decreases the affinity of the tag for the resin. While this procedure is generally used for the purification of recombinant proteins with an engineered affinity tag (such as a 6xHis-tag or Clontech's HAT tag), it can also be used for natural proteins with an inherent affinity for divalent cations.

5. Immunoaffinity Chromatography:

Immunoaffinity chromatography uses the specific binding of an antibody to the target protein to selectively purify the protein. The procedure involves immobilizing an antibody to a column material, which then selectively binds the protein, while everything else flows through. The protein can be eluted by changing the pH or the salinity. Because this method does not involve engineering in a tag, it can be used for proteins from natural sources.

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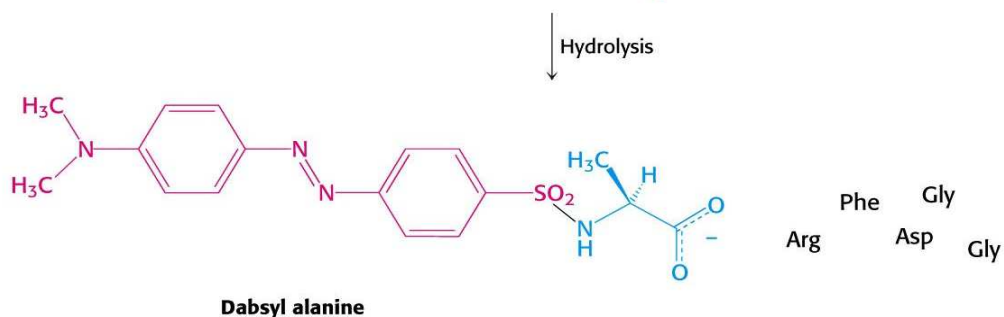
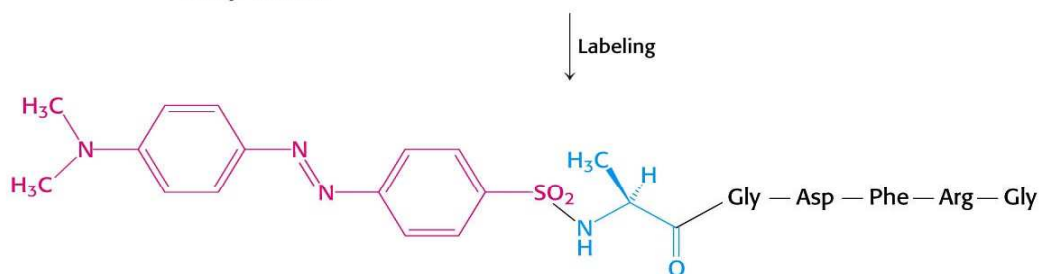
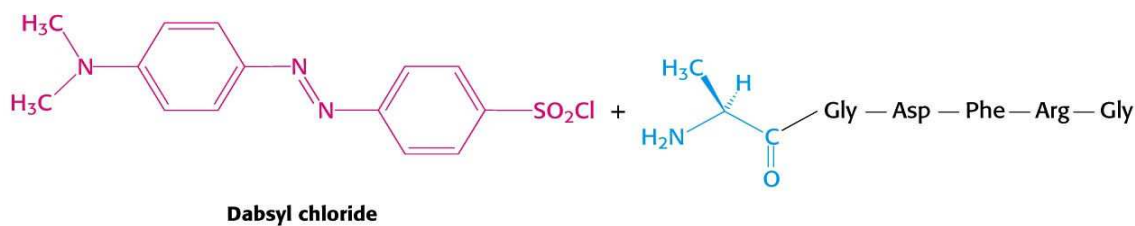
6. HPLC:

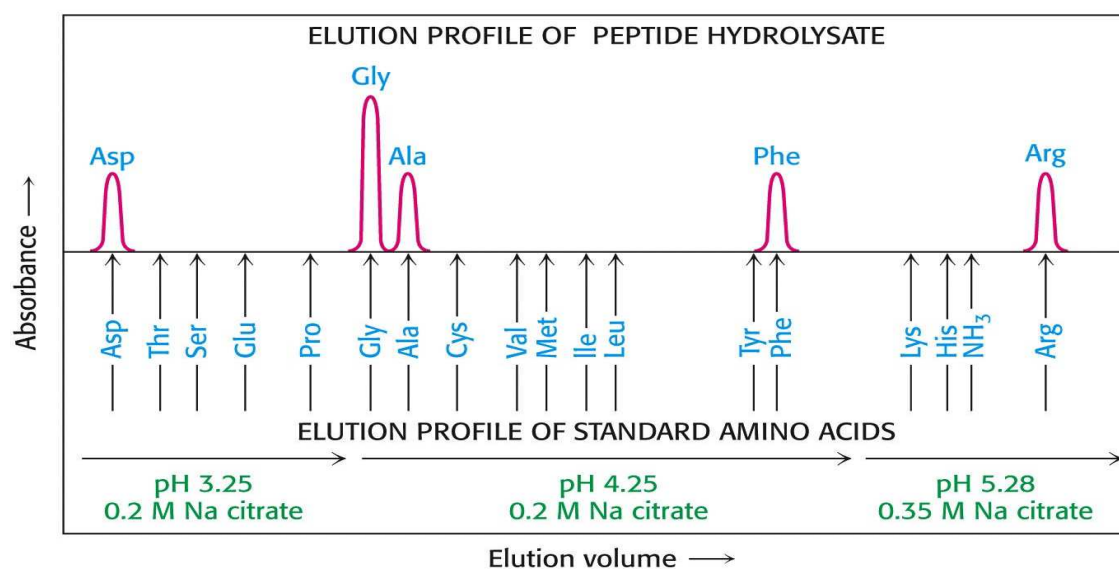
High performance liquid chromatography or high pressure liquid chromatography is a form of chromatography applying high pressure to drive the solutes through the column faster. This means that the diffusion is limited and the resolution is improved. The most common form is “reversed phase” HPLC, where the column material is hydrophobic.

The proteins are eluted by a gradient of increasing amounts of an organic solvent, such as acetonitrile. The proteins elute according to their hydrophobicity. After purification by HPLC the protein is in a solution that only contains volatile compounds, and can easily be lyophilized. HPLC purification frequently results in denaturation of the purified proteins and is thus not applicable to proteins that do not spontaneously refold.

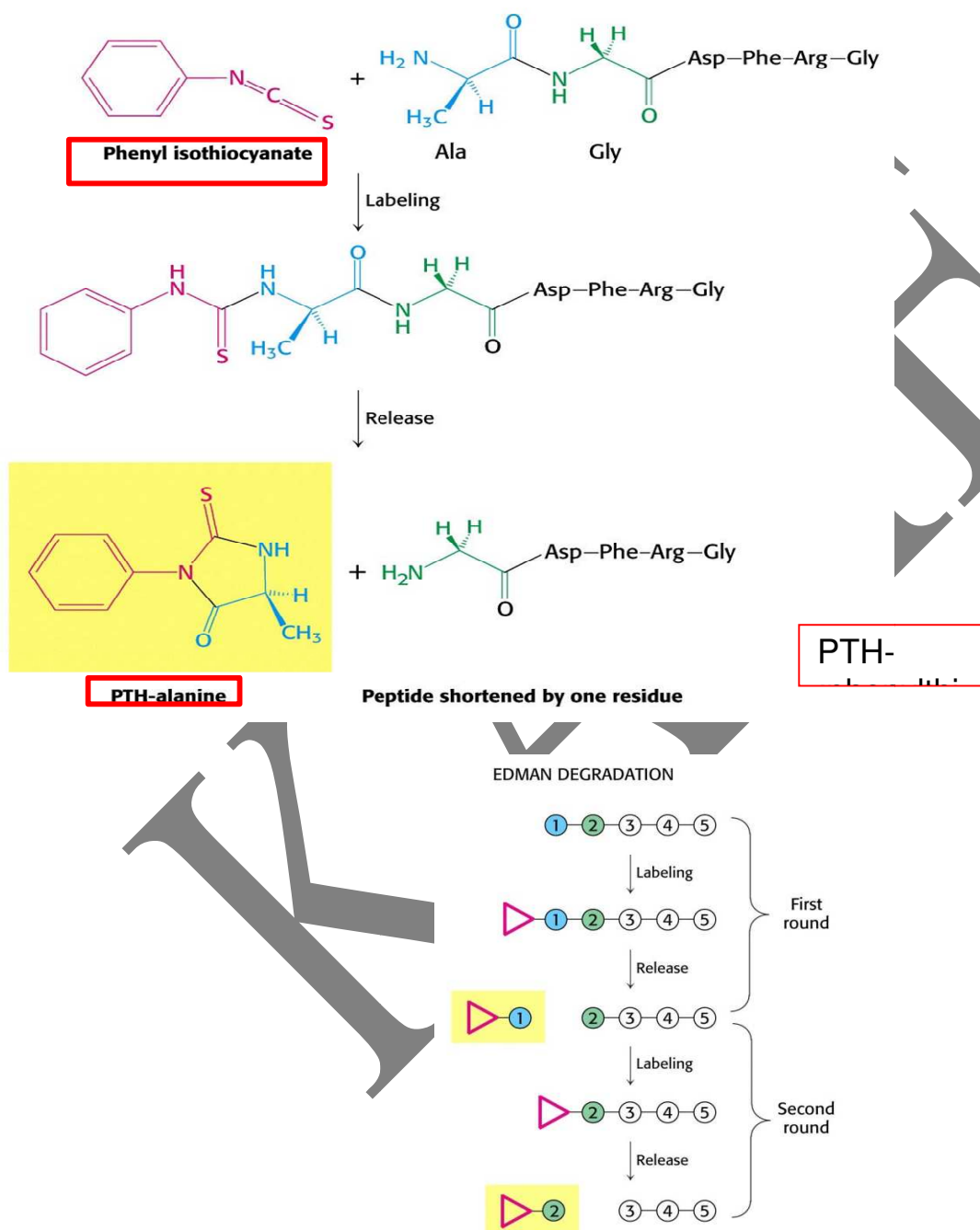
Primary sequence determination

Amino acid composition

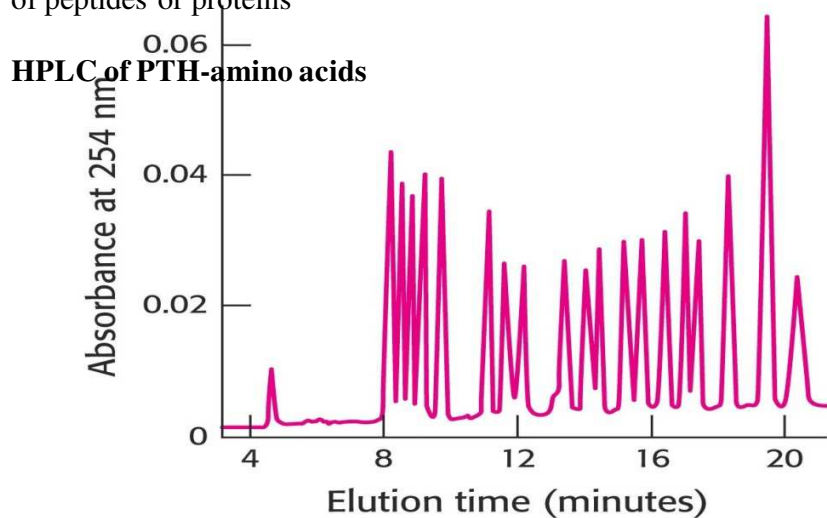




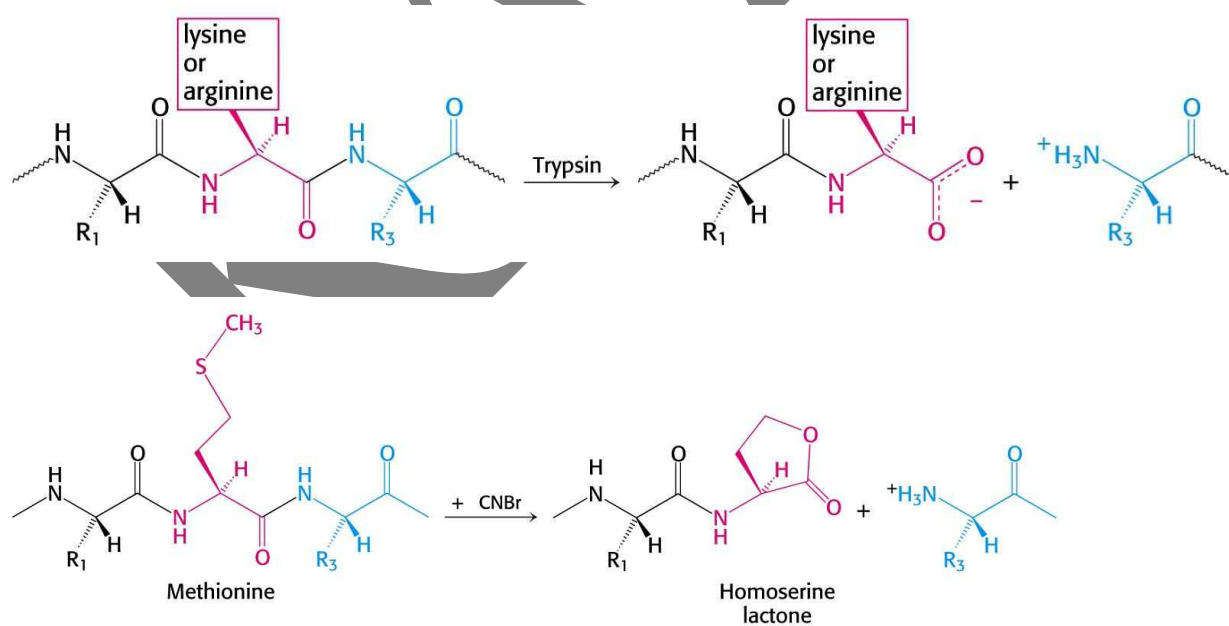
Edman degradation reaction



Many cycles of Edman degradation reveals the peptide sequence. Peptides of 50 amino acid residues can be sequenced easily. The gas-phase sequencer can analyze picomole quantities of peptides or proteins



Cleavage of polypeptides



Metal activated and metalloenzymes

Enzymes that require metal ions for their catalytic activity fall into two classes. They are the metal-activated enzymes and the metalloenzymes. The latter contain tightly bound metals that do not dissociate during isolation or dialysis of the enzyme under conditions where activity is retained. However, such metal ions can be removed under more drastic conditions, such as low pH. Bound metal ions can be involved with the maintenance of the structural integrity of enzymes, and they can participate in electrophilic catalysis.

Metal ions that are found in metalloenzymes include those of the first transition series of elements in the periodic table:

$Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}$ and Cu^{2+} , as well as Zn^{2+}, Mo^{2+} and Ca^{2+} . Examples of enzymes that contain metal ions are listed in Table 1. Metal ions involved with enzymes that participate in electron transport undergo redox reactions. Thus, the ionic forms of iron, copper, cobalt, and molybdenum can be $Fe^{2+}/Fe^{3+}, Cu^{+}/Cu^{2+}, Co^{2+}/Co^{3+}$, and Mo^{2+} to Mo^{6+} , respectively.



For convenience, these metal ions will be listed simply as bivalent ions. Fe is most commonly found as a heme complex in redox enzymes such as catalase and peroxidases (see Iron-Binding Enzymes). It also occurs as a component of iron-sulfur clusters in enzymes that are involved in one-electron transfer processes; *NADH dehydrogenase* and *succinate dehydrogenase* belong to

this group and are flavoEnzyme enzymes. Like Fe^{2+}, Cu^{2+} has multivalent oxidation states, and many Cu enzymes are either *oxidases* or *hydrolases* that utilize molecular oxygen. Co enzymes, such as *methylmalonyl-CoA mutase* and *ribonucleotide reductase*, have the cobalt atom bound within a corrin ring. Ni is rarely found as a component of metalloenzymes, but *urease* from jack bean is an exception. The occurrence of Mn and Ca in metalloenzymes is also somewhat rare (see Calcium-Binding Enzymes). Mg, the alkaline earth metal that is found so commonly in biological systems, does not play a role in the functioning of metalloenzymes, but is important in metal- activated enzymes (see below). By contrast, Zn is an important and widely utilized metal for electrophilic catalysis (see Zinc-Binding Enzymes). Not all enzymes that catalyze a particular reaction have the same requirement for a metal. Thus, fructose biphosphate aldolase from yeast and bacteria utilize Zn ions, whereas the same enzyme from muscle uses a Schiff Base intermediate to activate the substrate (1).

Table 1. Selected Examples of Metalloenzymes

Metal Ion	Enzyme
Ca^{2+}	amylase, galactosyltransferase, thermolysin

Co^{2+}	dioldehydrase, glycerol dehydratase, methylmalonyl-CoA mutase, ribonucleotide reductase
Cu^{2+}	cytochrome c oxidase, dopamine- β -hydroxylase, superoxide dismutase
Fe^{2+}	catalase, NADH dehydrogenase, nitrogenase, peroxidase, succinate dehydrogenase, xanthine oxidase
Mn^{2+}	arginase, histidine-ammonia lyase, pyruvate carboxylase
Mo^{2+}	nitrogenase, xanthine oxidase
Ni^{2+}	urease, Ni-Fe hydrogenase
Zn^{2+}	alcohol dehydrogenase, carbonic anhydrase, carboxypeptidase, superoxide dismutase, thermolysin

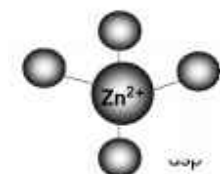
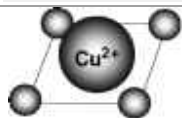
The largest group of metal-activated enzymes contains the *phosphotransferases* that catalyze the transfer of the terminal phosphoryl group of ATP to an acceptor molecule that can be an alcohol, carboxylic acid, nitrogenous compound, or a phosphorylated compound (see Kinase). Their essential requirement for a bivalent metal ion is always satisfied by Mg or Mn. However, other bivalent metal ions have been shown to activate some *phosphotransferases*. The role of bivalent metal ions in the activation of *phosphotransferases* is to form a MgATP complex that then acts as the true substrate for the reaction. Thus, the binary complex formed by the interaction of the enzyme and its nucleotide substrate is an enzyme-nucleotide-metal complex. Some *phosphotransferases* involve a second metal ion that is liganded by the enzyme as well as the substrate. Examples are *pyruvate kinase* and the biotin-containing enzymes that form carboxybiotin by the initial phosphorylation of bicarbonate to carboxy-phosphate. *Pyruvate kinase* also differs from most other *phosphotransferases* in its requirement for K and its inhibition, rather than activation, by Ca.

Enzymes that depend on metal ions as cofactors fall into two categories: metal-activated enzymes and metalloenzymes. As the name implies, metal-activated enzymes are prompted to greater catalytic activity by the presence of a mono- or divalent metal ion exterior to the Enzyme (in the assay medium). The metal may activate the substrate (e.g., Mg^{2+} with ATP), engage the enzyme directly, or enter into equilibrium with the enzyme exploiting its ionic charge to render a more favorable substrate binding or catalytic environment. Therefore, metal-activated enzymes require the metal to be present in excess, perhaps 2-10 times more than the enzyme concentration. Because the metal cannot be bound in a more permanent way, metal-activated enzymes typically lose activity during purification. An example is pyruvate kinase, which has a specific requirement for K^{+} and is inactivated by dialysis (diffusion through a semiporous membrane). Other examples of metal-activated enzymes are shown in Table 2.

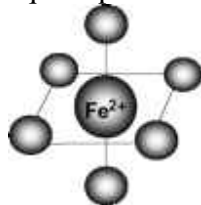
Metalloenzymes, in contrast, have a metal cofactor bound firmly to a specific region on the Enzyme surface. Some may even require more than one metal ion and in rare instances could be two different metals as, for example, in Cu_2Zn_2 superoxide dismutase. With few exceptions, trace metals fit into the picture as cofactors for metalloenzymes. Fe, Zn, Cu, and Mn, referred to as first transition series metals, are the most common. Their counterparts,

Table 2 Metal-activated enzymes and metalloenzymes		
Metal or metal cofactor	Enzyme	Function
Metal-activated enzymes		
K^{+}	Pyruvate kinase	Synthesize pyruvate
Mg^{2+}	Hexokinase	Phosphorylate
		glucose
	DNase	Cleave DNA
	RNase	Cleave RNA
	ATPase	Cleave ATP
Metalloenzymes		
Cu^{2+} , Zn^{2+}	Superoxide	Destroy superoxide
	dismutase	anion
Fe	Catalase	Destroy H_2O_2
Zn	Alcohol	Metabolize alcohol
	dehydrogenase	
	DNA polymerase	Synthesize DNA
Mn	Pyruvate	Synthesize
	carboxylase	oxaloacetate
	Arginase	Synthesize urea
Ca	Alpha amylase	Cleave glycogen,

	starch
--	--------



Square planar dsp^2
Square planar dsp^2



Octahedral d^2sp^3
Tetrahedral dsp^3

Figure 1 Some common geometries of metal complexes.

Mg, K, Ca, and Na, are not considered 'trace' and only in rare instances are these so-called macroelements strongly bound to the surface of enzymes. Tight binding precludes loss of the metal ion by dialysis or loss to weakly dissociating agents. Metalloenzymes, however, can lose their metal cofactor and hence be rendered inactive when treated with metal chelators that have a stronger binding affinity than the enzyme and out compete the enzyme for the metal ion. As prosthetic groups, metals in metalloenzymes have a stoichiometric relationship (metal ion-enzyme ratio) represented by a whole integer. Metalloenzymes seldom are primed to greater activity by adding its conjugate metal ion to the enzyme. Spatial geometry is also a concern. Metals in the first transition series metals (Mn, Fe, Co, Ni, Cu, Zn) must adhere to strict geometric configurations around the metal-binding site. Examples of the more common geometrical arrangements are shown in Figure 1. For metals in the first transition series one takes note of the 3d and 4s orbitals in assigning valence states and likely geometric shapes. Apart from those with Zn, enzymes with first transition series metals tend to be highly colorful; for example, the beautiful red color of hemoglobin (iron) or the blue color of ceruloplasmin (whose name means heavenly blue) associated with copper. Table 3 gives some examples of metalloenzymes and the specific metal each requires.

POSSIBLE QUESTIONS
UNIT – I

Two Marks

1. Define coenzymes.
2. What is an active site?
3. What is a catalytic triad?
4. What are the techniques used for to isolate enzymes?
5. What are the techniques used for to purify enzymes?
6. Explain lock and key hypothesis.
7. Define cofactor.
8. Name the classification of enzymes.

Essay type Questions

1. Discuss on coenzymes and co factor.
2. Write short notes on lock and key model and induced fit model.
3. Explain in detail about isolation, purification and characterization of enzymes.
4. Write short notes on active site and catalytic triad.
5. Comment on acid base and covalent catalysis.
6. Comment on nomenclature and classification of enzymes.
7. Explain the factors affecting enzyme activity in detail.
8. Explain about metal activated and metalloenzymes.
9. Comment on mechanism of enzyme action.

KARPAGAM ACADEMY OF HIGHER EDUCATION
DEPARTMENT OF BIOCHEMISTRY
I-M.Sc., BIOCHEMISTRY
ENZYMES AND MICROBIAL TECHNOLOGY (19BCP102)
MULTIPLE CHOICE QUESTIONS
UNIT I

S.No	Questions	Option A	Option B	Option C	Option D	Answer
1	Fischer's 'lock and key' model of the enzyme action implies that	The active site is complementary in shape to that of substance only after interaction.	The active site is complementary in shape to that of substance	Substrates change conformation prior to active site interaction	The active site is flexible and adjusts to substrate	The active site is complementary in shape to that of substance
2	An inducer is absent in the type of enzyme:	Allosteric enzyme	Constitutive enzyme	Co-operative enzyme	Isoenzymic enzyme	Constitutive enzyme
3	In reversible non-competitive enzyme activity inhibition	Vmax is increased	Km is increased	Km is decreased	Concentration of active enzyme is reduced	Concentration of active enzyme is reduced
4	Enzyme involved in joining together two substrates is	Glutamine synthetase	Aldolase	Gunaine deaminase	Arginase	Glutamine synthetase
5	The pH optima of most of the enzymes is	Between 2 and 4	Between 5 and 9	Between 8 and 12	Above 12	Between 5 and 9
6	Coenzymes are	Heat stable, dialyzable, non protein organic molecules	Soluble, colloidal, protein molecules	Structural analogue of enzymes	Different forms of enzymes	Heat stable, dialyzable, non protein organic molecules
7	An example of group transferring coenzyme is	NAD+	NADP+	FAD	CoA	CoA
8	An example of hydrogen transferring coenzyme is	CoA	NAD+	Biotin	TPP	NAD+
9	Coccarboxylase is	Thiamine pyrophosphate	Pyridoxal phosphate	Biotin	CoA	Biotin
10	Factors affecting enzyme activity:	Concentration	pH	Temperature	All options	All options
11	Alkaline phosphatase is present in	Liver	Bones	Intestinal mucosa	All options	All options

12	All of the following are zinc-containing enzymes except	Acid Phosphatase	Alkaline Phosphatase	Carbonic anhydrase	RNA polymerase	Acid Phosphatase
13	Which binding can provide more permanent linkages between enzyme and carrier	Physical adsorption	Ionic binding	Covalant binding	Peptide binding	Covalant binding
14	In Un competitive inhibitor	Increases Km and decreases Vmax value of the enzyme	Decreases both Km and Vmax value of the enzyme	Increases Vmax value of the enzyme	Increases Km value	Decreases both Km and Vmax value of the enzyme
15	Some enzymes act on closely related substrates which is known as	Relative substrate specificity	Broad specificity	Reaction specificity	Stereo specificity	Broad specificity
16	Coenzymes are often regarded as	First substrate	Second substrate	Third substrate	Fourth substrate	Second substrate
17	A nonprotein compound identified to bring catalysis in biological system.	DNA	RNA	Lipids	Carbohydrates	RNA
18	The ligands that were involved in regulation of enzyme activity is	Substrate	Effectors	Activators	None of the above	Effectors
19	Many enzymes require a non protein component for activity is	Cofactor	Holoenzyme	Iso enzyme	Coenzyme	Cofactor
20	Maximal velocity is obtained when	substrate is increased	Enzyme is saturated with substrate	pH is optimum	Temperature is optimum	substrate is increased
21	Fructose 2,3 phosphate is a allosteric activator of	Phosphofructo kinase 1	Fructose 1,6 diphosphate	Hexokinaase	Glucokinase	Phosphofructo kinase 1
22	The allosteric effector Pyruvate carboxilase is	Oxaloacetate	Acetyl CoA	Citrate	Beolin	Acetyl CoA
23	K enzymes show	Change in km	NoChange in V max	Change in V max	Change in km	NoChange in V max
24	Threonine deaminase is a	Allosteric Enzyme	Protease	Transaminase	All the above	Allosteric Enzyme

25	Competitive inhibitor	Increases k_m	Decreases k_m	Does not affect k_m/V_{max}	Increases V_{max}	Increases k_m
26	The substrate for lysozyme is	bacterial cell wall	viral capsid	cornea of the eye	lipopolysaccharides	bacterial cell wall
27	The aminoacids in the active site of Lysozyme are	Asp52 & Glu 35	Asp35 & Glu 52	Asp52 & Arg 35	Glu 35 & Arg 52	Asp52 & Glu 35
28	Lysozyme is a	Hydrolase	Isomerase	Transferase	Lyase	Hydrolase
29	The substrate of Lysozyme has repeating units of	NAM & NAM	NAM & NAG	NAG & NAG	NAG & NAM	NAM & NAG
30	Lysozyme cleaves the bond between carbon and oxygen of the residues	1&2	2&3	1&4	2&4	1&4
31	----- is the multifunctional enzyme	Lactate dehydrogenase	Fatty acid synthetase	Aminoacid oxidase	Lipase	Fatty acid synthetase
32	The catalytic amino acids of Lysozyme are	Asp 52,Gln 35	Asp 35,Gln 52	Ser 195,His 52	Gln270,Tyr 245	Asp 35,Gln 52
33	Modulator binding site is present in	all the enzymes	allosteric enzymes	ligases	isomerases	allosteric enzymes
34	Oxygen –hemoglobin saturation curve is	sigmoidal	rectangular hyperbolic	linear	semilogarithmic.	sigmoidal
35	One of the following is a multienzyme complex	lysozyme	superoxide dismutase	pyruvate dehydrogenase	ribozyme	pyruvate dehydrogenase
36	One of the following is a serine protease	lysozyme	chymotrypsin	ribonuclease	carboxy peptidase	chymotrypsin
37	A competitive inhibitor	increases k_m	decreases k_m	decreases k_m and V_{max}	increases V_{max}	decreases k_m and V_{max}
38	Irreversible covalent modification of enzyme brought about by	adenylation	noncompetitive inhibition	phosphorylation	partial proteolysis	phosphorylation
39	One of the following is not a serine protease	lysozyme	chymotrypsin	ribonuclease	carboxy peptidase	carboxy peptidase

40	A competitive inhibitor	increases k_m	decreases k_m	decreases k_m and V_{max}	increases V_{max}	increases k_m
41	Irreversible covalent modification of enzyme is brought about by	adenylation	noncompetitive inhibition	phosphorylation	partial proteolysis	phosphorylation
42	Who won the noble prize for the discovery that can act as an enzyme	Waston and Crick	Mary Stephenson	Thomas Cech	Emily Smyth	Thomas Cech
43	Example of an extracellular enzyme is	Lactate dehydrogenase	Cytochrome oxidase	Pancreatic lipase	Hexokinase	Pancreatic lipase
44	Enzymes, which are produced in inactive form in the living cells, are called	Papain	Lysozymes	Apoenzymes	Proenzymes	Proenzymes
45	An example of ligases is	Succinate thiokinase	Alanine racemase	Fumarase	Aldolase	Succinate thiokinase
46	An example of lyases is	Glutamine synthetase	Fumarase	Cholinesterase	Amylase	Fumarase
47	Activation or inactivation of certain key regulatory enzymes is accomplished by covalent modification of the amino acid:	Tyrosine	Phenylalanine	Lysine	Serine	Serine
48	The enzyme which can add water to a carbon-carbon double bond or remove water to create a double bond without breaking the bond is	Hydratase	Hydroxylase	Hydrolase	Esterase	Hydratase
49	In the feedback regulation, the end product binds at	Active site	Allosteric site	E-S complex	None of these	Allosteric site
50	Any molecule which can reversibly bind to an enzyme is termed a	Holoenzyme	Apoenzyme	Ligand	Activesite	Ligand
51	The substrate concentration to produce half maximal velocity is known as	K_m value	V_{max} value	Initial value	None of the above	K_m value
52	The substrate binds at the active site by	Hydrogen bonds	Non covalent bonds	Weak non covalent bonds	All the above	Weak non covalent bonds

53	The inhibitor binds at a site other than the active site on the enzyme surface is	Competitive inhibition	Non competitive inhibition	Un competitive inhibition	Irreversible inhibition	Irreversible inhibition
54	The inhibitor that inhibits Xanthine oxidase is	Aminopterin	Allopurinol	Sulphanilamide	Dicumarol	Allopurinol
55	The process of inhibiting the first step by the final product is	Feed back inhibition	Non competitive inhibition	Un competitive inhibition	Irreversible inhibition	Feed back inhibition
56	Proenzymes undergo irreversible covalent activation by break down of	Peptide bonds	Hydrogen bonds	Phosphate bonds	None of the above	Peptide bonds
57	The rate proportional to the concentration of two reactants is	First order reaction	Second order reaction	Zero order reaction	All the above	Second order reaction
58	For non competitive inhibition	K _m value is unchanged while V _{max} is lowered	K _m value is changed while V _{max} is lowered	K _m value is unchanged while V _{max} is lowered	K _m value is unchanged while V _{max} is increased	K _m value is unchanged while V _{max} is lowered
59	In competitive inhibition	K _m value decreases where as V _{max} remains unchanged	K _m value decreases where as V _{max} remains changed	K _m value decreases where as V _{max} remains unchanged	K _m value increases where as V _{max} remains unchanged	K _m value increases where as V _{max} remains unchanged

**UNIT II
SYLLABUS**

Enzyme Kinetics : Derivation of MM equation, LB plot, Eadie Hofstee plot and Hanes plot. Bisubstrate reactions-types of bi-bi reactions, differentiating bi substrate mechanisms-diagnostic plots, isotope exchange. Enzyme inhibition-Types and differentiation of competitive, uncompetitive and non-competitive inhibition, Allosteric inhibition, feed-back inhibition and regulation. Reversible covalent modification (glycogen phosphorylase); proteolytic cleavage (Zymogen); multi enzyme complex as regulatory enzymes (PDH); isoenzymes (LDH). Mechanism based inhibitors-antibiotics as inhibitors. Mechanism of action of enzymes - chymotrypsin and lysozyme. Enzyme based diagnostic techniques.

Derivation of MM equation

In biological systems, enzymes act as catalysts and play a critical role in accelerating reactions, anywhere from 10³ to 10¹⁷ times faster than the reaction would normally proceed. Enzymes are high-molecular weight proteins that act on a substrate, or reactant molecule, to form one or more products.

Enzymes are highly specific catalysts for biochemical reactions, with each enzyme showing a selectivity for a single reactant, or **substrate**. For example, the enzyme acetylcholinesterase catalyzes the decomposition of the neurotransmitter acetylcholine to choline and acetic acid. Many enzyme-substrate reactions follow a simple mechanism that consists of the initial formation of an enzyme-substrate complex, ESES, which subsequently decomposes to form product, releasing the enzyme to react again.

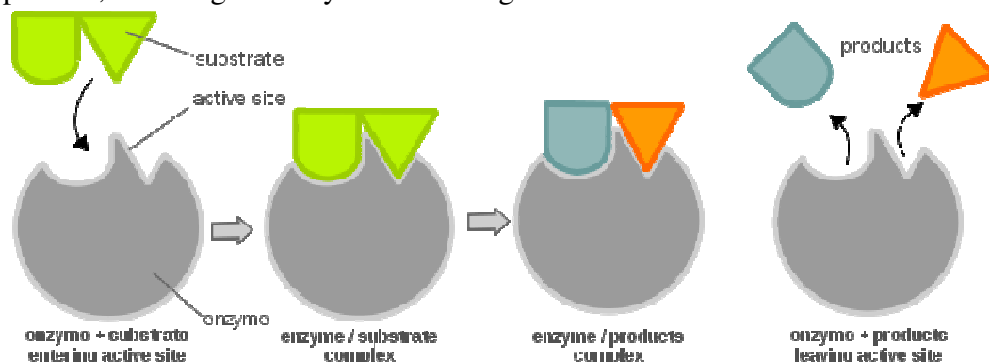
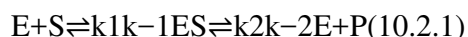


Figure : An enzyme catalyzes the reaction of two substrates and to form one product.

This is described within the following multi-step mechanism



where k_1 , k_{-1} , k_2 , and k_{-2} are rate constants. The reaction's rate law for generating the product $[P]$ is

$$\text{rate} = \frac{d[P]}{dt} = k_2[ES] - k_{-2}[E][P] \quad (10.2.2)$$

However, if we make measurement early in the reaction, the concentration of products is negligible, i.e.,

$$[P] \approx 0 \quad (10.2.3) \quad [P] \approx 0$$

and we can ignore the back reaction (second term in right side of Equation 10.2.2). Then under these conditions, the reaction's rate is

$$\text{rate} = d[P]/dt = k_2[ES] \quad (10.2.4)$$

To be analytically useful we need to write Equation 10.2.4 in terms of the reactants (e.g., the concentrations of enzyme and substrate). To do this we use the **steady-state approximation**, in which we assume that the concentration of ESES remains essentially constant. Following an initial period, during which the enzyme-substrate complex first forms, the rate at which ESES forms

$$d[ES]/dt = k_1[E][S] = k_1([E]_0 - [ES])[S] \quad (10.2.5)$$

is equal to the rate at which it disappears

$$-d[ES]/dt = k_{-1}[ES] + k_2[ES] \quad (10.2.6)$$

where $[E]_0$ is the enzyme's original concentration.

Combining Equations 10.2.5 and 10.2.6 gives

$$k_1([E]_0 - [ES])[S] = k_{-1}[ES] + k_2[ES] \quad (10.2.7)$$

which we solve for the concentration of the enzyme-substrate complex

$$[ES] = [E]_0[S] / (k_{-1} + k_2 + [S]) = [E]_0[S] / (K_m + [S]) \quad (10.2.8)$$

where K_m is the **Michaelis constant**. Substituting Equation 10.2.8 into Equation 10.2.4 leaves us with our final rate equation.

$$d[P]/dt = k_2[E]_0[S] / (K_m + [S]) \quad (10.2.9)$$

A plot of Equation 10.2.9, as shown in Figure 10.2.1, is instructive for defining conditions where we can use the rate of an enzymatic reaction for the quantitative analysis of an enzyme or substrate.

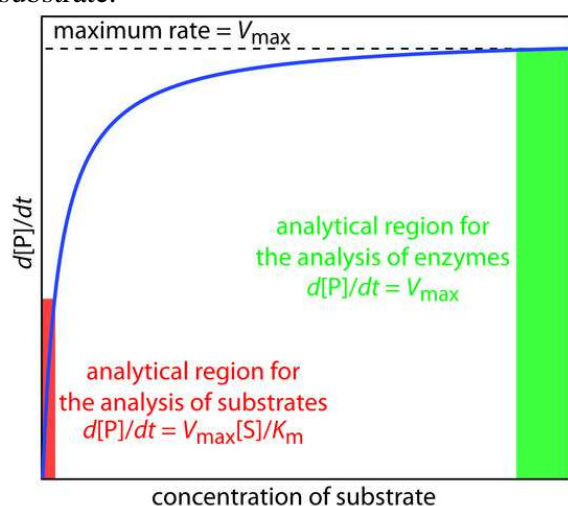


Figure : Plot of Equation 10.2.9 showing limits for the analysis of substrates and enzymes in an enzyme-catalyzed chemical kinetic method of analysis. The curve in the region highlighted

in red obeys equation 10.2.11 10.2.11 and the curve in the area highlighted in green follows Equation

For high substrate concentrations, where $[S] \gg K_m$, Equation 10.2.9 simplifies to $d[P]/dt = k_2[E]_0[S]/K_m + [S] \approx k_2[E]_0[S] = k_2[E]_0 V_{max}$ (10.2.10)

where V_{max} is the maximum rate for the catalyzed reaction. Under these conditions the reaction is zero-order in substrate and we can use V_{max} to calculate the enzyme's concentration, typically using a variable-time method. At lower substrate concentrations, where $[S] \ll K_m$, Equation 10.2.9 becomes

$$d[P]/dt = k_2[E]_0[S]/K_m + [S] \approx k_2[E]_0[S]/K_m = V_{max}[S]/K_m \quad (10.2.11)$$

The reaction is now first-order in substrate, and we can use the rate of the reaction to determine the substrate's concentration by a fixed-time method.

The Michaelis constant K_m , K_m is the substrate concentration at which the reaction rate is at half-maximum, and is an inverse measure of the substrate's affinity for the enzyme—as a small K_m indicates high affinity, meaning that the rate will approach V_{max} more quickly. The value of K_m is dependent on both the enzyme and the substrate, as well as conditions such as temperature and pH.

The Michaelis constant K_m is the substrate concentration at which the reaction rate is at half-maximum.

From the last two terms in Equation 10.2.11 10.2.11, we can express

V_{max} in terms of a **turnover number** (kcat):

$$V_{max} = k_{cat}[E]_0 \quad (10.2.12)$$

where $[E]_0$ is the enzyme concentration and k_{cat} is the turnover number, defined as the maximum number of substrate molecules converted to product per enzyme molecule per second. Hence, the turnover number is defined as the maximum number of chemical conversions of substrate molecules per second that a single catalytic site will execute for a given enzyme concentration $[E]_0$.

Determining V_m and K_m from experimental data can be difficult and the most common way is to determine initial rates, v_0 , from experimental values of $[P]$ or $[S]$ as a function of time. Hyperbolic graphs of v_0 vs. $[S]$ can be fit or transformed as we explored with the different mathematical transformations of the hyperbolic binding equation to determine K_d . These included:

- nonlinear hyperbolic fit (e.g., Figure 10.2.11 10.2.1)
- double reciprocal plot (e.g., Lineweaver–Burk plot discussed below)
- Eadie-Hofstee plot

Lineweaver–Burk [LB] plot

Another commonly-used plot in examining enzyme kinetics is the **Lineweaver–Burk plot**, in which the inverse of the reaction rate, $1/r$, is plotted against the inverse of the substrate concentration $1/[S]$. Rearranging Equation 10.2.10,

$$1/r = K_m/[S] + 1/k_2[E]_0 \quad (10.2.14)$$

the Lineweaver–Burk plot (or double reciprocal plot) is a graphical representation of the Lineweaver–Burk equation of enzyme kinetics, described by Hans Lineweaver and Dean Burk in 1934 (Figure 10.2.2). The Lineweaver–Burk plot results in a straight line with the slope equal to $K_m/k_2[E]_0$ and y-intercept equal to $1/k_2[E]_0$ which is $1/V_{max}$ via Equation 10.2.10.

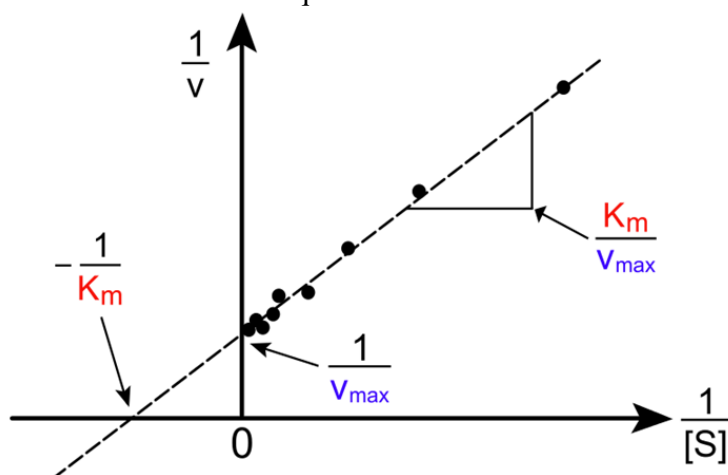


Figure : Lineweaver–Burk plot of Michaelis–Menten kinetics.

The plot provides a useful graphical method for analysis of the Michaelis–Menten equation:

$$V_o = V_{max}[S]/K_m + [S] \quad (10.2.15)$$

Taking the reciprocal gives

$$1/V = K_m/V_{max} + [S]/V_{max} = K_m/V_{max} \cdot 1/[S] + 1/V_{max} \quad (10.2.16)$$

where

- V is the reaction velocity (the reaction rate),
- K_m is the Michaelis–Menten constant,
- V_{max} is the maximum reaction velocity, and
- $[S]$ is the substrate concentration.

The Lineweaver–Burk plot was widely used to determine important terms in enzyme kinetics, such as K_m and V_{max} , before the wide availability of powerful computers and non-linear regression software. The y-intercept of such a graph is equivalent to the inverse of V_{max} ; the x-intercept of the graph represents $-1/K_m$. It also gives a quick, visual impression of the different forms of enzyme inhibition.

Eadie–Hofstee Plot

The Eadie–Hofstee plot is a graphical representation of enzyme kinetics in which reaction rate is plotted as a function of the ratio between rate and substrate concentration and can be derived from the Michaelis–Menten equation (10.2.9) by inverting and multiplying with V_{max} :

$$V_{max}v = V_{max}(K_m + [S]) \quad (10.2.17)$$

Rearrange:

$$V_{\max} = v K_m [S] + v [S] [S] = v K_m [S] + v (10.2.18)$$

Isolate v:

$$v = -K_m v [S] + V_{\max} (10.2.19)$$

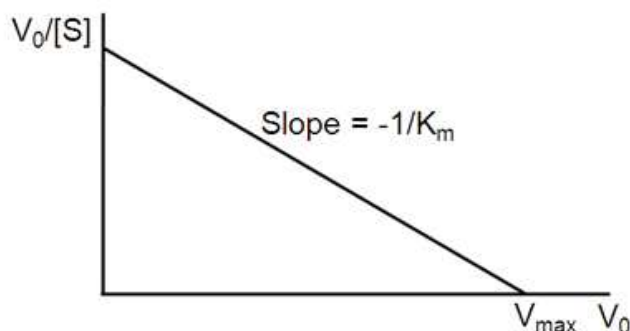


Figure 10.2.3 10.2.3: The Eadie-Hofstee plot is a more accurate linear plotting method with v is plotted against $v/[S]$.

A plot of v against $v/[S]$ will hence yield V_{\max} as the y-intercept, V_{\max}/K_m as the x-intercept, and K_m as the negative slope (Figure 10.2.3). Like other techniques that linearize the Michaelis–Menten equation, the Eadie-Hofstee plot was used historically for rapid identification of important kinetic terms like K_m and V_{\max} , but has been superseded by nonlinear regression methods that are significantly more accurate and no longer computationally inaccessible. It is also more robust against error-prone data than the Lineweaver–Burk plot, particularly because it gives equal weight to data points in any range of substrate concentration or reaction rate (the Lineweaver–Burk plot unevenly weights such points). Both Eadie-Hofstee and Lineweaver–Burk plots remain useful as a means to present data graphically.

Hanes–Woelf plot

In biochemistry, a **Hanes–Woelf plot** is a graphical representation of enzyme kinetics in which the ratio of the initial substrate concentration $[S]$ to the reaction velocity v is plotted against $[S]$. It is based on the rearrangement of the Michaelis–Menten equation shown below:

where K is the Michaelis–Menten constant and V is the maximum reaction velocity.

J B S Haldane stated that this method was due to Barnet Woelf. It was also used by Charles Samuel Hanes, even though he neither mentions nor cites Woelf. Hanes pointed out that the use of linear regression to determine kinetic parameters from this type of linear transformation is flawed, because it generates the best fit between observed and calculated values of $1/v$, rather than v .

The equation can be derived from the Michaelis–Menten equation as follows:

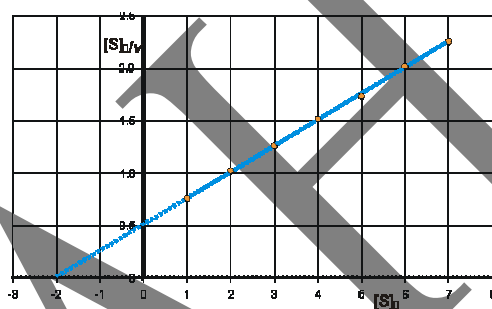
invert and multiply by $[S]$:

Rearrange:

As is clear from the equation, perfect data will yield a straight line of slope $1/V$, a y-intercept of K/V and an x-intercept of $-K$.

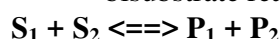
Like other techniques that linearize the Michaelis–Menten equation, the Hanes–Woolf plot was used historically for rapid determination of the important kinetic parameters K_m , V and V/K , but it has been superseded by nonlinear regression methods that are significantly more accurate and no longer computationally inaccessible. It remains useful, however, as a means to present data graphically. One drawback of the Hanes–Woolf approach is that neither ordinate nor abscissa represent independent variables: both are dependent on substrate concentration. As a result, the typical measure of goodness of fit, the correlation coefficient R , is not applicable. Hanes–Woolf (half-reciprocal) plot of $[S]_0/v$ against $[S]_0$ giving intercepts at K_m/V_{max} and K_m .

$$\frac{[S]_0}{v} = \frac{1}{V_{max}} \times [S]_0 + \frac{K_m}{V_{max}}$$



Bisubstrate Reactions

- ~ 60% of enzyme-catalyzed reactions have 2 substrates & 2 products
- bisubstrate reaction:



- Kinetics can be complex, but can be very informative about the mechanism.

- to understand the 2 different types of bisubstrate kinetic mechanisms:
 - sequential (single displacement) reactions, which can be of either of 2 subtypes:
 - ordered sequential
 - random sequential
 - "ping-pong" (double displacement) reactions
- to understand and be able to write kinetic mechanisms for different types of bisubstrate reactions using Cleland (W.W. "Mo" Cleland) terminology ("shorthand" diagrams for kinetic mechanisms)
 - Cleland terminology:
 - Reaction coordinate (progress of reaction) indicated by a line
 - Reactants and products are indicated by arrows "coming" and "going" from the reaction *above* the line.

CLASS: I M.Sc., BIOCHEMISTRY

COURSE NAME: ENZYMES AND MICROBIAL TECHNOLOGY

COURSE CODE: 19BCP102

**UNIT-II: ENZYME KINETICS
(BATCH-2019-2021)**

- Stable enzyme forms (designated E, E', etc. if there *are* different stable enzyme forms in the reaction) are written *below* the line ("stable enzyme form" = a form that can't convert to another stable enzyme form by itself)
- Number of reactants and number of products in reaction indicated as "uni" (1), "bi" (2), "ter" (3), and "quad" (4). For example, $A + B \rightarrow C$ would be "bi uni", and $A + B \rightarrow C + D$ would be "bi bi".
- to be able to distinguish between *sequential* and *ping-pong* kinetic mechanisms based on the patterns observed on double reciprocal (Lineweaver-Burk) plots

Types of bi-bi reactions

SEQUENTIAL vs. PING-PONG

Sequential kinetic mechanisms (single displacement reactions):

- All substrates must bind to enzyme before any product is released.
- A ternary complex (3 components: E, S₁ and S₂, all bound in same E•S₁•S₂ complex) must form before any chemistry can occur.
- 2 sub-types, depending on whether the substrates can bind randomly or must bind in a required order:
 - ordered sequential: substrate binding has to occur in a certain order to form the ternary complex
 - random sequential: the substrates can bind randomly -- doesn't matter which one binds first on the way to forming the ternary complex
- Ordered sequential kinetic mechanisms (single displacement reactions)
 - Second substrate's binding site isn't there or isn't available until first substrate binds.
 - e.g., many enzymes that use the coenzyme (cosubstrate) NAD⁺/NADH, such as lactate dehydrogenase, which catalyzes a redox reaction (so it's an oxidoreductase)
 - the coenzyme (cosubstrate) has to bind first, and the other substrate then can bind
 - product release is also ordered: the other product is released first, and other form of the coenzyme is released last.
- **EXAMPLE:** the lactate dehydrogenase reaction
- a very important enzyme in glucose metabolism -- reversible reaction in which pyruvate (an α -keto acid) is reduced to lactate (the corresponding α -hydroxy acid); the 2-electron donor is the coenzyme NADH; the products are the reduced product (lactate) and the oxidized coenzyme, NAD⁺
- an ordered bi bi reaction

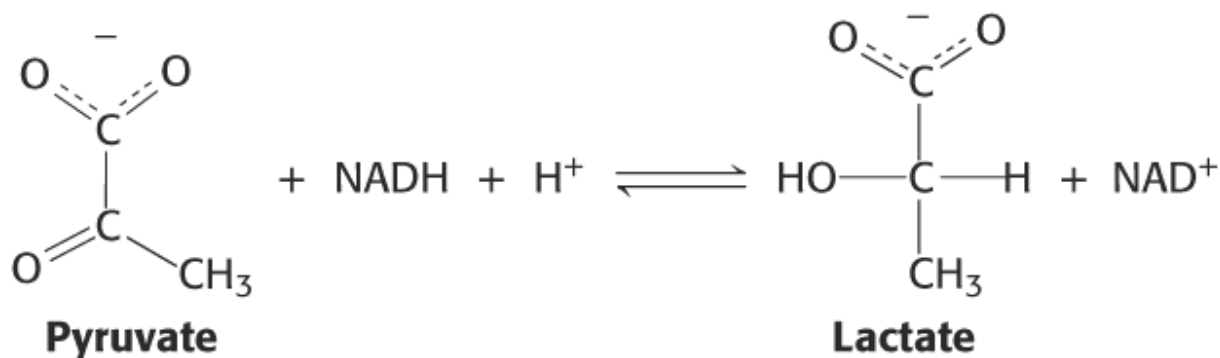


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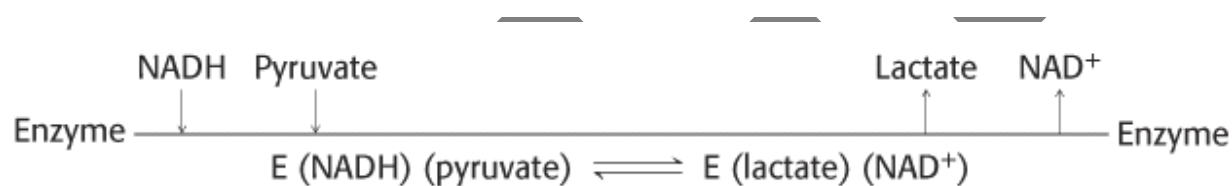
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**UNIT-II: ENZYME KINETICS
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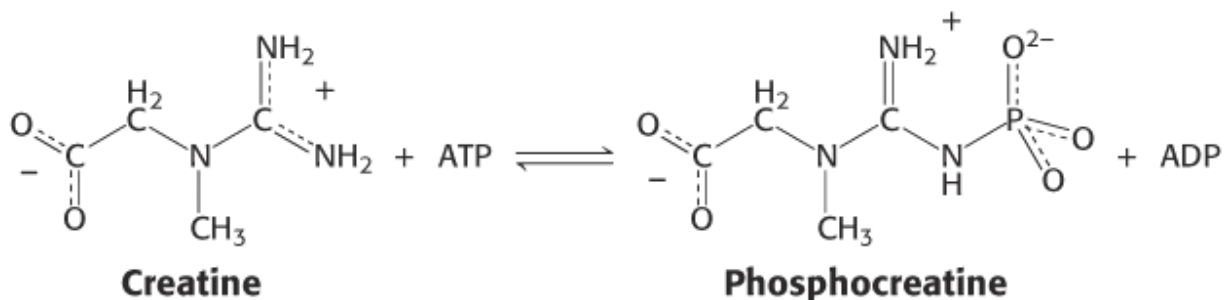


Differentiating bi substrate mechanisms-diagnostic plots

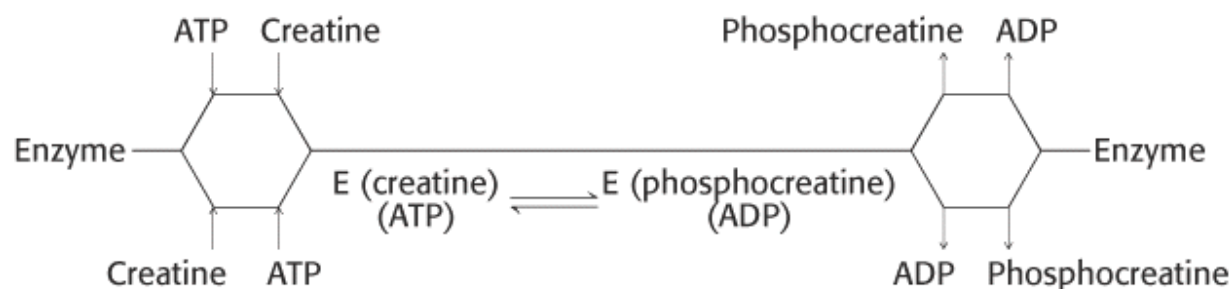
Kinetic mechanism of the LDH reaction (modified Cleland notation):



- Random sequential kinetic mechanisms
- Both substrates' binding sites are available on the free enzyme.
- EXAMPLE: the creatine kinase reaction (phosphoryl group transfer from ATP to creatine, or from phosphocreatine to ADP)
- a random bi bi reaction

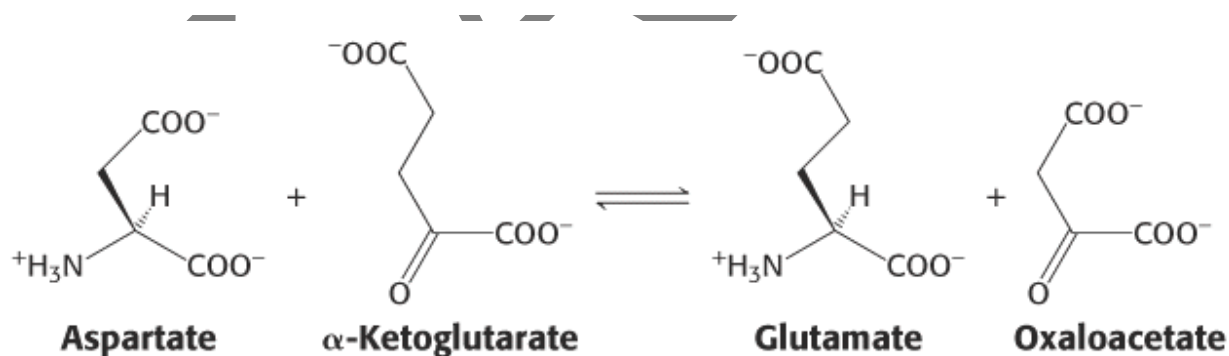


- **Kinetic mechanism of the creatine kinase reaction (modified Cleland notation):**
- Either creatine can bind first and then ATP, or vice versa; likewise, the order of product release is random.



Ping pong kinetic mechanisms (double displacement reactions)

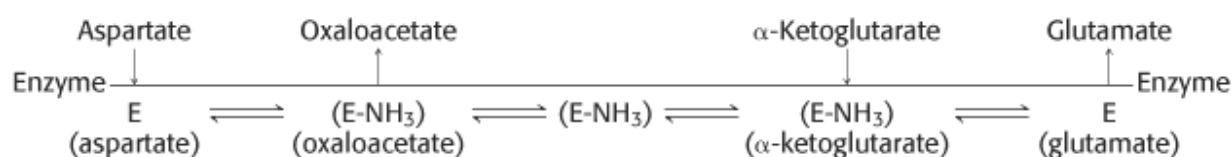
- No ternary complex is formed.
- One or more products are released *before* all substrates have been added.
- Substrates don't react directly with each other in active site of enzyme.
- 1st substrate binds and *reacts with enzyme*, converting enzyme to *another stable enzyme form (E')*, a Chemically modified form of the enzyme
- 1st product (the "remains" of first substrate) is released
- 2nd substrate binds to E' and *reacts with E'*, forming 2nd product and regenerating original stable enzyme form (E)
- 2nd product is released
- There are thus 2 half reactions in the kinetic mechanism.
- Example: the aspartate aminotransferase reaction (one of a number of metabolically very important *aminotransferase* reactions (enzymes sometimes called *transaminases*), that all use the coenzyme pyridoxal phosphate (PLP))



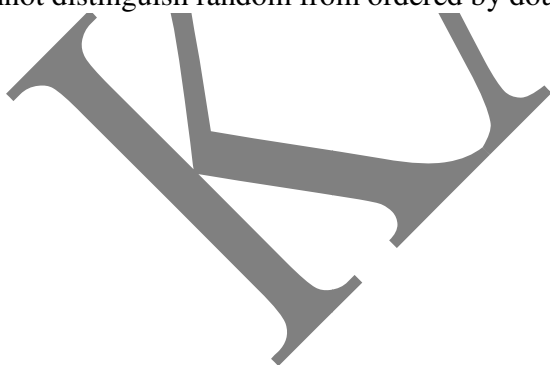
- a ping pong bi bi reaction
- The amino group donor substrate (an α -AMINO acid) transfers its amino group to PLP and the resulting product, an α -KETO acid, dissociates from the "modified enzyme"
- The second substrate, a different α -keto acid, which is to receive the amino group, binds to the modified enzyme, and the coenzyme transfers the amino group to the recipient α -keto acid, generating the second product of the reaction, a different α -amino acid.

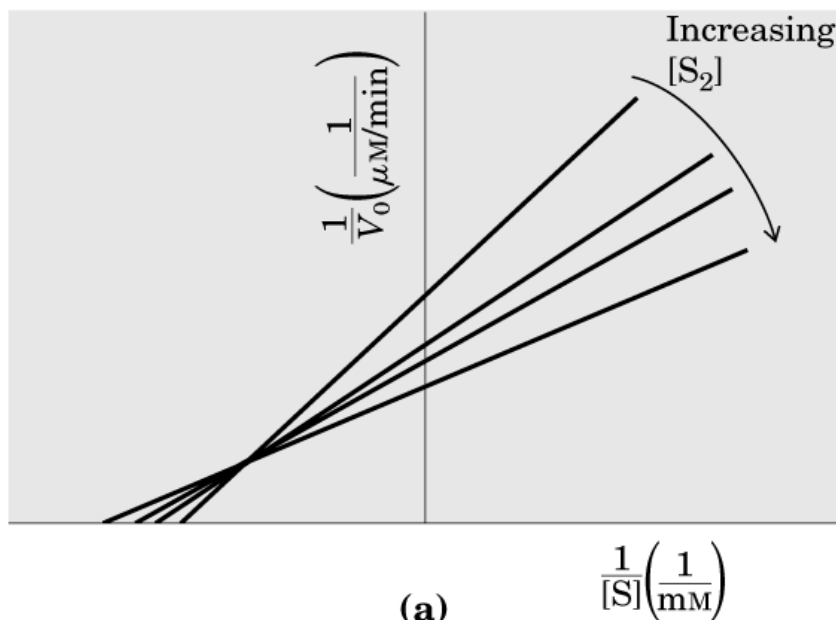
Kinetic mechanism of *aspartate aminotransferase*

- Identify the 2 half reactions, connected by the modified enzyme form, E-NH₃ (the amino group is actually "attached" to the pyridoxal cofactor on the enzyme.)

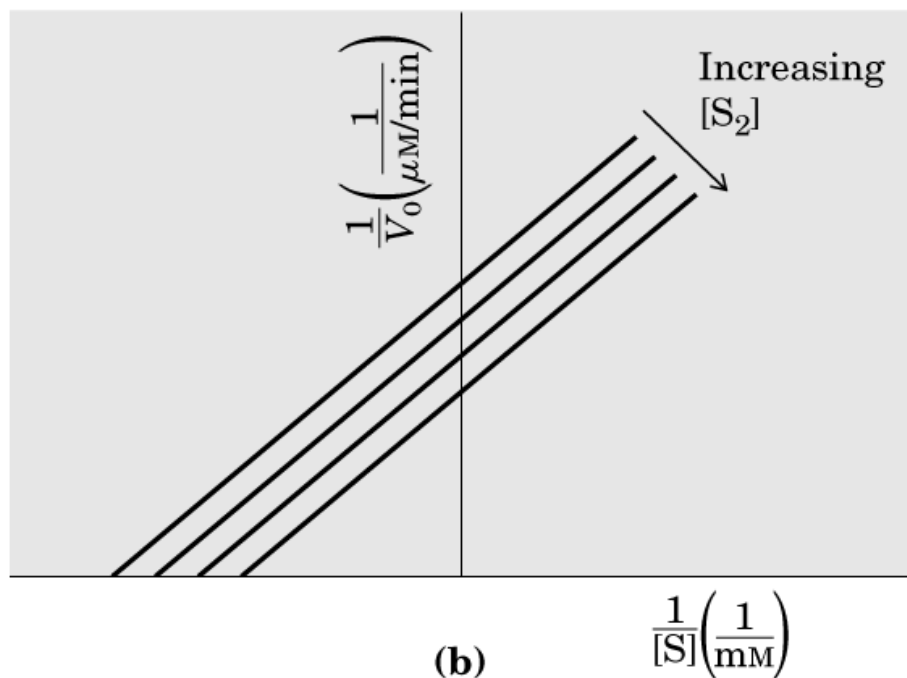


- Double reciprocal plots for bisubstrate reactions ($S_1 + S_2 \rightarrow \text{product(s)}$)
 - Concentration of substrate 1 is varied while the concentration of S_2 is held constant and velocities are measured.
 - This is repeated for several concentrations of S_2 .
 - $1/\text{velocity}$ as a function of $1/S_1$ is plotted as a straight line (Lineweaver-Burk/double reciprocal plot) for *each concentration of S_2* , generating *several separate lines*.
 - Pattern of those lines, specifically *whether or not they intersect* permits identification of the kinetic mechanism as *sequential or ping-pong*.
 - Intersecting* lines ($1/v_0$ vs. $1/S_1$, with a different line for each concentration of S_2) are diagnostic of a sequential kinetic mechanism.
- Fig: Intersecting lines indicate that a ternary complex is formed in the reaction. (Lines always intersect to *left* of the $1/v_0$ axis.)
- What is the overall order of this reaction?
- Start at line for the lowest $[S_2]$ value. At a *higher $[S_2]$* , are the *velocities* HIGHER or LOWER? Does *higher $[S_2]$* make the values of $1/v$ *higher or lower*?
- cannot distinguish random from ordered by double reciprocal plots

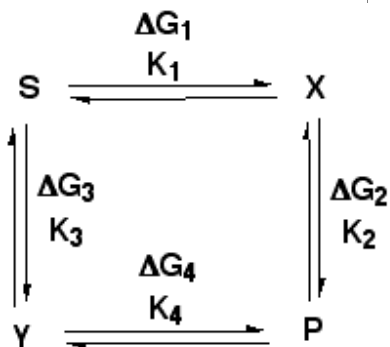




- Parallel lines ($1/v_0$ vs. $1/S_1$, with a different line for each concentration of S_2) are diagnostic of a ping-pong kinetic mechanism.
- Fig. Parallel lines indicate a ping-pong (double displacement) pathway. (No ternary complex is formed in the reaction.)
- What is the overall order of this reaction?
- Start at line for the lowest $[S_2]$ value. At a higher $[S_2]$, are the velocities HIGHER or LOWER? Does higher $[S_2]$ make the values of $1/v$ higher or lower?



- True V_{\max} for bisubstrate reactions is observed only at saturating concentrations of both S_1 and S_2 , and the true K_m value for one substrate is that required to give $1/2 V_{\max}$ when the other substrate is saturating.
- You can determine whether a particular kinetic mechanism is *consistent* with the data (so you can *eliminate* mechanisms that *aren't* consistent with the data).
- There can be many kinetic mechanisms all consistent with the data, so you cannot "prove" a specific mechanism is correct by steady-state kinetic studies.
- individual rate constants for different steps (need pre-steady state analysis, rapid kinetic measurements before steady state is reached).
- number or chemical nature of intermediates in the pathway (need other types of analysis, e.g. spectroscopic characterization of intermediates).



What is the overall free energy change for the $S \rightarrow P$ reaction ($\Delta G_{(S-P)}$) in terms of the component ΔG values going by way of "X"?

What is the overall free energy change for the $S \rightarrow P$ reaction ($\Delta G_{(S-P)}$) in terms of the component ΔG values going by way of "Y"?

- $\Delta G_{(S-P)}$ is *independent of the reaction pathway*, and ΔG values for coupled reactions in a reaction pathway are *additive*.

That means that

$$\Delta G_{(S-P)} = \Delta G_1 + \Delta G_2 = \Delta G_3 + \Delta G_4$$

- What is the overall equilibrium constant for the $S \rightarrow P$ reaction ($K_{(S-P)}$) in terms of the component K_{eq} values going by way of "X"?
- What is the overall equilibrium constant for the $S \rightarrow P$ reaction ($K_{(S-P)}$) in terms of the component K_{eq} values going by way of "Y"?
- K_{S-P} is *independent of the reaction pathway*, and K_{eq} values for coupled reactions in a reaction pathway are *multiplicative*.

That means that

$$K_{(S-P)} = K_1 \cdot K_2 = K_3 \cdot K_4$$

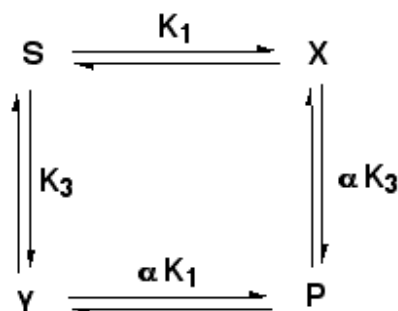
(On your own, try writing out the expressions for the various K_{eq} values in this "linked function" box in terms of the eq. concentrations of S, P, X and Y, and show that indeed the overall K_{eqS-P} value is the *product* of the 2 "component" equilibrium constants.)

- What if K_2 differs from K_3 by a factor of " α " (a constant)?

i.e., $K_2 = \alpha K_3$

THEN (to maintain the equality of $K_1 \cdot K_2 = K_3 \cdot K_4$, i.e., the fact that $K_{(S-P)}$ is what it is irrespective of the pathway),

$$K_4 = \alpha K_1$$



The equilibria are **LINKED** -- if you change K_{eq} for one reaction, you'll change another one by the same factor:

$$K_1 \cdot K_2 = K_3 \cdot K_4 \text{ becomes } K_1 \cdot \alpha K_3 = K_3 \cdot \alpha K_1$$

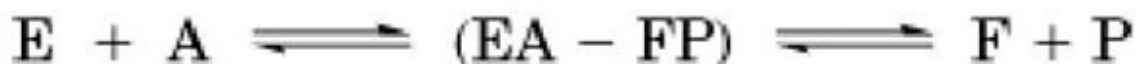
Obviously, you have to pay attention to what the *overall* reaction is, so you're maintaining equalities that make sense!

Isotope Exchange

Measuring the rate of exchange of isotopes between substrates and products in the presence of an enzyme has been very useful for determining the kinetic mechanisms of enzyme-catalyzed reactions. It relies on the incorporation of radioactive atoms from a substrate (or product) into the product (or substrate) under conditions where there is no net reaction. Thus, it differs from other kinetic techniques that rely on the measurement of the rates of product formation. The procedure that is utilized depends on the mechanism under study.

A single-site ping-pong mechanism consists of two partial reactions, so the overall reaction is a double displacement (see Kinetic mechanisms); First, substrate A is converted to P, leaving a

modified form of the enzyme that then converts substrate B to product Q. For the first partial reaction



enzyme concentration and that will occur only when the equilibrium of the partial reaction lies far to the right or the concentration of A is high. Usually, the concentration of enzyme that is used to detect a partial exchange reaction is low and in the micromolar region, so the release of P is small. In the absence of substrate B, the system is at thermodynamic equilibrium, and the A and P reactants are shuttling back and forth. Thus, on the addition of isotopically labeled P (or A), it will be converted to A (or P) and the isotope is incorporated into A (or P). Given sufficient time, the label will be distributed in the same proportions in A and P, and the specific radioactivities of A and P will be identical. The rate at which A is converted to P must be the same as that for the conversion of P to A. It is this partial exchange reaction in the absence of the other substrate that characterizes a ping-pong mechanism. But it is important to demonstrate that the exchange reaction occurs with both halves of the reaction, ie, with both A/P and with B/Q. If only one exchange reaction occurs, it could be due to the presence of an impurity in the enzyme preparation.

The equation for the initial rate of the exchange reaction observed between A and P, starting with labeled A, v_{A-P} , is given by the expression

$$v_{A-P} = \frac{(V_1 K_{ia}/K_a)AP}{K_{ia}P + K_{ip}A + AP}$$

Here, V_j is the maximum rate of the enzyme-catalyzed reaction, A and P are the concentrations of the two reactants, and K_a is the K_m of A. The equation has the same form as that for the initial velocity of a ping-pong mechanism (see Kinetic mechanisms), except that the denominator contains dissociation constants for the EA (K_{ja}) and FP (K_{ip}) complexes (1). In the absence of the formation of a dead-end FA complex (see Substrate Inhibition), a plot of $1/v$ against $1/A$ at different concentrations of P yields a family of parallel straight lines. Analysis of the kinetic data can give values for K_{ia} and K as well as for the apparent maximum velocity of the isotope

$$V_1 K_{ia}/K_a \text{ or } V_2 K_{ip}/K_p,$$

exchange reaction, which is equal to either

where V_2 is the maximum rate of the reverse reaction. An equation of the same form applies for measurement of the initial rate of the B-Q exchange:

$$v_{B-Q} = \frac{(V_1 K_{ib}/K_b)BQ}{K_{iq}B + K_{ib}Q + BQ}$$

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The maximum velocity of the exchange reaction, relative to the maximum velocities of the overall chemical reactions, depends on the values of the dissociation and Michaelis constants. But there is a relationship between the maximum rates of the chemical (V_1 and V_2) and exchange (V_{A-P} and V_{B-Q}) reactions (Eq. 3):

$$\frac{1}{V_{A-P}} + \frac{1}{V_{B-Q}} = \frac{1}{V_1} + \frac{1}{V_2}$$

A further relationship that holds for a single-site ping-pong mechanism is given in Equation 4:

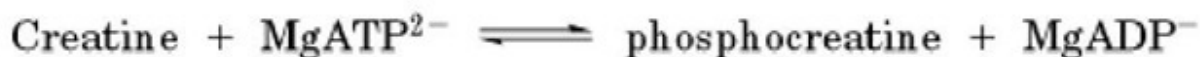
$$\frac{1}{V_{A-Q}} = \frac{1}{V_{A-P}} + \frac{1}{V_{B-Q}}$$

The addition of both substrates or both products to measure the rate of the overall exchange reaction would reduce the rate of exchange of the partial exchange reactions because of the formation of the FB and/or EQ complexes, which would reduce the concentration of the forms of enzyme that participate in the partial exchange reactions.

The procedure that is utilized for the study of exchange reactions with sequential reactions differs from that for a ping-pong mechanism, because sequential mechanisms do not exhibit partial exchange reactions. The technique is applied by allowing the sequential reaction to come to equilibrium and then disturbing that equilibrium by the addition of a small chemical concentration of a highly radiolabeled reactant. The initial rate of the exchange of the label from substrate to product is measured as a function of the concentration of a substrate-product pair that is increased in its equilibrium ratio. For the Bi-Bi reaction



K_{eq} is equal to $(P)(Q)/(A)(B)$ and it is possible to raise the concentration of any one of four pairs of reactants, P/A, P/B, Q/A, or Q/B, without disturbing the equilibrium. The exchange rate can be measured for any one of three possible pairs, but for the most part, exchanges are measured between like substrate-product pairs with the absolute concentration of the same pairs being raised in constant ratio. For the creatine kinase reaction the three possible exchanges would be creatine-phosphocreatine, ATP-phosphocreatine, and ATP-ADP. No atoms are exchanged between creatine and ADP.



The exchange patterns for ordered and rapid equilibrium, random mechanisms are given in Table 1. A straightforward ordered mechanism is characterized by the fact that the A-Q exchange is inhibited as the concentration of the B/P pair is increased (1). This result contrasts

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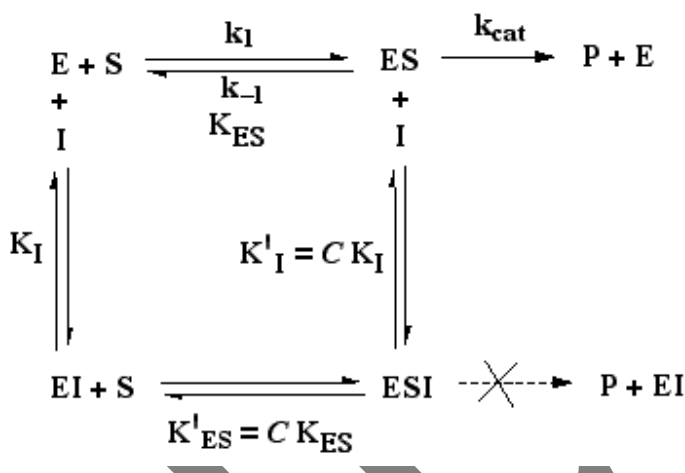
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with that for a rapid equilibrium, random mechanism where there is no inhibition of the A-Q exchange rate with increasing concentrations of the B/P pair. The latter mechanism is further characterized by the equality of all three exchange reactions, as demonstrated for the creatine kinase reaction (2). This will not be the case unless catalysis is the sole rate-limiting step of the reaction sequence (3). An enzyme that catalyzes a Bi-Bi reaction which conforms to a rapid equilibrium, random mechanism has distinct binding sites for each of the two substrates involved in the reaction. Thus, it becomes possible for one substrate and the product of the other substrate to be present on the enzyme at the same time. Two such dead-end complexes could form. The one involving the smaller substrate and smaller product (EBQ) will always form, whereas the other dead-end complex may, or may not, do so. Increasing the concentration of the reactant pair that is involved in dead-end complex formation will lead to inhibition of all three exchanges.

Enzyme inhibition

- **Inhibitor:** any compound that reduces the velocity of an enzyme-catalyzed reaction when present in the reaction mixture
- Use of enzyme inhibitors can often provide valuable information about the catalytic mechanism of an mechanism.
- Many drugs (some discussed below) are based on the use of enzyme inhibitors, e.g.,
 - Penicillin irreversibly (covalently) inhibits an enzyme involved in bacterial cell wall synthesis.
 - Ibuprofen and many other nonsteroidal antiinflammatory drugs (NSAIDs) are reversible competitive inhibitors of the cyclooxygenase activity of prostaglandin H₂ synthase; they bind to the channel leading to the active site, preventing substrate binding. (See cox2 if you're interested in some structural information about cyclooxygenase and inhibitor binding.)
- Inhibitors can be either reversible or irreversible.
 - Irreversible inhibitors bind very tightly (usually covalently) to the enzyme they inhibit.
 - Reversible inhibitors bind to and dissociate from the enzyme (with a measurable **dissociation equilibrium constant, K_I**).
 - $E \cdot I \rightleftharpoons E + I$
- 3 types of reversible inhibition, distinguishable by steady-state kinetics:
 - competitive inhibition
 - uncompetitive inhibition
 - noncompetitive inhibition (also called mixed inhibition)
- Reversible inhibition can be thought of in the context of a *linked function diagram (thermodynamic box)*, as follows:

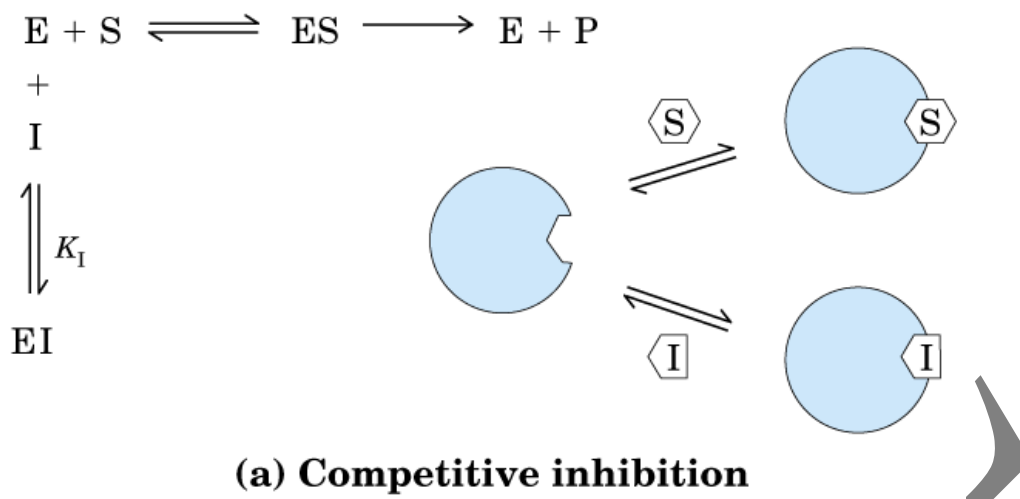
If binding of inhibitor alters the dissociation constant for the substrate from enzyme by a factor C (so $K'_{ES} = CK_{ES}$), then binding of substrate must alter the dissociation constant for inhibitor from the enzyme by the same factor C (so $K'_I = CK_I$).



In the following discussion, it is assumed that the rate of formation of P from ESI (ES with inhibitor also bound) is "zero" (operationally, so slow as to be negligible).

Competitive Inhibition

- E can bind S OR it can bind competitive I , but not both at the same time.
- α has such a large value that there is no measurable binding of both substrate and inhibitor at the same time. That doesn't prove that they can't bind at the same time, only that no simultaneous binding is detectable experimentally.
- The K_{dissoc} value for I from ESI (K'_I) and the K_{dissoc} value for S from ESI (K'_{ES}) are both too high to measure (C is a very large number), so we say that the binding of substrate and the binding of inhibitor are mutually exclusive as far as we can measure.
- From your knowledge of protein structure and ligand binding, what ways can you think of that might explain how binding of one ligand could prevent binding of another?

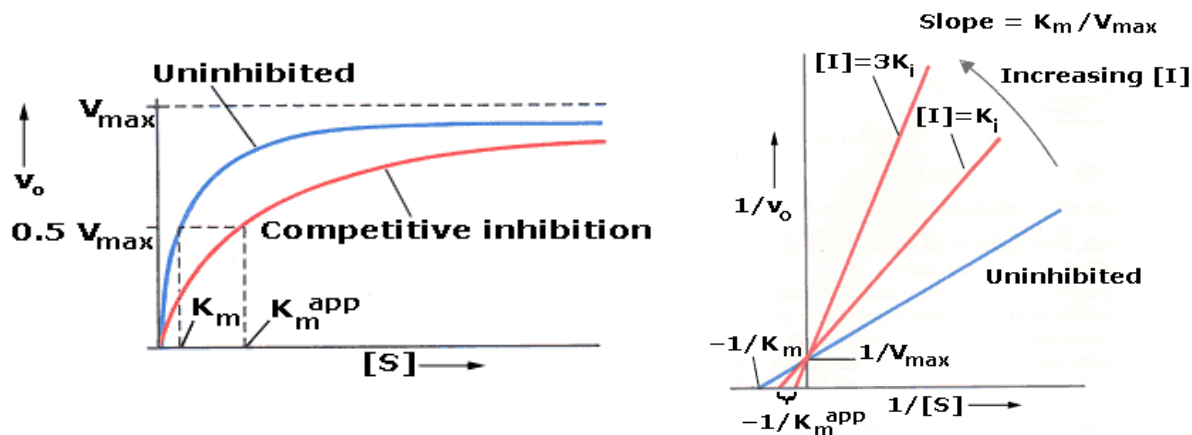


- Competitive inhibitor reduces velocity by effectively reducing the number of available active sites (the effective concentration of free [E]) -- some of the [E] is tied up in the (inactive) form, EI, thus increasing K_m to K_m^{app} by a factor of α

$$\alpha = \left(1 + \frac{[I]}{K_I}\right)$$

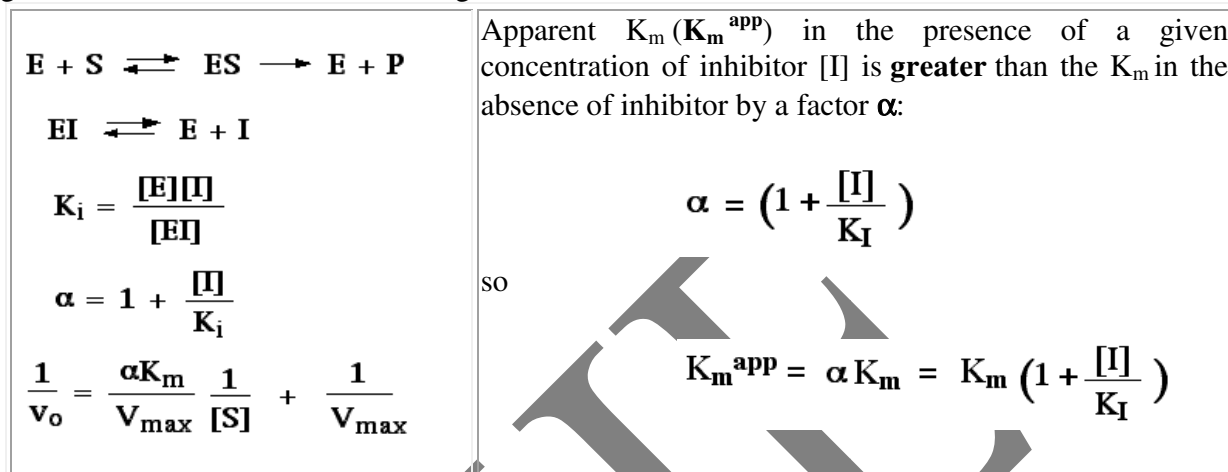
[S] (hyperbolic) and $1/v_o$ vs. $1/[S]$ in the absence and in the presence of a competitive inhibitor.

- On a Lineweaver-Burk (double reciprocal) plot in the presence of a competitive inhibitor
- x-intercepts give $-1/K_m^{app}$ ($= -1/\alpha K_m$ because $K_m^{app} = \alpha K_m$)
- y-intercept gives $1/V_{max}^{app}$ (but $V_{max}^{app} = V_{max}$ for a competitive inhibitor)
- slope is $K_m^{app}/V_{max} = \alpha K_m/V_{max}$



- Steady state kinetics for competitive inhibitor:**
- effect of competitive inhibitor = increase in apparent K_m in presence of inhibitor.

- The higher the inhibitor concentration, the greater the increase in K_m .



Is α greater than, or less than, 1?

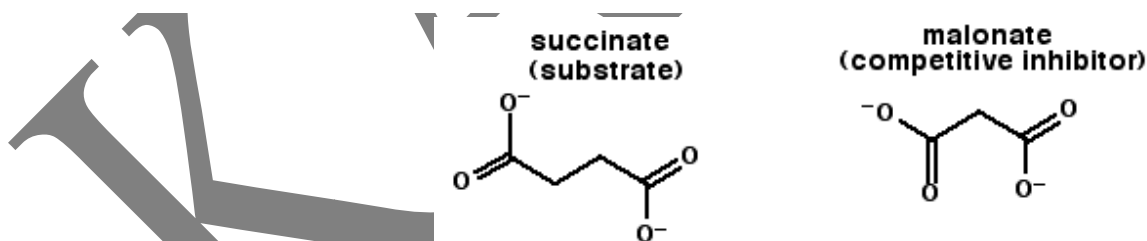
Thus, is K_m^{app} greater than, or less than K_m ?

What happens to the velocity in the presence of a competitive inhibitor as $[S]$ gets very high (approaches infinity, i.e., $1/[S]$ approaches zero)?

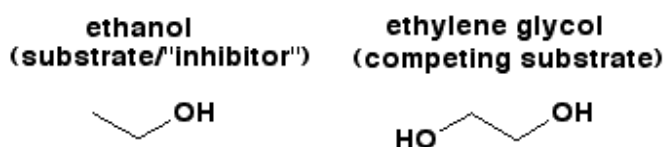
- High $[S]$ overcomes the effect of the inhibitor, so V_{max} is unaffected by a competitive inhibitor. (Note that the $1/v$ intercept, $1/V_{max}$, is unchanged by the competitive inhibitor.)

Examples:

- succinate dehydrogenase:



- alcohol dehydrogenase:



- Often (but *not always*), inability to bind both ligands at once is due to inhibitor binding at same site as substrate -- both ligands obviously cannot occupy the same site at the same time! Such examples often involve inhibitors that are structural analogs of the substrate.

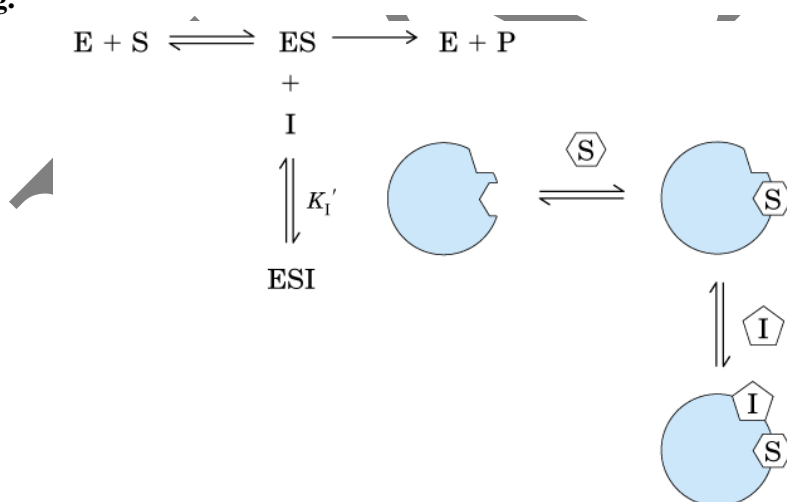
- However, there are examples of competitive inhibitors that bind to a different site, preventing substrate binding by another means, such as a conformational change.
- To cite an example of competitive ligand binding with which you're already familiar: Hemoglobin is of course not an enzyme, but 2,3-bisphosphoglycerate binds in central cavity of tetramer in T state, effectively preventing oxygen binding to all 4 hemes on that tetramer, so it *behaves* as a competitive inhibitor of oxygen binding, but binds to an entirely different site from the oxygen binding sites.

Uncompetitive inhibition

- Uncompetitive I binds only to the ES complex, and cannot bind (*detectibly*) to the free enzyme E.
- a ligand (I) that binds *only* to the ES complex will have the effect of *increasing* the apparent binding affinity of E for S (shifting the E + S binding equilibrium toward ES), which results in a decrease in the apparent K_m for substrate.
- K'_I is the dissociation equilibrium constant for the ESI complex, $ESI \rightleftharpoons ES + I$, so

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

Fig.



(b) Uncompetitive inhibition

- Uncompetitive inhibitors affect the "uninhibited" V_{max} and K_m by exactly the same factor (α'), where

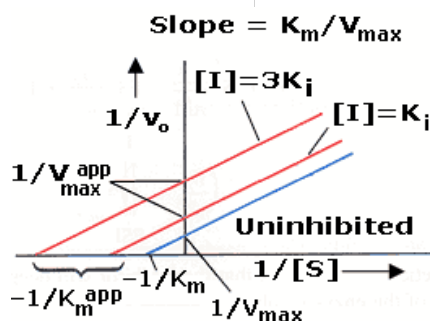
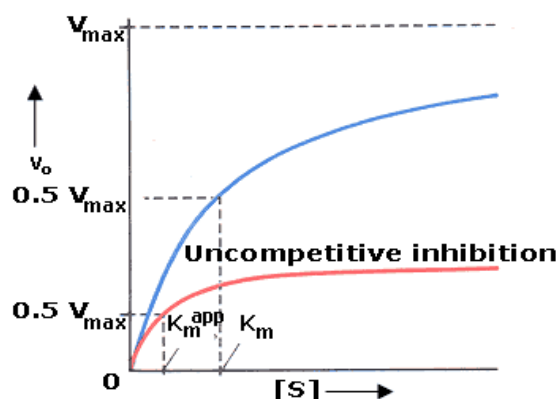
$$\alpha' = \left(1 + \frac{[I]}{K'_I}\right)$$

- V_{\max} is decreased to V_{\max}^{app} and
- also K_m is decreased to K_m^{app} (by the same factor), so
- the *slope* of a Lineweaver-Burk plot, K_m/V_{\max} , is unaffected by the presence of the inhibitor.
- Thus in the presence of increasing concentrations of inhibitor, a Lineweaver-Burk plot shows a family of parallel lines.

[S] (hyperbolic) and $1/v_o$ vs. $1/[S]$ (linear) in the absence and in the presence of an uncompetitive inhibitor.

On Lineweaver-Burk (double reciprocal) plot in the presence of an uncompetitive inhibitor

- x-intercepts give $-1/K_m^{\text{app}}$ (where $K_m^{\text{app}} = K_m/\alpha'$)
- y-intercepts give $1/V_{\max}^{\text{app}}$ (where $V_{\max}^{\text{app}} = V_{\max}/\alpha'$)
- slopes are the same in presence and absence of inhibitor because slope = $K_m/V_{\max} = K_m^{\text{app}}/V_{\max}^{\text{app}}$ (α' cancels out in expression for slope)



- Steady state kinetics for uncompetitive inhibitor:
 - Uncompetitive inhibitor reduces velocity by making the ESI complex catalytically inactive, thus effectively reducing the concentration of the active [ES] (some of the [ES] is tied up in the (inactive) form, ESI),
 - decreasing K_m by a factor of α' to K_m^{app} , AND ALSO decreasing V_{\max} by a factor of α' to V_{\max}^{app} .

$E + S \rightleftharpoons ES \longrightarrow E + P$ $ESI \rightleftharpoons ES + I$ $K'_i = \frac{[ES][I]}{[ESI]}$ $\alpha' = 1 + \frac{[I]}{K'_i}$ $\frac{1}{v_o} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{\alpha'}{V_{max}}$	<p>Apparent K_m (K_m^{app}) and apparent V_{max} (V_{max}^{app}) in the presence of a given concentration of inhibitor $[I]$ are BOTH less than the K_m in the absence of inhibitor by a factor α':</p> $\alpha' = \left(1 + \frac{[I]}{K'_i}\right)$ <p>so</p> $K_m^{app} = \frac{K_m}{\alpha'} = \frac{K_m}{1 + \frac{[I]}{K'_i}}$
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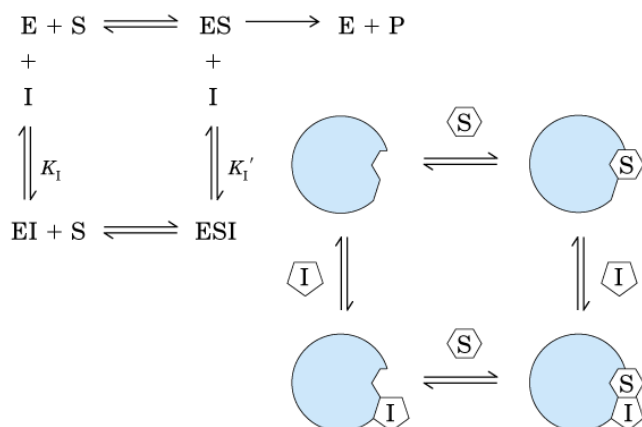
- Is α' greater than, or less than, 1? Thus, is K_m^{app} greater than, or less than K_m ?

Apparent V_{max} (V_{max}^{app}) in the presence of a given concentration of uncompetitive inhibitor $[I]$ is **less than** the V_{max} in the absence of inhibitor by the factor α' , so uninhibited V_{max} **must be divided by α'** .
Increasing the substrate concentration does not overcome the effect of the uncompetitive inhibitor.

$$V_{max}^{app} = \frac{V_{max}}{\alpha'} = \frac{V_{max}}{1 + \frac{[I]}{K'_i}}$$

Mixed inhibition (noncompetitive inhibition)

- A mixed inhibitor
 - binds to a site other than the active site, and
 - reduces the rate of product formation, and
 - can bind to either E or to ES, not necessarily with the same affinity
 - Note: In real life, mixed inhibitors usually affect BOTH K_m and V_{max} .
 - Fig.



(c) Mixed inhibition

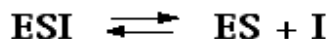
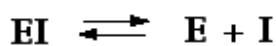
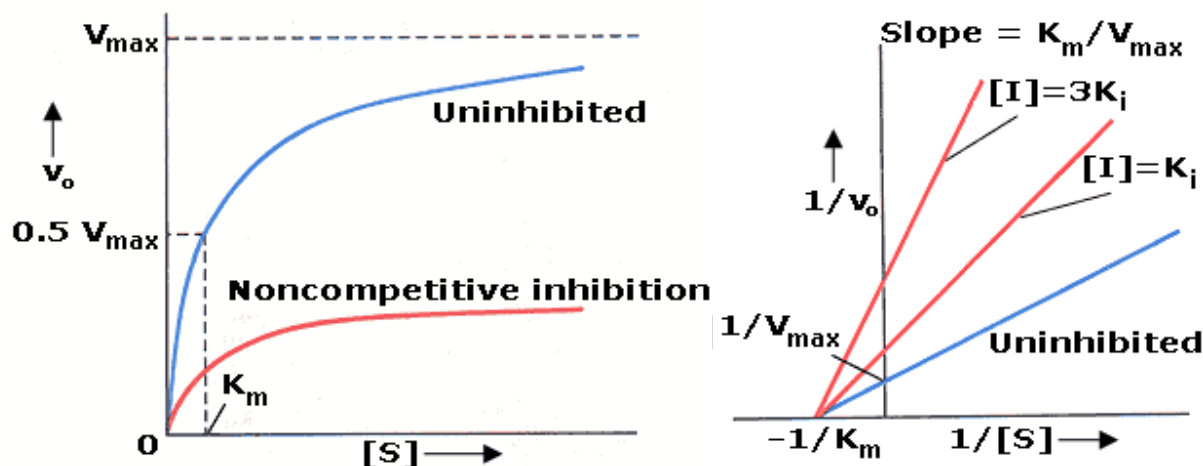
○ **Steady state kinetics for mixed inhibition**

- A mixed inhibitor reduces velocity by both tying up enzyme active sites in the forms of both EI and ESI, and also making the ESI complex catalytically inactive,
- thus *changing K_m by a factor of α/α' to K_m^{app} , and decreasing V_{max} by a factor of α' to V_{max}^{app}* .
- "pure" noncompetitive inhibition
- a special case of mixed inhibition in which E and ES both bind I with the *same affinity*, so
- $\alpha = \alpha'$, so
- $K_I = K_I'$ and $K_{ES} = K_{ES}'$
- K_m is not affected by I.
- "pure" noncompetitive inhibition (shown on plots below)
- rarely encountered in real life
- does not affect K_m ($K_m^{app} = K_m$) because $\alpha = \alpha'$.
- reduces the velocity of the reaction by reducing the concentrations of both E_{free} and ES by equivalent fractions, so V_{max} is reduced by a factor α' .

Figs, v_o vs. $[S]$ (hyperbolic) and $1/v_o$ vs. $1/[S]$ (linear) in the absence and in the presence of a pure noncompetitive (mixed) inhibitor.

- On Lineweaver-Burk (double reciprocal) plot in the presence of a pure noncompetitive inhibitor (on right below):

- x-intercept gives $-1/K_m^{app}$ (where $K_m^{app} = K_m$ because $\alpha = \alpha'$)
- y-intercepts give $1/V_{max}^{app}$ (where $V_{max}^{app} = V_{max}/\alpha'$)
- Mixed inhibition slopes are actually K_m^{app}/V_{max}^{app} , which $= (\alpha K_m/\alpha')/(V_{max}/\alpha')$; the α' values cancel so slopes $= \alpha K_m/V_{max}$;
because $\alpha = \alpha'$ for pure noncompetitive inhibition, slopes for pure noncompetitive inhibition $= K_m/V_{max}^{app}$



"Pure" noncompetitive inhibition:

$$K_i = \frac{[E][I]}{[EI]} = K'_i = \frac{[ES][I]}{[ESI]}$$

$$\alpha = 1 + \frac{[I]}{K_i} = \alpha' = 1 + \frac{[I]}{K'_i}$$

$$\frac{1}{v_o} = \frac{\alpha K_m}{V_{max}} \frac{1}{[S]} + \frac{\alpha'}{V_{max}}$$

$$\alpha' = \left(1 + \frac{[I]}{K'_i} \right)$$

Apparent V_{max} (V_{max}^{app}) in the presence of a given concentration of uncompetitive inhibitor $[I]$ is less than the V_{max} in the absence of inhibitor by the factor α' , so uninhibited V_{max} must be divided by α' .

$$V_{max}^{app} = \frac{V_{max}}{\alpha'} = \frac{V_{max}}{1 + \frac{[I]}{K'_i}}$$

Increasing the substrate concentration does not overcome the effect of the noncompetitive inhibitor.

• **SUMMARY OF EFFECTS OF REVERSIBLE INHIBITORS ON V_{max} AND K_m**

Type of Inhibition	V_{max}^{app}	K_m^{app}
None	V_{max}	K_m
Competitive	V_{max}	αK_m
Uncompetitive	V_{max} / α'	K_m / α'
Mixed PURE noncompetitive (special case of Mixed)	V_{max} / α' V_{max} / α'	$\alpha K_m / \alpha'$ $K_m (\alpha = \alpha')$

Irreversible or covalent inhibition

- involves chemical modification of the protein

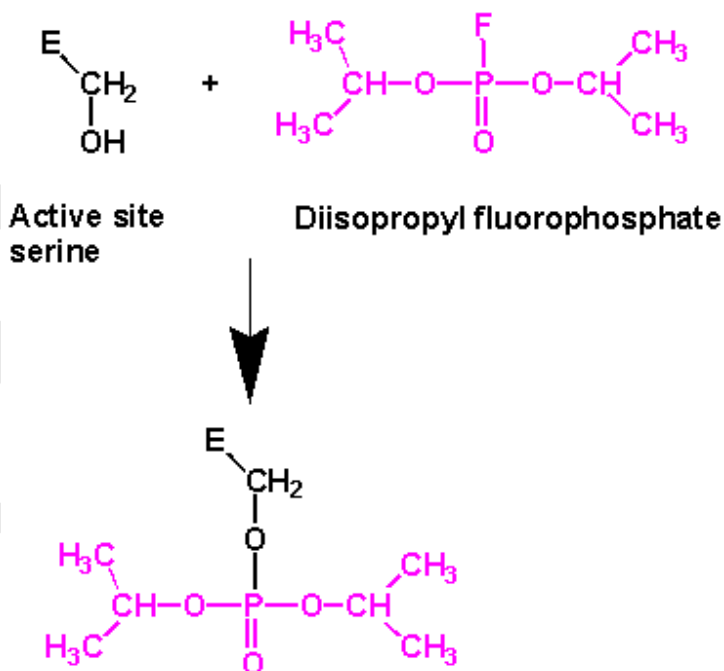
Example 1: irreversible inhibition of enzymes that have active site Ser residues (required in the catalytic mechanism)

reagent diisopropyl fluorophosphate, DIFP, also known as diisopropyl phosphofluoridate

reaction shown at right

DIFP inhibits the digestive proteases chymotrypsin and trypsin (was useful in identifying the active site Ser in the structure and chemical mechanism)

also a potent nerve toxin -- it covalently inhibits acetylcholine esterase, an enzyme with an active site Ser that's required for the *breakdown of the neurotransmitter acetylcholine at synapses*.



- Example 2: irreversible inhibition of the cyclooxygenase activity of prostaglandin H_2 synthase by aspirin
 - acetylates a Ser residue that is NOT catalytically required; the covalent modification blocks *access* of the substrate arachidonate to the active site.
 - See Chime routine cox2 for some structural information about cyclooxygenase and inhibitor binding.

- Other nonsteroidal antiinflammatory drugs (e.g., ibuprofen) bind noncovalently in the same channel into the cyclooxygenase active site, so also block substrate binding, but aren't covalent inhibitors.

Allosteric inhibition

Sometimes it has been found that when a series of reactions is catalysed by a number of enzymes in sequence, the accumulation of the final end-product may cause inhibition in the activity of the first enzyme of the series. This inhibition due to a compound (final end product) which is totally different in structure from the substrate of the enzyme is called as allosteric inhibition or feedback inhibition and such an enzyme is called as allosteric enzyme.

This type of inhibition takes place due to the presence of allosteric site on the surface of the allosteric enzyme away from the active site. The final end-product molecule fits in the allosteric site and in some way brings about a change in shape of the enzyme so that the active site of the enzyme becomes unfit for making complex with its substrate. The allosteric inhibition is reversible. When the concentration of the final end product in the cell falls, it leaves the allosteric site, and the activity of the allosteric enzyme is restored.

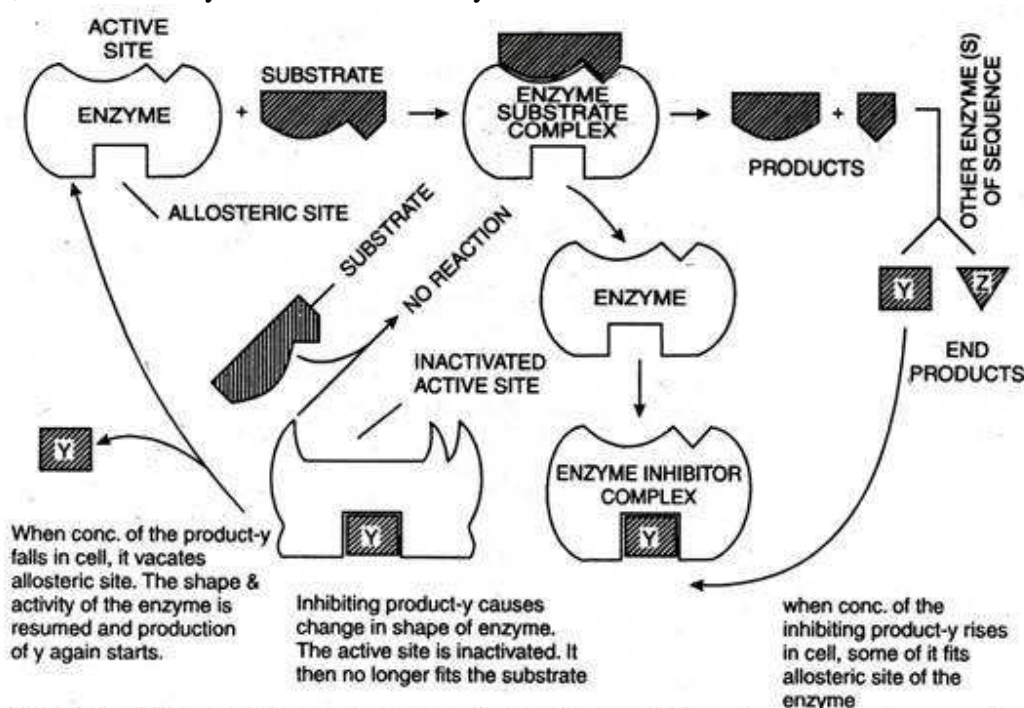


Fig. 10.11. Diagrammatic representation of allosteric inhibition of an enzyme by a product of the reaction sequence.

Allosteric inhibition is shown diagrammatically in Fig.

One of the classical and first discovered examples of allosteric inhibition is furnished by the bacterial enzyme system of *E. coli* which catalyses the conversion of L-Threonine into L-Isoleucine involving 5 different enzymes in sequence viz., 1. Threonine dehydratase 2. Acetolactate synthase 3. Ketoacid reductoisomerase 4. Dihydroxy acid dehydratase and 5. Transaminase.

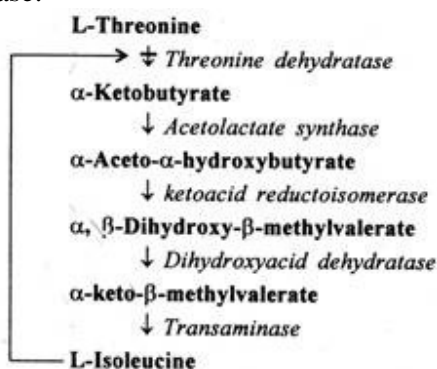


Fig. 10.12. Schematic representation of biosynthesis of Isoleucine from Threonine and allosteric inhibition of Threonine dehydratase by Isoleucine in *E. coli*.

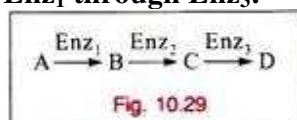
In this sequence, only the first enzyme i.e., threonine dehydratase is inhibited by Isoleucine which is the end-product of this sequence. The activity of this enzyme is neither inhibited by any other intermediate of the sequence, nor is any other enzyme of this sequence inhibited by Isoleucine.

The inhibition of the first enzyme threonine dehydratase is reversible. When the concentration of isoleucine in the cells increases the activity of this enzyme is decreased so that production of isoleucine falls. But, when isoleucine concentration decreases, the activity of threonine dehydratase increases and the production of isoleucine in the cells is restored'.

Feedback Inhibition

The changes in the concentration of substrates, coenzymes, activators or inhibitors affect the catalytic efficiency of an enzyme. Feedback inhibition inhibits the activity of an enzyme early in the biosynthetic pathway.

In the biosynthetic reaction, sequences leading from A to D is catalysed by enzymes Enz₁ through Enz₃:



A high concentration of D will inhibit conversion of A to B. D is specifically able to bind to and inhibit Enz₁. D thus acts as a negative allosteric effector or feedback inhibitor of Enz₁. This feedback inhibition on an early enzyme by an end product of its own biosynthesis achieves regulation of synthesis of D.

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Frequently the feedback inhibitor is the last small molecule before a macromolecule, e.g, amino acid before proteins or nucleotides before nucleic acid.

Feedback inhibition is a mechanism for regulation of many bacterial and mammalian enzymes, e.g., dietary cholesterol restricts the synthesis of cholesterol from acetate in mammalian tissues. The feedback regulation is not involved in feedback inhibition of an early enzyme of cholesterol biosynthesis.

An early enzyme (HMG-CoA reductase) is affected, but the mechanism involves curtailment by cholesterol or a cholesterol metabolite of the expression of the gene that code for the formation of HMG-CoA reductase (i.e. enzyme repression). The catalytic activity of HMG-CoA reductase is not effected by the direct addition of cholesterol.

The kinetics of feedback inhibition may be competitive, non-competitive, mixed etc. It is the common path of regulation of a biosynthetic pathway.

In cumulative feedback inhibition, the inhibitory effect of two or more end products on a single regulatory enzyme is strictly additive.

Complete inhibition occurs only when two or more end products are present in excess in multivalent feedback inhibition.

In cooperative feedback inhibition, a single end product present in excess inhibits the regulatory enzyme.

Control of Enzyme Synthesis –Induction:

For a molecule to be metabolized or for an inducer to act, it first must enter the cell. In some cases, a specific transport system or permease is needed. Permeases have many properties in common with enzymes and perform functions like cytochromes in electron transport.

Escherichia coli grown on glucose will not ferment lactose due to the absence of specific permeases for a β -galactoside (Lactose) and of β -galactosidase. If lactose is added to the medium, both the permease and the β -galactosidases are induced and the culture will now ferment lactose.

The inducer (lactose) is a substrate for the induced proteins, the permease and the β -galactosidase. Enzymes whose concentration in a cell is independent of an added inducer are termed constitutive enzymes.

The structural genes which specify a group of catabolic enzymes comprise an operon. All the enzymes of that operon are induced by a single inducer. This process is known as coordinate induction.

Control of Enzyme Synthesis –Repression and De-repression:

In bacteria, the presence of the synthesized particular amino acid in the culture medium prevents new synthesis of that amino acid via repression. A small molecule such as histidine or leucine, acting as a co-repressor, can ultimately block the synthesis of the enzymes involved in its own synthesis.

The genetic information coding for the biosynthesis of enzymes is again expressed after the removal or exhaustion of an essential biosynthetic intermediate from the medium. This is termed as de-repression.

Regulation

Needs and conditions vary from cell to cell and change in individual cells over time. For instance, stomach cells need different enzymes than fat storage cells, skin cells, blood cells, or nerve cells. Also, a digestive cell works much harder to process and break down nutrients during the time that follows a meal as compared with many hours after a meal. As these cellular demands and conditions changes, so do the amounts and functionality of different enzymes.

Because enzymes guide and regulate the metabolism of a cell, they tend to be carefully controlled. In this article, we'll take a look at factors that can affect or control enzyme activity. These include pH and temperature, as well as:

- **Regulatory molecules.** Enzyme activity may be turned "up" or "down" by activator and inhibitor molecules that bind specifically to the enzyme.

Regulatory molecules

Enzymes can be regulated by other molecules that either increase or reduce their activity. Molecules that increase the activity of an enzymes are called **activators**, while molecule that decrease activity of an enzyme are called **inhibitors**.

There are many kinds of molecules that block or promote enzyme function, and that affect enzyme function by different routes.

Competitive vs. noncompetitive

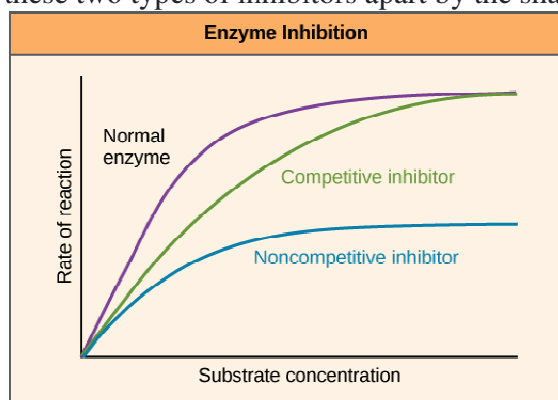
In many well-studied cases, an activator or inhibitor's binding is reversible, meaning that the molecule doesn't permanently attach to the enzyme. Some important types of drugs act as reversible inhibitors. For example, the drug tipranivir, which is used to treat HIV, is a reversible inhibitor.¹ It blocks activity of a viral enzyme that helps the virus make more copies of itself.

Reversible inhibitors are divided into groups based on their binding behavior. We won't discuss all of the types here, but we will look at two important groups: competitive and noncompetitive inhibitors.

- An inhibitor may bind to an enzyme and block binding of the substrate, for example, by attaching to the active site. This is called **competitive inhibition**, because the inhibitor "competes" with the substrate for the enzyme. That is, only the inhibitor or the substrate can be bound at a given moment.
- In **noncompetitive inhibition**, the inhibitor doesn't block the substrate from binding to the active site. Instead, it attaches at another site and blocks the enzyme from doing its job. This inhibition is said to be "noncompetitive" because the inhibitor and substrate can both be bound at the same time.
- If an inhibitor is competitive, it will decrease reaction rate when there's not much substrate, but can be "out-competed" by lots of substrate. That is, the enzyme can still reach its maximum reaction rate given enough substrate. In that case, almost all the active sites of almost all the enzyme molecules will be occupied by the substrate rather than the inhibitor.
- If an inhibitor is noncompetitive, the enzyme-catalyzed reaction will never reach its normal maximum rate even with a lot of substrate. This is because the enzyme molecules with the

noncompetitive inhibitor bound are "poisoned" and can't do their job, regardless of how much substrate is available.

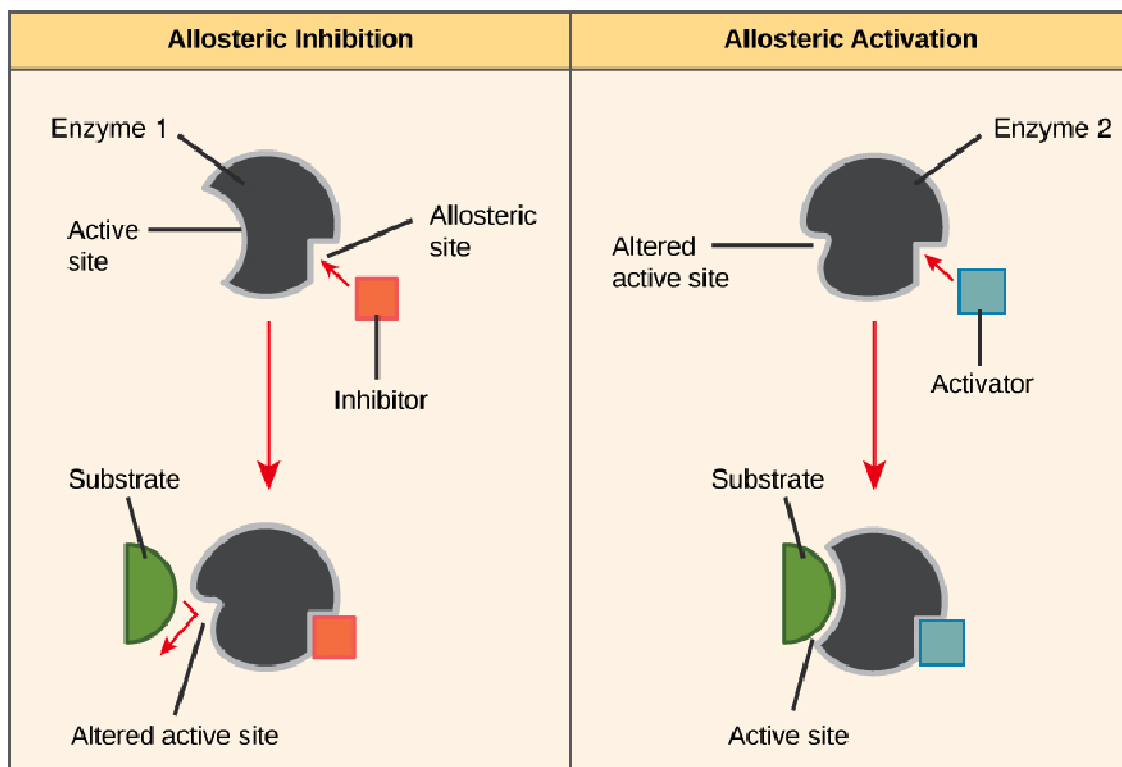
On a graph of reaction velocity (y-axis) at different substrate concentrations (x-axis), you can tell these two types of inhibitors apart by the shape of the curves:



This plot shows rate of reaction versus substrate concentration for an enzyme in the absence of inhibitor, and for enzyme in the presence of competitive and noncompetitive inhibitors. Both competitive and noncompetitive inhibitors slow the rate of reaction, but competitive inhibitors can be overcome by high concentrations of substrate, whereas noncompetitive inhibitors cannot.

Allosteric regulation

Allosteric regulation, broadly speaking, is just any form of regulation where the regulatory molecule (an activator or inhibitor) binds to an enzyme someplace other than the active site. The place where the regulator binds is called the **allosteric site**.



The left part of this diagram shows allosteric inhibition. The allosteric inhibitor binds to an enzyme at a site other than the active site. The shape of the active site is altered so that the enzyme can no longer bind to its substrate.

The right part of this diagram shows allosteric activation. The allosteric activator binds to an enzyme at a site other than the active site. The shape of the active site is changed, allowing substrate to bind at a higher affinity.

Pretty much all cases of noncompetitive inhibition (along with some cases of competitive inhibition, the ones where the inhibitor binds elsewhere than the active site) are forms of allosteric regulation.

However, some enzymes that are allosterically regulated have a set of unique properties that set them apart. These enzymes, which include some of our key metabolic regulators, are often given the name of **allosteric enzymes**²². Allosteric enzymes typically have multiple active sites located on different protein subunits. When an allosteric inhibitor binds to an enzyme, all active sites on the protein subunits are changed slightly so that they work less well.

There are also allosteric activators. Some allosteric activators bind to locations on an enzyme other than the active site, causing an increase in the function of the active site. Also, in a process called **cooperativity**, the substrate itself can serve as an allosteric activator: when it binds to one active site, the activity of the other active sites goes up.³

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superscript This is considered allosteric regulation because the substrate affects active sites far from its binding site.

Cofactors and coenzymes

Many enzymes don't work optimally, or even at all, unless bound to other non-protein helper molecules called **cofactors**. These may be attached temporarily to the enzyme through ionic or hydrogen bonds, or permanently through stronger covalent bonds. Common cofactors include inorganic ions such as iron (Fe^{2+}) and magnesium (Mg^{2+}) . For example, the enzyme that builds DNA molecules, DNA polymerase, requires magnesium ions to function.

Coenzymes are a subset of cofactors that are organic (carbon-based) molecules. The most common sources of coenzymes are dietary vitamins. Some vitamins are precursors to coenzymes and others act directly as coenzymes. For example, vitamin C is a coenzyme for several enzymes that take part in building the protein collagen, a key part of connective tissue.

Enzyme compartmentalization

Enzymes are often compartmentalized (stored in a specific part of the cell where they do their job) -- for instance, in a particular organelle. Compartmentalization means that enzymes needed for specific processes can be kept in the places where they act, ensuring they can find their substrates readily, don't damage the cell, and have the right microenvironment to work well.

For instance, digestive enzymes of the lysosome work best at a pH around 5.05, which is found in the acidic interior of the lysosome (but not in the cytosol, which has a pH of about 7.27). Lysosomal enzymes have low activity at the pH of the cytosol, which may serve as "insurance" for the cell: even if a lysosome bursts and spills its enzymes, the enzymes will not begin digesting the cell, because they will no longer have the right pH to function.

Feedback inhibition of metabolic pathways

In the process of **feedback inhibition**, the end product of a metabolic pathway acts on the key enzyme regulating entry to that pathway, keeping more of the end product from being produced.

This may seem odd – why would a molecule want to turn off its own pathway? But it's actually a clever way for the cell to make just the right amount of the product. When there's little of the product, the enzyme will not be inhibited, and the pathway will go full steam ahead to replenish the supply. When there's lots of the product sitting around, it will block the enzyme, preventing the production of new product until the existing supply has been used up.



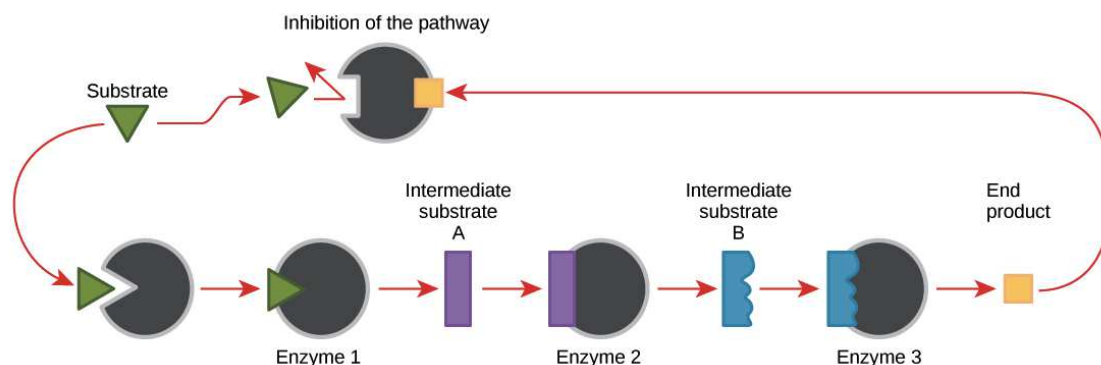


Diagram illustrating feedback inhibition. The end product of a multi-step metabolic pathway binds to an allosteric site on the enzyme that catalyzes the committed step of the pathway, reducing the enzyme's activity. This regulation helps slow the pathway down when levels of the end product are already high (when more is not needed).

Typically, feedback inhibition acts at the **first committed step** of the pathway, meaning the first step that's effectively irreversible. However, feedback inhibition can sometimes hit multiple points along a pathway as well, particularly if the pathway has lots of branch points. The pathway steps regulated by feedback inhibition are often catalyzed by allosteric enzymes.⁶

For example, the energy carrier molecule ATP is an allosteric inhibitor of some of the enzymes involved in cellular respiration, a process that makes ATP to power cellular reactions. When there is lots of ATP, this feedback inhibition keeps more ATP from being made. This is useful because ATP is an unstable molecule. If too much ATP were made, much of it might go to waste, spontaneously breaking back down into its components (ADP and P_i).

ADP, on the other hand, serves as a positive allosteric regulator (an allosteric **activator**) for some of the same enzymes that are inhibited by ATP. For instance, ADP may act by binding to an enzyme and changing its shape so that it becomes more active.⁷

Thanks to this pattern of regulation, when ADP levels are high compared to ATP levels, cellular respiration enzymes become very active and will make more ATP through cellular respiration.

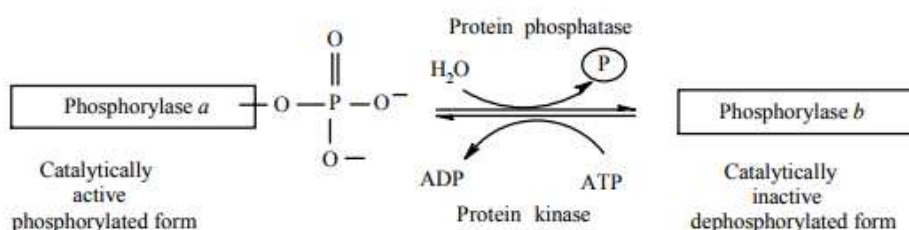
Reversible covalent modification (glycogen phosphorylase)

This is also one of the major ways of controlling the enzyme activity to exercise a regulatory control over metabolism. In this the enzyme protein gets activated or inhibited by undergoing through a covalent modification. These modifications are reversible and require two enzymes. Depending on the metabolic milieu of the cell one enzyme incorporates a covalently linked group and the other enzyme removes it from the enzyme protein whose activity is being controlled.

Fig. 8: A schematic presentation of covalent modifications of an enzyme protein. The following are some well established examples of covalent modification: 1. Glycogen phosphorylase: Activated by phosphorylation of enzyme protein. The enzyme liberates glucose-1-phosphate from glycogen in muscle. A glucose residue at the non-reducing end of the chain is removed by breaking the glycosidic bond involving a phosphoric acid molecule. Thus, a molecule of glucose-1-phosphate is released that acts as a source of energy and glycogen chain becomes shorter by one glucose unit at each step as shown below:



The enzyme phosphorylase in active form is phosphorylated and called phosphorylase a. Under the conditions where the breakdown of glycogen is not needed this active enzyme is converted into inactive form called phosphorylase b. These two forms of enzyme are interconvertible with the help of two enzymes; a protein phosphatase and a protein kinase as shown below:



In this enzyme catalyzed interconversion of phosphorylase into inactive and active forms, respectively by a protein phosphatase and a protein kinase, the incorporation of phosphate group takes place at the —OH group of amino acid serine in regulatory protein.

Proteolytic cleavage (Zymogen)

The type of mechanism is exemplified by conversion into active form of digestive enzymes e.g. trypsin and chymotrypsin. These enzymes are synthesized in pancreas in their inactive forms called trypsinogen and chymotrypsinogen, respectively. The general name for such catalytically inactive forms is zymogen or proenzyme.

The zymogen form of the enzymes is slightly longer and this inactive form is broken down by the action of a protease to result the formation of active enzyme. The zymogen form on synthesis in pancreas is secreted in pancreatic juice which then brings it to duodenum where first the N-terminal end of trypsinogen is removed by a protease (enteropeptidase) of duodenum. This

results in formation of active trypsin which subsequently activates other zymogens to form respective active enzymes (Fig.9). The process is called zymogen activation.

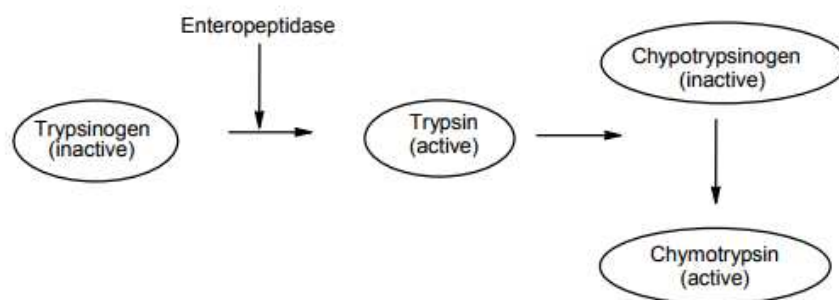


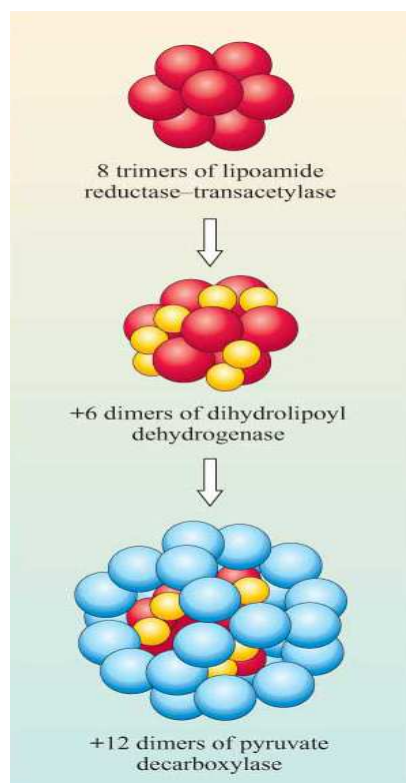
Fig: Sequence of zymogen activation

It should be noted that the secretion of inactive zymogen forms of these enzymes is a protective mechanism to safeguard pancreas from enzymic active form otherwise this would pose a serious crisis for the organ. The zymogen activation cannot be reversed.

Multi enzyme complex as regulatory enzymes (Pyruvate dehydrogenase)

In free solution, the rate of an enzyme-catalysed reaction depends on the concentration of the enzyme and the concentration of its substrate. For an enzyme operating at suboptimal concentrations, the reaction is said to be *diffusion-limited*, since it depends on the random collision of the enzyme and substrate. If we consider a metabolic pathway, the product of one reaction is the substrate for the next enzyme in the pathway. Direct transfer of a metabolite from one enzyme to another would avoid dilution of the metabolite in the bulk aqueous environment and would increase the rate of reaction.

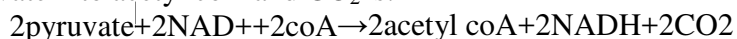
In the cell, enzymes of a particular pathway are frequently organised spatially so that such metabolic channelling can occur. Some enzymes are associated with other enzymes involved in a particular pathway to form multienzyme complexes. For the enzymes in such complexes, the diffusion of the substrate is not rate-limiting. Pyruvate dehydrogenase is a complex of three different enzymes that collectively catalyse the oxidation of pyruvate as described previously. In fact, in eukaryotic cells, most enzymes do not diffuse freely in the cytosol but are effectively concentrated in particular parts of the cell along with other enzymes or proteins involved in related processes. Concentration of enzymes in this way can be achieved by specific protein-protein interactions.



The oxidation of pyruvate occurs in the mitochondria of the cell. The mitochondria is an organelle in the cell. It is considered the "powerhouse" of the cell. Pyruvate is transported there via pyruvate translocase. Pyruvate dehydrogenase is a multi-enzyme complex that uses three enzymes:

1. E₁: Pyruvate dehydrogenase which uses thiamine pyrophosphate (TPP) as its prosthetic group.
2. E₂: Dihydrolipoyl transacetylase which uses lipoamide and coenzyme A (also known as coASH) as its prosthetic groups.
3. E₃: Dihydrolipoyl dehydrogenase which uses flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NAD⁺) as its cofactors.

Note: Prosthetic groups are molecules that are covalently bonded to an enzyme. The net reaction of converting pyruvate into acetyl coA and CO₂ is:



The Process

This is a five step process.

1. Step A: Pyruvate is decarboxylated by pyruvate dehydrogenase with help from TPP.
2. Step B: The reactive carbon (between the N and the S of the five membered ring) of the TPP is oxidized and transferred as the acetyl group to lipoamide (which is the prosthetic

group of the dihydrolipoyl transacetylase). This forms hydroxyethyl-TPP. An H^+ ion is required for the intermediate to give off CO_2 .

3. Step C: E_2 (dihydrolipoyl transacetylase with cofactor lipoamide) oxidizes hydroxyethyl- to acetyl- and then transfers acetyl- to CoA, forming acetyl-CoA.
4. Step D: Acetyl CoA was made in the previous step. However, the process is incomplete. The E_2 is still attached to the acetyl CoA molecule. So, E_3 (dihydrolipoyl dehydrogenase) oxidizes the thiol groups of the dihydrolipoamide back to lipoamide.
5. Step E: As a side reaction, NAD^+ becomes reduced to NADH.

Isozymes (LDH)

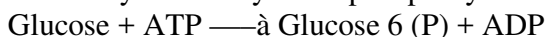
Isozymes or Isoenzymes are proteins with different structure which catalyze the same reaction. Frequently they are oligomers made with different polypeptide chains, so they usually differ in regulatory mechanisms and in kinetic characteristics.

From the physiological point of view, isozymes allow the existence of similar enzymes with different characteristics, “customized” to specific tissue requirements or metabolic conditions.

One example of the advantages of having isoenzymes for adjusting the metabolism to different conditions and/ or in different organs is the following:

Glucokinase and Hexokinase are typical examples of isoenzymes. In fact, there are four Hexokinases: I, II, III and IV. Hexokinase I is present in all mammalian tissues, and Hexokinase IV, aka Glucokinase, is found mainly in liver, pancreas and brain.

Both enzymes catalyze the phosphorylation of Glucose:



Hexokinase I has a low K_m and is inhibited by glucose 6 (P). Glucokinase is not inhibited by Glucose 6 (P) and its K_m is high. These two facts indicate that the activity of glucokinase depends on the availability of substrate and not on the demand of the product.

Since Glucokinase is not inhibited by glucose 6 phosphate, in conditions of high concentrations of glucose this enzyme continues phosphorylating glucose, which can be used for glycogen synthesis in liver. Additionally, since Glucokinase has a high K_m , its activity does not compromise the supply of glucose to other organs; in other words, if Glucokinase had a low K_m , and since it is not inhibited by its product, it would continue converting glucose to glucose 6 phosphate in the liver, making glucose unavailable for other organs (remember that after meals, glucose arrives first to the liver through the portal system).

Since isoenzymes have different tissue distributions, their study is an important tool in assessing the damage to specific organs.

Examples of the diagnostic use of isoenzymes are the study of Lactate dehydrogenase and Creatine Kinase.

Lactate Dehydrogenase (LDH)

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It is formed by the association of five peptide chains of two different kinds of monomers: M and H.

The variants seen in humans are:

LDH1: M M M M (abundant in heart, brain erythrocytes; around 33% of serum LDH)

LDH2: M M M H (abundant in heart, brain erythrocytes; around 45% of serum LDH)

LDH3: M M H H (abundant in brain, kidneys, lung; around 18 % of serum LDH)

LDH4: M H H H ((abundant in liver, skeletal muscle, kidney; around 3% of serum LDH)

LDH5: H H H H ((abundant in liver, skeletal muscle, ileum; around 1 % of serum LDH)

In myocardial infarction, Total LDH increases, and since heart muscle contains more LDH1 than LDH2, LDH1 becomes greater than LDH2 between 12 and 24 hours, after the infarction, so the ratio LDH1/LDH2 becomes higher than 1 and will stay flipped for several days.

An increase of LDH 5 in serum is seen in different hepatic pathologies: cirrhosis, hepatitis and others. An increase of LDH5 in heart diseases usually indicates secondary congestive liver involvement.

Mechanism based inhibitors - antibiotics as inhibitors

inhibitor not especially reactive until it binds to enzyme active site

- Enzyme treats inhibitor as a substrate, but chemical structure of inhibitor results in *covalent inactivation* of enzyme by an intermediate generated in the mechanism of catalysis, so active site ends up irreversibly modified.
- (Enzyme "commits suicide" by trying to catalyze the reaction with the inhibitor as substrate.)
- Mechanism-based inhibitors are *very* useful as drugs, because they're specific for only the enzyme with the unique catalytic capability to generate the reactive species *in its active site*, so few side effects
- Examples:

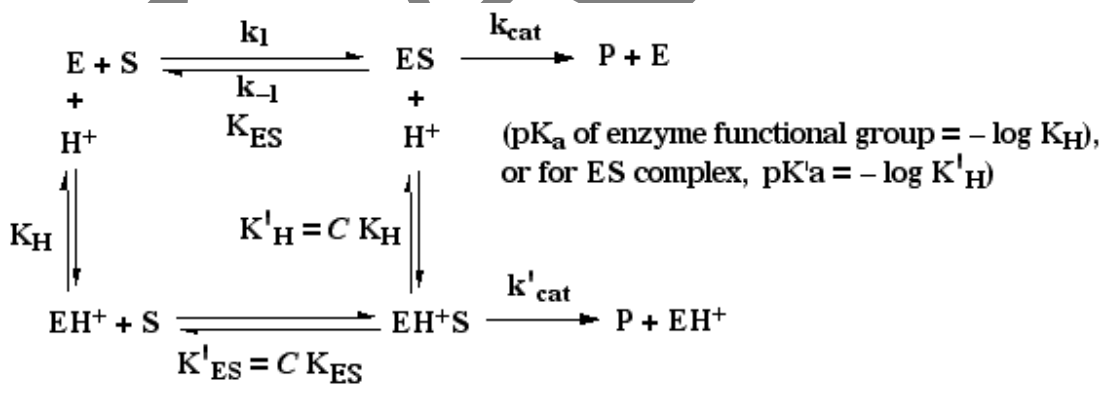
Penicillin:

- blocks formation of peptide crosslinks in peptidoglycan component of bacterial cell walls (so no equivalent enzyme in eukaryotes, minimizing side effects)
- targets an enzyme required for bacterial cell wall synthesis, a *transpeptidase*
- enzyme binds penicillin (or an analogous β -lactam antibiotic) and catalytic mechanism generates a covalent penicilloyl-enzyme derivative that can't break down to form product and regenerate free enzyme the way the normal intermediate would.
- drugs for African sleeping sickness (African trypanosomiasis, caused by a trypanosome, a single-celled eukaryote)
- target enzyme = *ornithine decarboxylase (ODC)*, enzyme required for first step in biosynthesis of polyamines needed for DNA packaging
- mammalian cells degrade ODC and make new enzyme rapidly and continuously, so inhibiting host enzyme isn't much of a problem; trypanosomes make a *stable* enzyme that *isn't* rapidly degraded and replaced, so covalent inhibition affects the parasite much more than the host cells.

- Drugs (substrate analogs like difluoromethylornithine) form a covalent bond to the pyridoxal phosphate cofactor in the enzyme active site the way the normal substrate ornithine does, but as the drug is decarboxylated, a fluorine (excellent leaving group) departs as a F^- ion, generating a highly reactive fluoromethylornithine derivative of the cofactor that can be attacked by a nucleophilic group on the enzyme to displace a 2nd F^- , so the drug derivative ends up irreversibly attached to both the enzyme and the cofactor.
- Development of such drugs requires understanding the microbiology of the pathogen, an understanding of metabolic pathways in order to choose a target enzyme (and in this case, also understanding the difference in turnover rate of the target enzyme itself between host and pathogen), and of course an understanding of enzymology, the chemical mechanism of the enzyme. (It also helps to know the structure of the enzyme, in particular the structure of its active site, to design a drug that's a good "fit".)

Effect of pH on enzyme-catalyzed reactions

- Alteration in pH is not usually an important regulatory mechanism in biological systems, but the effect of pH can be highly informative about the mechanism -- see linked function diagram below..
- Changing pH can *increase* or *decrease* rate of an enzyme-catalyzed reaction by changing state of ionization (protonation/deprotonation) of one or more specific functional groups, which can be either on the enzyme:
 - pH can affect catalytic activity (k_{cat}).
 - pH can affect substrate binding (K_{ES}).
 - pH can affect the structure or stability of the enzyme in a less specific way (usually only a problem at extremes of pH)
- or on the substrate: state of ionization of substrate can have an effect either on k_{cat} or on K_{ES} .



- (Analogous linked functions diagram could be drawn to illustrate effect of state of ionization of substrate on pK_a values.)
- "pH-rate profile" = plot of velocity vs. pH (but one could also look at effect of pH on K_m , or explicitly on V_{max} , or V_{max} / K_m , rather than just velocity at a fixed substrate concentration)

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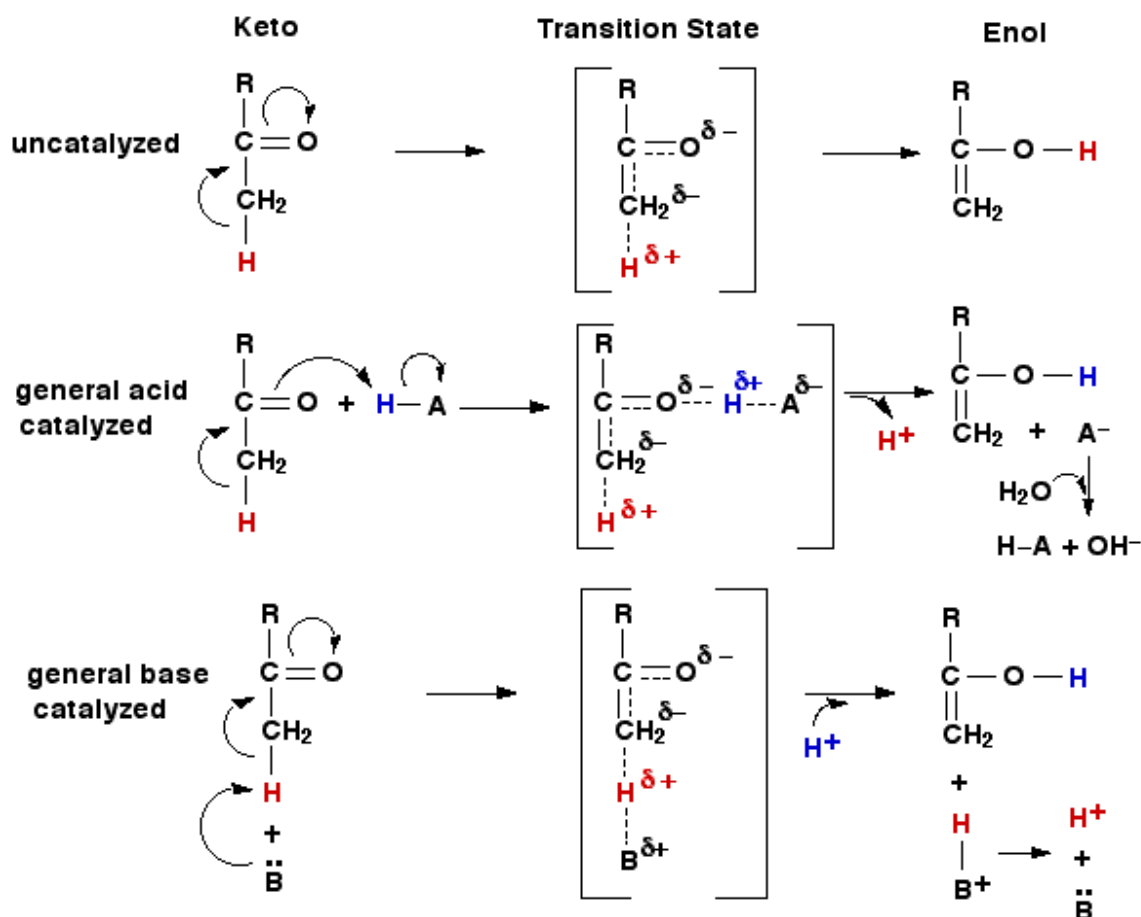
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- Bell-shaped pH-rate profile below: there must be 2 ionizable groups whose state of ionization affects velocity.

Mechanism of action of enzymes

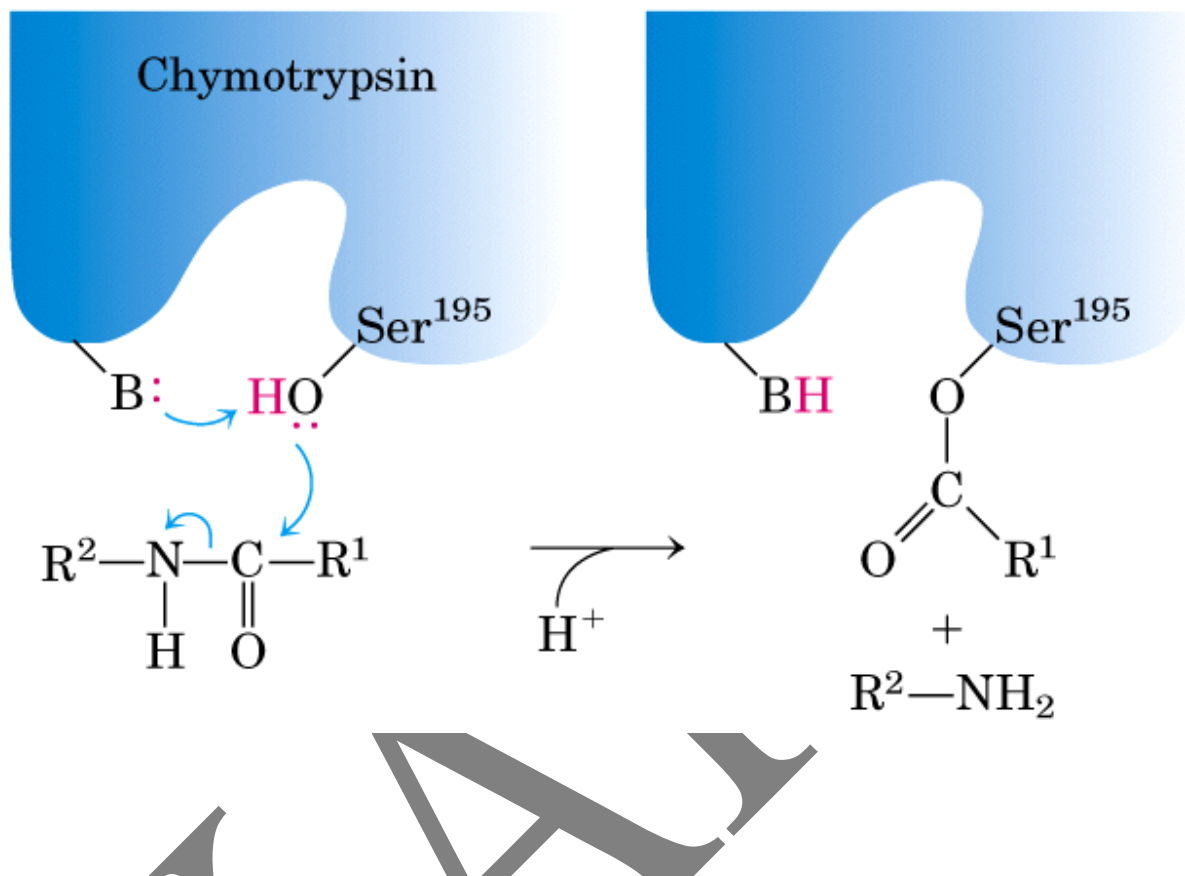
Chymotrypsin

- very often-used mechanism in enzyme reactions, e.g., hydrolysis of ester/ peptide bonds, phosphate group reactions, addition to carbonyl groups, etc.
- Enzyme *avoids unstable charged intermediates in reaction* (which would have high free energies) by having groups appropriately located to
 - donate a proton (act as a *general acid*), or
 - accept a proton (abstract a proton, act as a *general base*)
- If a group *donates* a proton (acts as a general acid) in chemical mechanism, it has to *get a proton (a different one!) back* (act as a general base) by end of catalytic cycle, and vice versa.
- Protein functional groups that can function as general acid/base catalysts:
 - His imidazole
 - -amino group
 - -carboxyl group
 - thiol of Cys
 - R group carboxyls of Glu, Asp
 - -amino group of Lys
 - aromatic OH of Tyr
 - guanidino group of Arg
- Obviously, pH influences state of protonation of enzyme functional groups, so catalytic activity of enzymes using general acid-base catalysis is *sensitive to pH*.
- Example: Mechanisms of uncatalyzed, acid-catalyzed, and base-catalyzed keto-enol tautomerization
(redrawn from Fig. 11-6, Voet, Voet & Pratt, *Fundamentals of Biochemistry*, 1999)
- General acid catalysis: partial proton transfer from an acid lowers the free energy of the high-free energy carbanionlike transition state of the keto-enol tautomerization.
- Alternatively, the rate can be increased by partial proton abstraction by a base.
- *Concerted acid-base catalyzed reactions* involve both processes occurring simultaneously.



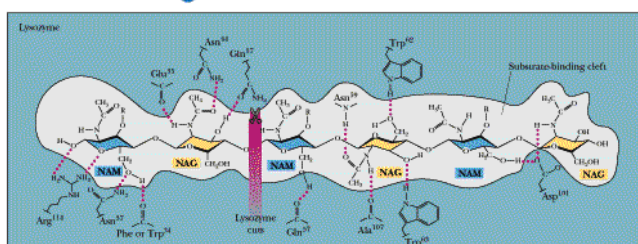
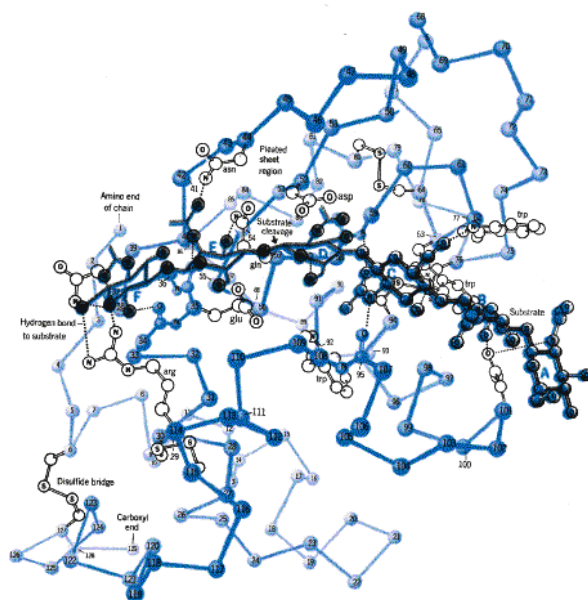
6. Covalent catalysis (also sometimes called nucleophilic catalysis):

- very often used in enzyme mechanisms
- rate enhancement by the transient formation of a catalyst-substrate covalent bond
- side chains of His, Cys, Asp, Lys and Ser can participate in covalent catalysis by acting as nucleophiles
- coenzymes pyridoxal phosphate and thiamine pyrophosphate function mainly by covalent catalysis
- Example of covalent catalysis coupled to general acid-base catalysis: Fig. First step in chymotrypsin-catalyzed peptide bond hydrolysis -- *general base catalyzed formation of covalent acyl-enzyme intermediate*



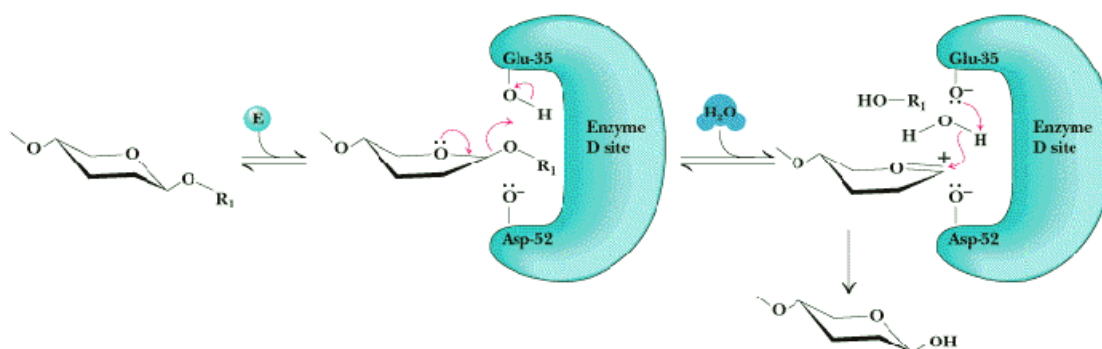
Lysozyme

- Lysozyme catalyzes the hydrolysis of the $\beta(1 \rightarrow 4)$ linkage between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) in bacterial cell wall polysaccharides and also the $\beta(1 \rightarrow 4)$ -linked poly NAG in chitin.
- 6 sugar residues of the polysaccharide substrate fit into the active site as shown in the figure below (structure of lysozyme and of its active site) -- cleavage is between the 4th and 5th residues *from the right* in active site diagram at bottom of figure.
- 4th sugar residue (adjacent to bond to be cleaved) has to be distorted out of its preferred chair conformation to fit into active site, and the inference is that the distorted structure is closer to the structure of the transition state for hydrolysis -- enzyme uses some of the favorable binding energy from binding the *rest* of the substrate structure to raise the substrate closer to the transition state, *stabilizing the transition state relative to the ES complex*.
- Fig. Structure and active site of lysozyme



- Hydrolysis reaction is accelerated also by participation of Glu³⁵ and Asp⁵².
- Bell-shaped pH-activity profile for lysozyme suggests involvement of ionizable groups with pK_a values of about 5.9 and 4.5.
- Glu³⁵ is in a nonpolar environment and has a higher than expected pK_a, and has to be *protonated* at start of reaction because it first must *donate* a proton to the oxygen of the leaving group from hydrolysis (an alcohol), and subsequently accepts a proton from H₂O, the 2nd substrate.
- Asp⁵² has a lower pK_a, 4.5, close to expected value for an R group carboxyl, and it must be *unprotonated* for activity because it's required to stabilize carbonium ion intermediate (+) formed when the alcohol leaves, until carbonium ion can react with H₂O, completing hydrolysis reaction:

Fig. Lysozyme mechanism



The lysozyme mechanism illustrates

- Transition state stabilization
- Acid-base catalysis
- Electrostatic catalysis
- Several residues in the protein participate in substrate binding.
- The binding of NAM4 in the chair conformation is unfavorable.
- But the binding of residue 4 in the half-chair conformation is favorable (preferential transition state binding).
- Hydrolysis involves acid-base catalysis:
- Glu35 serves as a proton donor to the oxygen of the leaving alcohol.
- The resulting carbonium ion (+) is stabilized by the ionized side chain of Asp52 (electrostatic catalysis) until it can react with water. (lysozyme)
- Many glycosidases utilize a covalent intermediate in their mechanism. Here is a good example of transition state stabilization using a bacterial glycosidase.
- Another excellent example that clearly shows how transition state stabilization works is found in the structure of this transferase.

Enzyme based diagnostic techniques

The measurement of the serum levels of numerous enzymes has been shown to be of diagnostic significance. This is because the presence of these enzymes in the serum indicates that tissue or cellular damage has occurred resulting in the release of intracellular components into the blood. Hence, when a physician indicates that he/she is going to assay for liver enzymes, the purpose is to ascertain the potential for liver cell damage. Commonly assayed enzymes are the amino transferases: alanine transaminase, ALT (sometimes still referred to as serum glutamate-pyruvate aminotransferase, SGPT) and aspartate aminotransferase, AST (also referred to as serum glutamate-oxaloacetate aminotransferase, SGOT); lactate dehydrogenase, LDH; creatine kinase, CK (also called creatine phosphokinase, CPK); gamma-glutamyl transpeptidase, GGT. Other enzymes are assayed under a variety of different clinical situations. Many enzymes are involved

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(BATCH-2019-2021)**

in the clinical diagnoses of various diseases in human and veterinary medicine. These enzymes facilitate or enhance rapid diagnoses of these diseases. These enzymes could be classified into many classes. They are:

Alkaline phosphatase: Alkaline phosphatases were the earliest serum enzymes to be recognized to have clinical significance, when in the 1920s, it was discovered that they increase in bone and liver diseases. Since then, they have been the subject of more publications than any other enzyme. Alkaline phosphates are a group of isoforms which hydrolyse many types of phosphate esters, whose natural substrate or substrates are unknown. The term '>alkaline' refers to the optimal alkaline pH of this class of phosphatases *in vitro*. In both humans and animals, the major sources of ALPs are the liver, bone, kidney and placenta. In humans, it is involved in bone and hepatobiliary diseases. ALPs are also of diagnostic importance in animal diseases. Total serum ALP activity has diagnostic value in the hepatic and bone diseases in dogs and cats. It is of little value in hepatic diseases of horses and ruminants because of the broad range of reference values against which the patients' values must be compared. The range of serum ALP value in goats may be 10-fold with no evidence of hepatic damage. Values within the individual are fairly constant for sequential evaluation.

Creatine kinase: Creatine kinase isozymes are the most organ-specific serum enzymes in clinical use. They catalyse the reversible phosphorylation of creatine by ATP to form creatine phosphate, the major storage form of high-energy phosphate required by muscle. Creatine kinases are found in many parts of the body like the heart, brain, skeletal muscle and smooth muscle but they have their highest specific activity in the skeletal muscle. In humans, Creatine kinase is associated with myocardial infarction and muscle diseases. Increase in Creatine kinase in cerebrospinal fluid has been associated with a number of disorders in dogs, cats, cattle and horses. The Creatine kinase are such sensitive indicators of muscle damage that, generally, only large increases in serum activity are of clinical significance.

Alanine aminotransferase: It was formerly known as Glutamic Pyruvate Transaminase; (GPT). It catalyses the reversible transamination of L-alanine and 2-oxoglutarate to pyruvate and glutamate in the cytoplasm of the cell. ALT can be found in the liver, skeletal muscle and heart. The greatest specific activity of ALT in primates, dogs, cats, rabbits and rats is in the liver. It is a well established, sensitive liver-specific indicator of damage. However, ALT in the tissues of pigs, horses, cattle, sheep or goats is too low to be of diagnostic value. It is used as an indicator of hepatopathy in toxicological studies which use small laboratory rodents as well as dogs.

Aspartate aminotransferase: It was formerly called Glutamic Oxaloacetic Transaminase; (GOT). It catalyses the transamination of L-aspartate and 2-oxoglutarate to oxaloacetate and glutamate. AST is found in skeletal muscle, heart, liver, kidney and erythrocytes and is associated with myocardial, hepatic parenchymal and muscle diseases in humans and animals. The presence of AST in so many tissues make their serum level a good marker of soft tissue but precludes its use as an organ-specific enzyme. Red blood cells contain a large amount of AST which leaks into plasma before haemolysis is seen.

Sorbitol dehydrogenase (SDH): It is also called L-iditol dehydrogenase; (IDH). It catalyses the reversible oxidation of D-sorbitol to D-fructose with the cofactor NAD. The plasma activity is

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low in dog and horse plasma but appreciably greater in cattle, sheep, and goat serum. Aside from the testes, it is found in appreciable amounts only in hepatocytes. As a result of this, an increase in plasma SDH is consistent with hepatocyte damage. SDH is liver specific in humans and all species of animals and hepatic injury appears to be the only source of increased SDH activity. Although SDH is liver specific in all species, the already established usage of ALT in dogs and cats has limited SDH as a diagnostic indicator of hepatocellular damage to horses, cattle, sheep and goats.

Lactate dehydrogenases (LDH): It catalyses the reversible oxidation of pyruvate to L(+) lactate with the cofactor NAD. The equilibrium favours lactate formation, but the preferred assay method is in the direction of pyruvate because pyruvate has an inhibitory effect on LDH. Lactate dehydrogenase has isoenzymes. LDH can be found in the heart, liver, erythrocyte, skeletal muscle, platelets and lymph nodes. In humans, it is involved in myocardial infarction, haemolysis and liver disease. LDH isoenzyme profiles were the first isoenzyme profiles used in clinical veterinary medicine in an attempt to detect specific organ damage. The introduction of more highly organ-specific procedures has resulted in LDH no longer being in common use in veterinary medicine

Cholinesterase (ChE): Serum cholinesterase (ChE) activity is composed of two distinct cholinesterases. The major substrate is acetylcholine, the neurotransmitter found at the myoneural junction. Acetylcholinesterase (AChE; EC 3.1.1.7) found at the myoneural junction is the true ChE and is essential in hydrolyzing acetylcholine so that the junction can be reestablished and prepared for additional signals. The myoneural junction AChE is also found in Red Blood Cells (RBC), mouse, pig, brain and rat liver. Only a small amount of AChE is found in plasma. The ChE of plasma is a pseudocholine sterase, butylcholinesterase (ButChE; EC 3.1.1.8), which hydrolyses butyrylcholine four times faster than acetylcholine and is also located in white matter of the brain, liver, pancreas and intestinal mucosa. Decreases in ButChE have been reported in humans with acute infection, muscular dystrophy, chronic renal disease and pregnancy, as well as insecticide intoxication.

Lipase: Serum pancreatic lipases (EC 3.1.1.3; triacylglycerol lipase) catalyse the hydrolysis of triglycerides preferentially at the 1 and 3 positions, releasing two fatty acids and a 2-monoglyceride. Lipase can be found in the pancreas and hepatobiliary tract and is involved in pancreatitis and hepatobiliary disease.

Amylase: Amylases are calcium-dependent metalloenzymes that randomly catalyze the hydrolysis of complex carbohydrates, e.g., glycogen at the -1-4 linkages. The products of this action are maltose and limit dextrins. The enzyme is a Ca_2^+ metalloenzyme which requires one of a number of activator ions such as Cl^- or Br^- . Amylase can be found in the salivary glands, pancreas and ovaries and is used as a diagnostic aid for pancreatitis.

Glutamyl transferase: This is a carboxypeptidase which cleaves C-terminal glutamyl groups and transfers them to peptides and other suitable acceptors. It is speculated that GGT is associated with glutathione metabolism. The major sources are the liver and kidney and are involved in hepato biliary disease and alcoholism. Cholestatic disorders of all species examined result in increased serum GGT activity.

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Trypsin: Trypsins are serum proteases which hydrolyse the peptide bonds formed by lysine or arginine with other **amino acids**. The pancreas as the zymogen trypsinogen, which is converted to trypsin by intestinal enterokinase or trypsin itself, secretes them.

Glutathione peroxidases: These are metalloenzymes containing four atoms of selenium per molecule of enzyme. They catalyze the oxidation of reduced glutathione by peroxide to form water and oxidized glutathione. Because of the high concentration of selenium in glutathione peroxidases, there is a good direct correlation between the amount of red blood cell GPx activity and the selenium concentration of other organs. Other enzymes with disease diagnosis applications are acid phosphatase (ACP), found in prostate and erythrocytes and are used in diagnosis of prostate carcinoma. Aldolase (ALD), found in skeletal muscle and heart and involved in muscle disease. Glutamate dehydrogenase (GLDH), found in the liver is used to diagnose hepatic parenchymal disease. Hydroxybutyrate dehydrogenase (HBD), which is the heart form of lactate dehydrogenase is involved in myocardial infarction. Just as enzyme assay is used to diagnose diseases in humans and animals, it may also be applied to the investigation of diseases in plants. For example, it has been found that an injury (either mechanical or pathogenic) results in a marked, localized increase in the activity of glucose-6-phosphate dehydrogenase, but not of glucose phosphate isomerase, indicating diversion of glucose breakdown from glycolysis to the pentose phosphate pathway.

Enzymes used in immunoassays

Enzymes may also be used as an alternative to radioisotopes as markers in immunoassays have been used for the determination of a variety of proteins and hormones. The role of enzymes in immunoassay used to replace radioisotopes as markers, since they are not hazardous to health and can be detected by techniques which are more generally available. Any enzyme with a sensitive and convenient assay procedure can be used for this purpose. Two common examples of enzyme immunoassay (EIA) procedures are enzyme-linked immunosorbent assay (ELISA) and Enzyme-Multiplied Immunoassay Test (EMIT). ELISA is a highly sensitive assay that can be used to detect either antigen or antibody. Applications of ELISA include diagnostics for noninfectious diseases involving hormones, drugs, serum components, oncofetal proteins, or autoimmune diseases, as well as diagnostics for infectious diseases caused by bacterial, viral, mycotic or parasitic organisms. The enzymes frequently used in ELISA are Horseradish peroxidase, alkaline phosphatase and β -galactosidase. In EMIT, the activity of malate dehydrogenase is assayed by standard enzyme methodology for the detection of thyroxine by Enzyme-labeled immunoassay.

Enzymes acted as therapeutic agents

In a few cases enzymes have been used as drugs in the therapy of specific medical problems. Streptokinase is an enzyme mixture prepared from streptococcus. It is useful in clearing blood clots that occur in the lower extremities. Streptokinase activates the fibrinolytic proenzyme plasminogen that is normally present in plasma. The activated enzyme is plasmin is a serine protease like trypsin that attacks fibrin, cleaving it into several soluble components. Another enzyme of therapeutic importance is asparaginase. Asparaginase therapy is used for some types of adult leukemia. Tumor cells have a nutritional requirement for asparagine and must scavenge

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it from the host's plasma. By administering asparaginase i.e., the host's plasma level of asparagine is markedly depressed, which results in depressing the viability of the tumor. Enzyme replacement in individuals that are genetically deficient in a particular enzyme are also applications of enzymes as therapeutic agents. Also, enzymes such as u-plasminogen activator, formerly known as urokinase, extracted from human urine, can be infused into the blood stream of patients at risk from a pulmonary embolism (a fragment of a blood-clot lodging in the pulmonary artery): these enzymes stimulate a cascade system responsible for the production of active plasmin, a proteolytic enzyme which digests fibrin, the main structural component of blood-clots. Some enzymes may also be used to restrict the growth of cancer cells by depriving them of essential nutrients: for example, Lasparaginase may be used in the treatment of several types of leukaemia, since the tumour cells, in contrast to normal cells, have a requirement for exogenous Lasparagine. Another example of therapeutic application of enzymes is the use of immobilized enzymes as components of artificial kidney machines, which are used to remove urea and other waste products from the body, where kidney disease prevents this being done by natural processes. Urea enters the machine from the blood, by dialysis (termed haemodialysis) and is converted to CO_2 and NH_4^+ by immobilized urease; toxic NH_4^+ is then either trapped on ion exchange resins or incorporated into glutamate by the action of immobilized glutamate dehydrogenase linked to alcohol dehydrogenase to ensure coenzyme recycling, before the fluid is returned to the blood stream.

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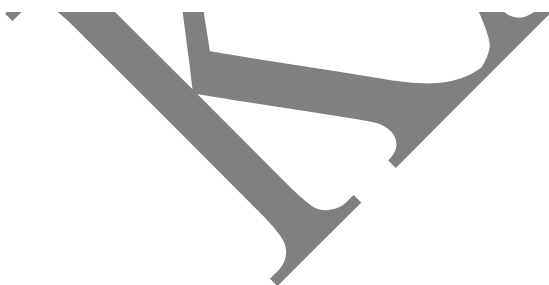
POSSIBLE QUESTIONS

Two marks

1. Differentiate competitive and uncompetitive inhibition.
2. What is an isoenzyme?
3. Name any three antibiotics.
4. Define antibiotic.
5. Define MM equation.
6. What is a multienzyme complex?
7. Define enzyme inhibition.
8. Define proteolytic cleavage.

Essay type questions

1. Write short notes on multienzyme complex and isoenzymes.
2. Discuss on enzyme based diagnostic techniques.
3. Write short notes on differentiating bi square mechanisms.
4. Comment on antibiotics as inhibitors.
5. Write a brief note on LB plot, Hofstee plot and Hanes plot.
6. Write short notes for the following. i) Proteolytic cleavage
ii) Multi enzyme complex
7. Discuss on the derivation of Michaelis-Menten equation
8. Write short notes on enzyme based diagnostic techniques.
9. Explain about enzyme inhibition.
10. Comment on mechanism of action of enzymes.



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I-M.Sc., BIOCHEMISTRY
ENZYMES AND MICROBIAL TECHNOLOGY (19BCP102)
MULTIPLE CHOICE QUESTIONS
UNIT II

S.No	Questions	Option A	Option B	Option C	Option D	Answer
1	The enzyme aspartokinase is regulated by the phenomena of	Compartmentation	Enzyme mutiplicity	Induction and repression	Feed forward stimulation	Feed forward stimulation
2	The quarternary T_R conformational change in heamoglobin is due to the	changes in primary structure	changes in secondary structure	changes in tertiary structure	all options	all options
3	Which of the following is not a serine protease	Papain	Chymotrypsin	Trypsin	Carboxypeptiase	Chymotrypsin
4	The sigmoidal kinetics enzyme due to	presence of subunits in the enzyme	binding of one substrate modulates the binding of the next substrate molecule	the allosteric inhibitor prevents binding of substrate by competing for active site	the inhibitor denatures the enzyme.	presence of subunits in the enzyme
5	The enzyme specific activity is expressed as	Moles per second per mg protein	Moles per liter	Moles per minute	Grams per liter	Moles per liter
6	The thermostability of an enzyme can increased by	adding disulfied bonds	changing non-essential asparagine residues	changing non-essential glutamine residues	all the above	adding disulfied bonds

7	Which is the negative heterotropic effector of O ₂ binding haemoglobin	BPG	CO ₂	H ⁺	All the above	All the above
8	High specificity of peptide bonds on COO side of the cationic amino acid found in enzyme.	carboxypeptidase	chymotrypsin	trypsin	pepsin	chymotrypsin
9	A sigmoidal plot of substrate concentration ([S]) versus reaction velocity (V) may indicate	Michaelis-Menten kinetics	Co-operative binding	Competitive inhibition	Non-competitive inhibition	Co-operative binding
10	The K _m of the enzyme giving the kinetic data as below is	-0.50	-0.25	0.25	0.33	0.33
11	Allosteric inhibitor usually increases the sigmoidal nature of	MM plot	LB plot	Eadie plot	Hanes plot	MM plot
12	K _m and rate of the reactions are	directly proportional	inversely proportional	independent with each other	not related	inversely proportional
13	The non-protein, inorganic part of the	cofactor	coenzymes	metallo enzymes	holoenzymes	cofactor

	enzyme is known as					
14	The steady state theory was proposed by	Michaelis and mentan	Hill	Eadie	Brigges and Handane	Michaelis and mentan
15	A competitive inhibitor	increases km	decreases km	decreases km and Vmax	increases Vmax	increases km
16	At half-maximal velocity the substrate concentration will be equal to	Km value	Km/2	100Km	All the above	Km value
17	The binding of oxygen to hemoglobin is an example of	-ve cooperativity	feed back inhibition	single amplification	feed forward stimulation	feed back inhibition
18	The T state of haemoglobin is stabilized by	hydrogen bonding	C-terminal salt bridges	hydrophobic interaction	vander Waals' forces.	vander Waals' forces.
19	The T___R conformational shift is triggered by	Movement of Fe (II) into the hemo plane	Network of salt bridges	Iron-oxygen bonds	None of the above	Movement of Fe (II) into the hemo plane
20	The lineweaver-burk plot is	linear	Sigmoidal	Hyperbolic	Parabolic	linear
21	Multiple forms of the same enzymes are	Zymogens	Isoenzymes	Proenzymes	Pre-enzymes	Isoenzymes

	known as					
22	A competitive inhibitor of an enzyme has which of the following properties?	It is frequently a feedback inhibitor	It becomes covalently attached to an enzyme	It decreases the V_{max}	It interferes with substrate binding to the enzyme	It interferes with substrate binding to the enzyme
23	How many different proteins may be present in normal LDH?	One	Two	Three	Four	Four
24	The number of enzymes known is about	10,000	100	50	26	50
25	Part of enzyme which combines with nonprotein part to form functional enzyme is	Apoenzyme	Coenzyme	Prosthetic group	None of these	Prosthetic group
26	Who got Nobel Prize in 1978 for working on enzymes?	Koshland	Arber and Nathans	Nass and Nass	H.G. Khorana	Koshland
27	Site of enzyme synthesis in a cell is	Ribosomes	RER	Golgi bodies	all options	RER

28	Which enzyme is concerned with transfer of electrons?	Desmolase	Hydrolase	Dehydrogenase	Transaminase	Desmolase
29	The best example of extracellular enzymes (exoenzyme) is	Nucleases	Digestive enzymes	Succinic dehydrogenase	None of these	Succinic dehydrogenase
30	A metabolic pathways is a	Route taken by chemicals	Sequence of enzyme facilitated chemical reactions	Route taken by an enzyme from one reaction to another	Sequence of origin of organic molecules	Sequence of enzyme facilitated chemical reactions
31	The pH optima for sucrase is	5.0–7.0	5.8–6.2	5.4–6.0	8.6	5.0–7.0
32	The substrate for amylase is	Cane sugar	Starch	Lactose	Ribose	Starch
33	Lineweaver – Burk double reciprocal plot is related to	Substrate concentration	Enzyme activity	Temperature	Both (A) and (B)	Both (A) and (B)
34	Enzymes which are always present in an organism are known as	Inducible enzymes	Constitutive enzymes	Functional enzymes	Apoenzymes	Constitutive enzymes
35	Inactive precursors of enzymes are known as	Apoenzymes	Coenzymes	Proenzymes	Holoenzymes	Proenzymes
36	Which of the following is a proenzyme?	Carboxypeptidase	Aminopeptidase	Chymotrypsin	Pepsinogen	Pepsinogen

37	An inorganic ion required for the activity of an enzyme is known as	Activator	Cofactor	Coenzyme	None of these	Cofactor
38	The first enzyme found to have isoenzymes was	Alkaline Phosphatase	Lactate dehydrogenase	Acid Phosphatase	Creatine kinase	Lactate dehydrogenase
39	Enzymes accelerate the rate of reactions by	Increasing the equilibrium constant of reactions	Increasing the energy of activation	Decreasing the energy of activation	Decreasing the free energy change of the reaction	Decreasing the energy of activation
40	Kinetics of an allosteric enzyme are explained by	Michaelis-Menten equation	Lineweaver-Burk plot	Hill plot	all options	Hill plot
41	Covalent modification of an enzyme usually involves phosphorylation / dephosphorylation of	Serine residue	Proline residue	Hydroxylysine residue	Hydroxyproline residue	Serine residue
42	V _{max} of an enzyme may be affected by	pH	Temperature	Non-competitive inhibitors	all options	all options
43	In enzyme assays, all the following are kept constant except	Substrate concentration	Enzyme concentration	pH	Temperature	Enzyme concentration

44	If the substrate concentration is much below the K_m of the enzyme, the velocity of the reaction is	Directly proportional to substrate concentration	Not affected by enzyme concentration	Nearly equal to V_{max}	Inversely proportional to substrate concentration	Directly proportional to substrate concentration
45	Different isoenzymes of an enzyme have the same	Amino acid sequence	Michaelis constant	Catalytic activity	all options	Catalytic activity
46	Ribozymes:	RNA enzyme	Non-protein enzymes	Catalyst function	all options	all options
47	All the following are coenzymes except	Ubiquinone	CoA	Pyruvate dehydrogenase	Lipoic acid	Pyruvate dehydrogenase
48	Which of the following is not a cofactor?	Mg	Iron	Cu	Methylcobalamine	Methylcobalamine
49	Maximum enzyme activity is observed at	Acidic pH	Neutral pH	Basic pH	Optimum pH	Optimum pH
50	The following reaction is characteristic of what type of enzymes? $2H_2O_2 \rightarrow 2H_2O + O_2$	Peroxides	Catalase	Dehydrogenase	Copper containing oxidases	Catalase

51	The kinetic effect of purely competitive inhibitor of an enzyme	Increases K_m without affecting V_{max}	Decreases K_m without affecting V_{max}	Increases V_{max} without affecting K_m	Decreases V_{max} without affecting K_m	Increases K_m without affecting V_{max}
52	Isoenzymes are	Chemically, immunologically and electrophoretically different forms of an enzyme	Different forms of an enzyme similar in all properties	Catalysing different reactions	Having the same quaternary structures like the enzymes	Chemically, immunologically and electrophoretically different forms of an enzyme
53	Isoenzymes can be characterized by	Proteins lacking enzymatic activity that are necessary for the activation of enzymes	Proteolytic enzymes activated by hydrolysis	Enzymes with identical primary structure	Similar enzymes that catalyse different reaction	Proteolytic enzymes activated by hydrolysis
54	The isoenzymes of LDH	Differ only in a single amino acid	Differ in catalytic activity	Exist in 5 forms depending on M and H monomer contents	Occur as monomers	Exist in 5 forms depending on M and H monomer contents
55	The isoenzymes LDH5 is elevated in	Myocardial infarction	Peptic ulcer	Liver disease	Infectious diseases	Liver disease
56	On the third day of onset of acute myocardial infarction	Serum AST	Serum CK	Serum LDH	Serum ALT	Serum LDH

	the enzyme elevated is					
57	LDH1 and LDH2 are elevated in	Myocardial infarction	Liver disease	Kidney disease	Brain disease	Myocardial infarction
58	Acute pancreatitis is characterised by	Lack of synthesis of zymogen enzymes	Continuous release of zymogen enzymes into the gut	Premature activation of zymogen enzymes	Inactivation of zymogen enzymes	Premature activation of zymogen enzymes
59	The pH optima for salivary analyse is	6.6–6.8	2.0–7.5	7.9	8.6	6.6–6.8
60	The pH optima for pancreatic analyse is	4	7.1	7.9	8.6	8.6

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COURSE CODE: 19BCP102

**UNIT-III: IMMOBILIZATION OF ENZYMES
(BATCH-2019-2021)**

UNIT III
SYLLABUS

Immobilization of enzymes: Methods of immobilization - adsorption, covalent binding, entrapment, membrane confinement. Effect of immobilization on enzyme. Use of enzymes in clinical diagnosis and industry. Enzyme engineering. Artificial enzymes and synzymes, Abzymes, ribozymes, enzymes in organic solvents.

Biosensors - Glucose oxidase, Cholesterol oxidase, Urease and antibiotics as biosensors

Immobilization of enzymes (or cells) refers to the technique of confining/anchoring the enzymes (or cells) in or on an inert support for their stability and functional reuse. By employing this technique, enzymes are made more efficient and cost-effective for their industrial use. Some workers regard immobilization as a goose with a golden egg in enzyme technology. Immobilized enzymes retain their structural conformation necessary for catalysis.

There are several advantages of immobilized enzymes:

- Stable and more efficient in function.
- Can be reused again and again.
- Products are enzyme-free.
- Ideal for multi-enzyme reaction systems.
- Control of enzyme function is easy.
- Suitable for industrial and medical use.
- Minimize effluent disposal problems.

There are however, certain disadvantages also associated with immobilization.

- The possibility of loss of biological activity of an enzyme during immobilization or while it is in use.
- Immobilization is an expensive affair often requiring sophisticated equipment.

Immobilized enzymes are generally preferred over immobilized cells due to specificity to yield the products in pure form. However, there are several advantages of using immobilized multi-enzyme systems such as organelles and whole cells over immobilized enzymes. The immobilized cells possess the natural environment with cofactor availability (and also its regeneration capability) and are particularly suitable for multiple enzymatic reactions.

Methods of Immobilization

The commonly employed techniques for immobilization of enzymes are—adsorption, entrapment, covalent binding and cross-linking.

Adsorption

Adsorption involves the physical binding of enzymes (or cells) on the surface of an inert support. The support materials may be inorganic (e.g. alumina, silica gel, calcium phosphate gel, glass) or organic (starch, carboxymethyl cellulose, DEAE-cellulose, DEAE-sephadex).

Adsorption of enzyme molecules (on the inert support) involves weak forces such as vander Waals forces and hydrogen bonds (Fig. 21.3). Therefore, the adsorbed enzymes can be easily removed by minor changes in pH, ionic strength or temperature. This is a disadvantage for industrial use of enzymes.

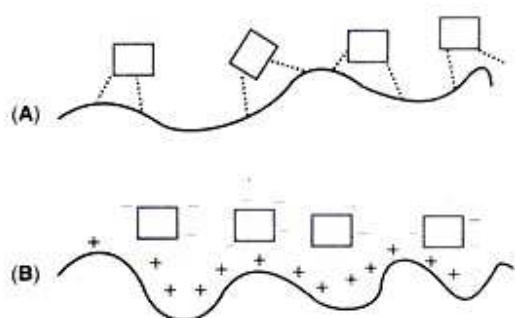


Fig. 21.3 : Immobilization of enzymes by adsorption
(A) By van der Waals forces (B) By hydrogen bonding (Note : Coloured blocks represent enzymes)

Covalent Binding:

Immobilization of the enzymes can be achieved by creation of covalent bonds between the chemical groups of enzymes and the chemical groups of the support (Fig. 21.5). This technique is widely used. However, covalent binding is often associated with loss of some enzyme activity. The inert support usually requires pretreatment (to form pre-activated support) before it binds to enzyme. The following are the common methods of covalent binding.

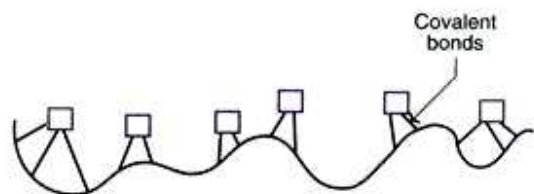


Fig. 21.5 : A general representation of immobilization of enzymes by covalent binding (Note : coloured blocks represent enzymes).

1. Cyanogen bromide activation:

The inert support materials (cellulose, sepharose, sephadex) containing glycol groups are activated by CNBr, which then bind to enzymes and immobilize them (Fig. 21.6A).

2. Diazotization:

Some of the support materials (amino benzyl cellulose, amino derivatives of polystyrene, aminosilanized porous glass) are subjected to diazotization on treatment with NaNO_2 and HCl. They, in turn, bind covalently to tyrosyl or histidyl groups of enzymes (Fig. 21.6B).

3. Peptide bond formation:

Enzyme immobilization can also be achieved by the formation of peptide bonds between the amino (or carboxyl) groups of the support and the carboxyl (or amino) groups of enzymes (Fig. 21.6C). The support material is first chemically treated to form active functional groups.

4. Activation by bi- or poly-functional reagents:

Some of the reagents such as glutaraldehyde can be used to create bonds between amino groups of enzymes and amino groups of support (e.g. aminoethylcellulose, albumin, amino alkylated porous glass). This is depicted in Fig. 21.6D.

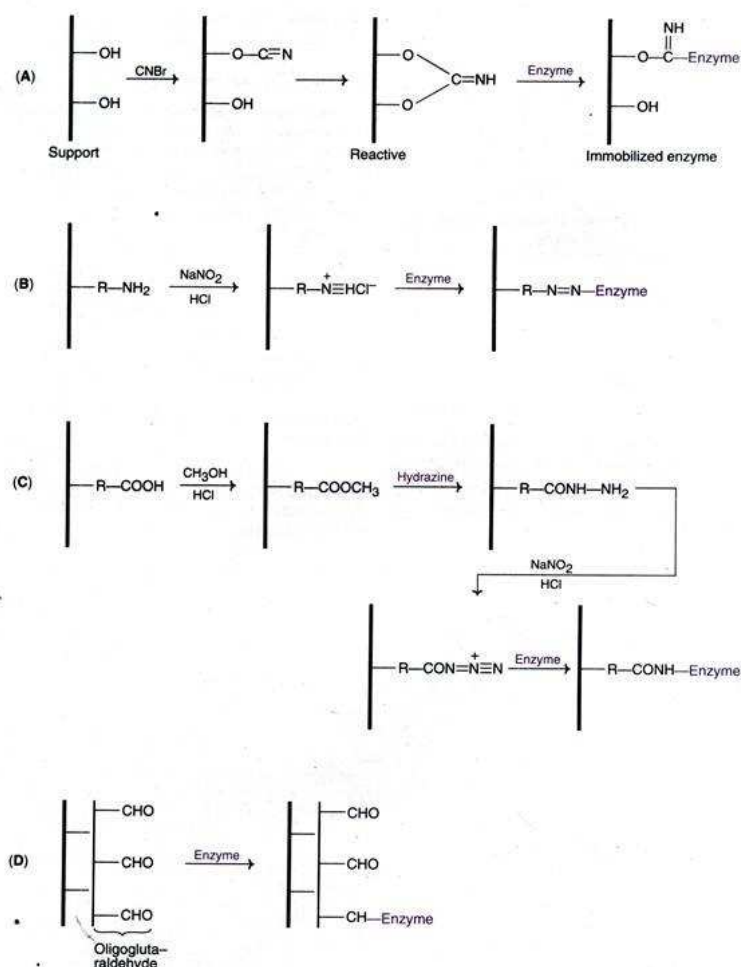


Fig. 21.6 : Immobilization of enzymes by covalent binding (A) Cyanogen bromide activation, (B) Diazotization, (C) Peptide bond formation, (D) Activation by bifunctional agent.

Entrapment

Enzymes can be immobilized by physical entrapment inside a polymer or a gel matrix. The size of the matrix pores is such that the enzyme is retained while the substrate and product molecules

pass through. In this technique, commonly referred to as lattice entrapment, the enzyme (or cell) is not subjected to strong binding forces and structural distortions.

Some deactivation may however, occur during immobilization process due to changes in pH or temperature or addition of solvents. The matrices used for entrapping of enzymes include polyacrylamide gel, collagen, gelatin, starch, cellulose, silicone and rubber. Enzymes can be entrapped by several ways.

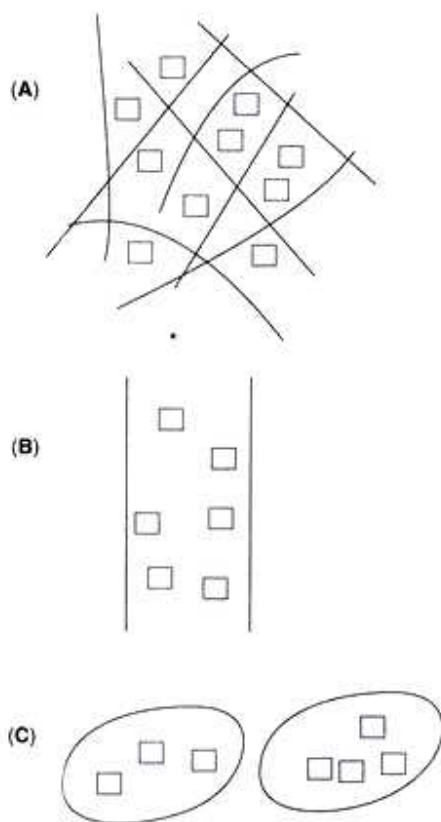


Fig. 21.4 : Immobilization of enzymes by entrapment
(A) Inclusion in gels (B) Inclusion in fibres
(C) Inclusion in microcapsules (Note : Coloured blocks represent enzymes)

1. Enzyme inclusion in gels:

This is an entrapment of enzymes inside the gels (Fig. 21.4A).

2. Enzyme inclusion in fibres:

The enzymes are trapped in a fibre format of the matrix (Fig. 21.4B).

3. Enzyme inclusion in microcapsules:

In this case, the enzymes are trapped inside a microcapsule matrix (Fig. 21.4C). The hydrophobic and hydrophilic forms of the matrix polymerise to form a microcapsule containing enzyme

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molecules inside. The major limitation for entrapment of enzymes is their leakage from the matrix. Most workers prefer to use the technique of entrapment for immobilization of whole cells. Entrapped cells are in use for industrial production of amino acids (L-isoleucine, L-aspartic acid), L-malic acid and hydroquinone.

Membrane confinement

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 ($> 20 \text{ m}^2 \text{ L}^{-1}$) 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

Microencapsulation:

Microencapsulation is a type of entrapment. It refers to the process of spherical particle formation wherein a liquid or suspension is enclosed in a semipermeable membrane. The membrane may be polymeric, lipoidal, lipoprotein-based or non-ionic in nature. There are three distinct ways of microencapsulation.

1. Building of special membrane reactors.
2. Formation of emulsions.
3. Stabilization of emulsions to form microcapsules.

Microencapsulation is recently being used for immobilization of enzymes and mammalian cells. For instance, pancreatic cells grown in cultures can be immobilized by microencapsulation. Hybridoma cells have also been immobilized successfully by this technique.

Cross-Linking:

The absence of a solid support is a characteristic feature of immobilization of enzymes by cross-linking. The enzyme molecules are immobilized by creating cross-links between them, through the involvement of poly-functional reagents. These reagents in fact react with the enzyme molecules and create bridges which form the backbone to hold enzyme molecules (Fig. 21.7). There are several reagents in use for cross-linking. These include glutaraldehyde, diazobenzidine, hexamethylene diisocyanate and toluene di-isothiocyanate.

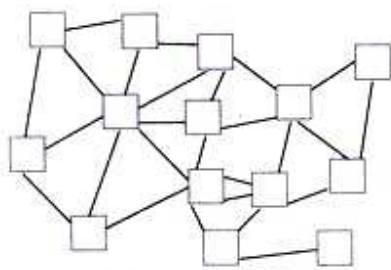


Fig. 21.7 : Immobilization of enzyme molecules by cross linking.

Glutaraldehyde is the most extensively used cross-linking reagent. It reacts with lysyl residues of the enzymes and forms a Schiff's base. The cross links formed between the enzyme and glutaraldehyde are irreversible and can withstand extreme pH and temperature. Glutaraldehyde cross-linking has been successfully used to immobilize several industrial enzymes e.g. glucose isomerase, penicillin amidase. The technique of cross-linking is quite simple and cost-effective. But the disadvantage is that it involves the risk of denaturation of the enzyme by the poly-functional reagent.

Choice of Immobilization Technique:

The selection of a particular method for immobilization of enzymes is based on a trial and error approach to choose the ideal one. Among the factors that decide a technique, the enzyme catalytic activity, stability, regenerability and cost factor are important.

Immobilization of L-amino acid acylase:

L-Amino acid acylase was the first enzyme to be immobilized by a group of Japanese workers (Chibata and Tosa, 1969). More than 40 different immobilization methods were attempted by this group. Only three of them were found to be useful. They were covalent binding to iodoacetyl cellulose, ionic binding to DEAE-Sephadex and entrapment within polyacrylamide.

Stabilization of Soluble Enzymes:

Some of the enzymes cannot be immobilized and they have to be used in soluble form e.g. enzymes used in liquid detergents, some diagnostic reagents and food additives. Such enzymes can be stabilized by using certain additives or by chemical modifications. The stabilized enzymes have longer half-lives, although they cannot be recycled. Some important methods of enzyme stabilization are briefly described.

Solvent Stabilization:

Certain solvents at low concentrations stabilize the enzymes, while at high concentrations the enzymes get denatured e.g. acetone (5%) and ethanol (5%) can stabilize benzyl alcohol dehydrogenase.

Substrate Stabilization:

The active site of an enzyme can be stabilized by adding substrates e.g. starch stabilizes α-amylase; glucose stabilizes glucose isomerase.

Stabilization by Polymers:

Enzymes can be stabilized, particularly against increased temperature, by addition of polymers such as gelatin, albumin and polyethylene glycol.

Stabilization by Salts:

Stability of metalloenzymes can be achieved by adding salts such as Ca, Fe, Mn, Cu and Zn e.g. proteases can be stabilized by adding calcium.

Stabilization by Chemical Modifications:

Enzymes can be stabilized by suitable chemical modifications without loss of biological activity. There are several types of chemical modifications.

a. Addition of poly-amino side chains e.g. polytyrosine, polyglycine.

b. Acylation of enzymes by adding groups such as acetyl, propionyl and succinyl.

Stabilization by Rebuilding:

Theoretically, the stability of the enzymes is due to hydrophobic interactions in the core of the enzyme. It is therefore, proposed that enzymes can be stabilized by enhancing hydrophobic interactions. For this purpose, the enzyme is first unfold and then rebuilt in one of the following ways (Fig. 21.8).

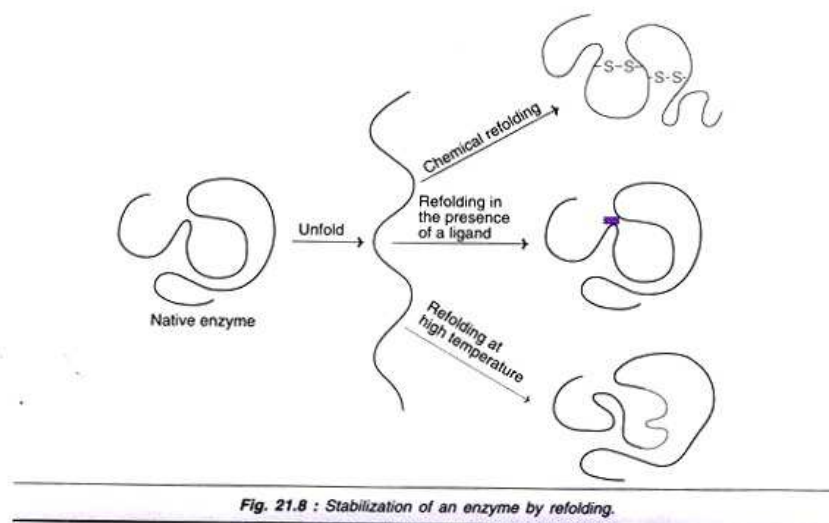


Fig. 21.8 : Stabilization of an enzyme by refolding.

1. The enzyme can be chemically treated (e.g. urea and a disulfide) and then refolded.
2. The refolding can be done in the presence of low molecular weight ligands.
3. For certain enzymes, refolding at higher temperatures (around 50°C) stabilize them.

Stabilization by Site-Directed Mutagenesis:

Site-directed mutagenesis has been successfully used to produce more stable and functionally more efficient enzymes e.g. subtilisin E.

Immobilization of Cells:

Immobilized individual enzymes can be successfully used for single-step reactions. They are, however, not suitable for multi-enzyme reactions and for the reactions requiring cofactors. The whole cells or cellular organelles can be immobilized to serve as multi-enzyme systems. In addition, immobilized cells rather than enzymes are sometimes preferred even for single

reactions, due to cost factor in isolating enzymes. For the enzymes which depend on the special arrangement of the membrane, cell immobilization is preferred.

Immobilized cells have been traditionally used for the treatment of sewage. The techniques employed for immobilization of cells are almost the same as that used for immobilization of enzymes with appropriate modifications. Entrapment and surface attachment techniques are commonly used. Gels, and to some extent membranes, are also employed.

Immobilized Viable Cells:

The viability of the cells can be preserved by mild immobilization. Such immobilized cells are particularly useful for fermentations. Sometimes mammalian cell cultures are made to function as immobilized viable cells.

Immobilized Non-viable Cells:

In many instances, immobilized non-viable cells are preferred over the enzymes or even the viable cells. This is mainly because of the costly isolation and purification processes. The best example is the immobilization of cells containing glucose isomerase for the industrial production of high fructose syrup. Other important examples of microbial biocatalysts and their applications are given in Table 21.5.

TABLE 21.5 Selected examples of immobilized cells (to bring out one or two enzyme reactions) in industrial applications	
<i>Immobilized microorganism (microbial biocatalyst)</i>	<i>Application(s)</i>
<i>Escherichia coli</i>	For the synthesis of L-aspartic acid from fumaric acid and NH_3
<i>Escherichia coli</i>	For the production of L-tryptophan from indole and serine
<i>Pseudomonas</i> sp	Production of L-serine from glycine and methanol
<i>Saccharomyces cerevisiae</i>	Hydrolysis of sucrose
<i>Saccharomyces</i> sp	Large scale production of alcohol
<i>Zymomonas mobilis</i>	Synthesis of sorbitol and gluconic acid from glucose and fructose
<i>Anthrobacter simplex</i>	Synthesis of prednisolone from hydrocortisone
<i>Pseudomonas chlororaphis</i>	Production of acrylamide from acrylonitrile
<i>Humicola</i> sp	For the conversion of rifamycin B to rifamycin S
Bacteria and yeasts (several sp)	In biosensors

Limitations of Immobilizing Eukaryotic Cells:

Prokaryotic cells (particularly bacterial) are mainly used for immobilization. It is also possible to immobilize eukaryotic plant and animal cells. Due to the presence of cellular organelles, the metabolism of eukaryotic cells is slow. Thus, for the industrial production of biochemical, prokaryotic cells are preferred. However, for the production of complex proteins (e.g. immunoglobulin's) and for the proteins that undergo post- translational modifications, eukaryotic cells may be used.

Effect of Immobilization on Enzyme

Enzyme immobilization is frequently associated with alterations in enzyme properties, particularly the kinetic properties of enzymes.

Some of them are listed below:

1. There is a substantial decrease in the enzyme specificity. This may be due to conformational changes that occur when the enzyme gets immobilized.

2. The kinetic constants K_m and V_{max} of an immobilized enzyme differ from that of the native enzyme. This is because the conformational change of the enzyme will affect the affinity between enzyme and substrate.

Immobilized Enzyme Reactors:

The immobilized enzymes cells are utilized in the industrial processes in the form of enzyme reactors. They are broadly of two types — batch reactors and continuous reactors. The frequently used enzyme reactors are shown in Fig. 21.9.

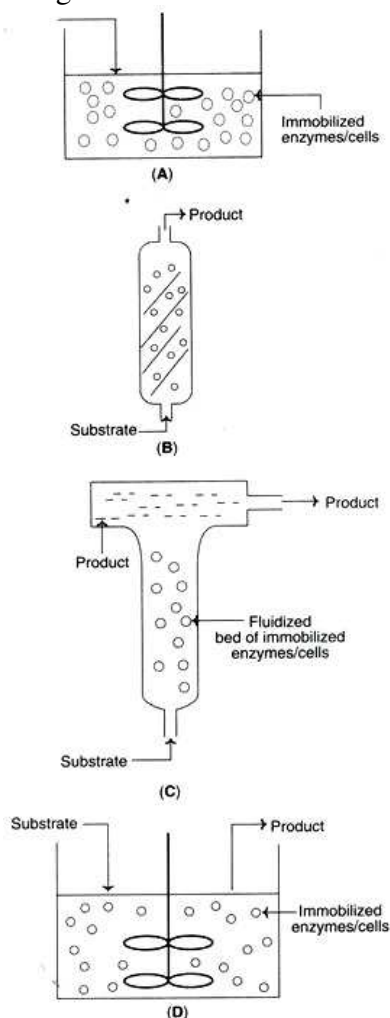


Fig. 21.9 : Immobilized enzyme (cell) reactors
(A) Batch stirred tank reactor, (B) Packed bed reactor,
(C) Fluidized bed reactor, (D) Continuous stirred tank reactor.

CLASS: I M.Sc., BIOCHEMISTRY

COURSE NAME: ENZYMES AND MICROBIAL TECHNOLOGY

COURSE CODE: 19BCP102

**UNIT-III: IMMOBILIZATION OF ENZYMES
(BATCH-2019-2021)**

Batch Reactors:

In batch reactors, the immobilized enzymes and substrates are placed, and the reaction is allowed to take place under constant stirring. As the reaction is completed, the product is separated from the enzyme (usually by denaturation).

Soluble enzymes are commonly used in batch reactors. It is rather difficult to separate the soluble enzymes from the products; hence there is a limitation of their reuse. However, special techniques have been developed for recovery of soluble enzymes, although this may result in loss of enzyme activity.

Stirred tank reactors:

The simplest form of batch reactor is the stirred tank reactor (Fig. 21.9A). It is composed of a reactor fitted with a stirrer that allows good mixing, and appropriate temperature and pH control. However, there may occur loss of some enzyme activity. A modification of stirred tank reactor is basket reactor. In this system, the enzyme is retained over the impeller blades. Both stirred tank reactor and basket reactor have a well-mixed flow pattern.

Plug flow type reactors:

These reactors are alternatives to flow pattern type of reactors. The flow rate of fluids controlled by a plug system. The plug flow type reactors may be in the form of packed bed or fluidized bed (Fig. 21.9B and 21.9C). These reactors are particularly useful when there occurs inadequate product formation in flow type reactors. Further, plug flow reactors are also useful for obtaining kinetic data on the reaction systems.

Continuous Reactors:

In continuous enzyme reactors, the substrate is added continuously while the product is removed simultaneously. Immobilized enzymes can also be used for continuous operation. Continuous reactors have certain advantages over batch reactors. These include control over the product formation, convenient operation of the system and easy automation of the entire process. There are mainly two types of continuous reactors-continuous stirred tank reactor (CSTR) and plug reactor (PR). A diagrammatic representation of CSTR is depicted in Fig. 21.9D. CSTR is ideal for good product formation.

Membrane Reactors:

Several membranes with a variety of chemical compositions can be used. The commonly used membrane materials include polysulfone, polyamide and cellulose acetate. The biocatalysts (enzymes or cells) are normally retained on the membranes of the reactor. The substrate is introduced into reactor while the product passes out. Good mixing in the reactor can be achieved by using stirrer (Fig. 21.10A). In a continuous membrane reactor, the biocatalysts are held over membrane layers on to which substrate molecules are passed (Fig. 21.10B).

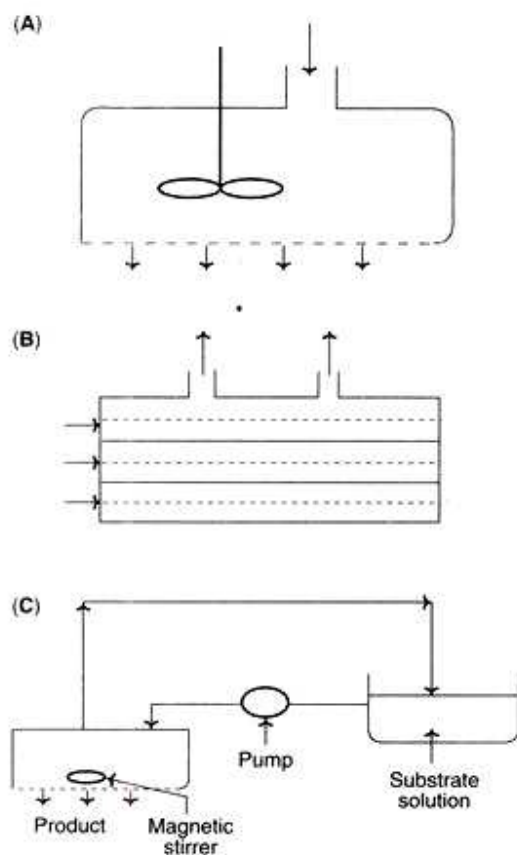


Fig. 21.10 : Membrane reactors (A) Batch membrane reactor, (B) Continuous membrane reactor, (C) Recycle membrane reactor (Coloured lines indicate membranes).

In a recycle model membrane reactor, the contents (i.e. the solution containing enzymes, cofactors, and substrates along with freshly released product) are recycled by using a pump (Fig. 21.10C). The product passes out which can be recovered.

Use of enzymes in clinical diagnosis and industry

Immobilized enzymes and cells are very widely used for industrial, analytical and therapeutic purpose, besides their involvement in food production and exploring the knowledge of biochemistry, microbiology and other allied specialties. A brief account of the industrial applications of immobilized cells is given in Table 21.5.

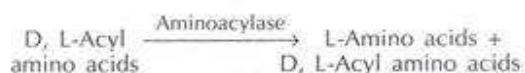
Manufacture of Commercial Products:

A selected list of important immobilized enzymes and their industrial applications is given in Table 21.6. Some details on the manufacture of L-amino acids and high fructose syrup are given hereunder.

TABLE 21.6 A selected list of important immobilized enzymes and their industrial applications	
<i>Immobilized enzyme</i>	<i>Application(s)</i>
Aminoacylase	Production of L-amino acids from D, L-acyl amino acids
Glucose isomerase	Production of high fructose syrup from glucose (or starch)
Amylase	Production of glucose from starch
Invertase	Splitting of sucrose to glucose and fructose
β -Galactosidase	Splitting of lactose to glucose and galactose
Penicillin acylase	Commercial production of semi-synthetic penicillins
Aspartase	Production of aspartic acid from fumaric acid
Fumarase	Synthesis of malic acid from fumaric acid
Histidine ammonia lyase	Production of urocanic acid from histidine
Ribonuclease	Synthesis of nucleotides from RNA
Nitrilase	Production of acrylamide from acrylonitrile

Production of L-Amino Acids:

L-Amino acids (and not D-amino acids) are very important for use in food and feed supplements and medical purposes. The chemical methods employed for their production result in a racemic mixture of D- and L-amino acids. They can be acylated to form D, L-acyl amino acids. The immobilized enzyme aminoacylase (frequently immobilized on DEAE sephadex) can selectively hydrolyse D, L-acyl amino acids to produce L-amino acids.



The free L-amino acids can be separated from the un-hydrolysed D-acyl amino acids. The latter can be racemized to D, L-acyl amino acids and recycled through the enzyme reactor containing immobilized aminoacylase. Huge quantities of L-methionine, L-phenylalanine, L-tryptophan and L-valine are produced worldwide by this approach.

Production of High Fructose Syrup:

Fructose is the sweetest among the monosaccharides, and has twice the sweetening strength of sucrose. Glucose is about 75% as sweet as sucrose. Therefore, glucose (the most abundant monosaccharide) cannot be a good substitute for sucrose for sweetening. Thus, there is a great demand for fructose which is very sweet, but has the same calorific value as that of glucose or sucrose.

High fructose syrup (HFS) contains approximately equivalent amounts of glucose and fructose. HFS is almost similar to sucrose from nutritional point of view. HFS is a good substitute for sugar in the preparation of soft drinks, processed foods and baking.

High fructose syrup can be produced from glucose by employing an immobilized enzyme glucose isomerase. The starch containing raw materials (wheat, potato, corn) are subjected to hydrolysis to produce glucose. Glucose isomerase then isomerizes glucose to fructose (Fig. 21.11). The product formed is HFS containing about 50% fructose. (Note: Some authors use the term high fructose corn syrup i.e. HFCS in place of HFS).

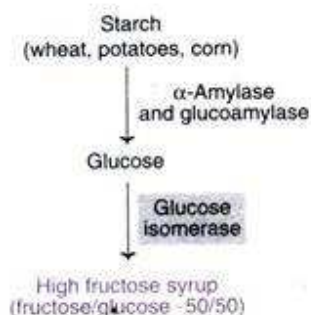


Fig. 21.11 : Production of high fructose syrup from starch (glucose isomerase is the immobilized enzyme).

Glucose isomerase:

This is an intracellular enzyme produced by a number of microorganisms. The species of *Arthrobacter*, *Bacillus* and *Streptomyces* are the preferred sources. Being an intracellular enzyme, the isolation of glucose isomerase without loss of biological activity requires special and costly techniques. Many a times, whole cells or partly broken cells are immobilized and used.

Immobilized Enzymes and Cells- Analytical Applications:

In Biochemical Analysis:

Immobilized enzymes (or cells) can be used for the development of precise and specific analytical techniques for the estimation of several biochemical compounds. The principle of analytical assay primarily involves the action of the immobilized enzyme on the substrate.

A decrease in the substrate concentration or an increase in the product level or an alteration in the cofactor concentration can be used for the assay. A selected list of examples of immobilized enzymes used in the assay of some substances is given in Table 21.7. Two types of detector systems are commonly employed.

TABLE 21.7 Selected examples of immobilized enzymes used in analytical biochemistry

<i>Immobilized enzyme</i>	<i>Substance assayed</i>
Glucose oxidase	Glucose
Urease	Urea
Cholesterol oxidase	Cholesterol
Lactate dehydrogenase	Lactate
Alcohol oxidase	Alcohol
Hexokinase	ATP
Galactose oxidase	Galactose
Penicillinase	Penicillin
Ascorbic acid oxidase	Ascorbic acid
L-Amino acid oxidase	L-Amino acids
Cephalosporinase	Cephalosporin
Monoamine oxidase	Monoamine

Thermistors are heat measuring devices which can record the heat generated in an enzyme catalysed reaction. Electrode devices are used for measuring potential differences in the reaction system. In the Fig. 21.12, an enzyme thermistor and an enzyme electrode, along with a specific urease electrode are depicted.

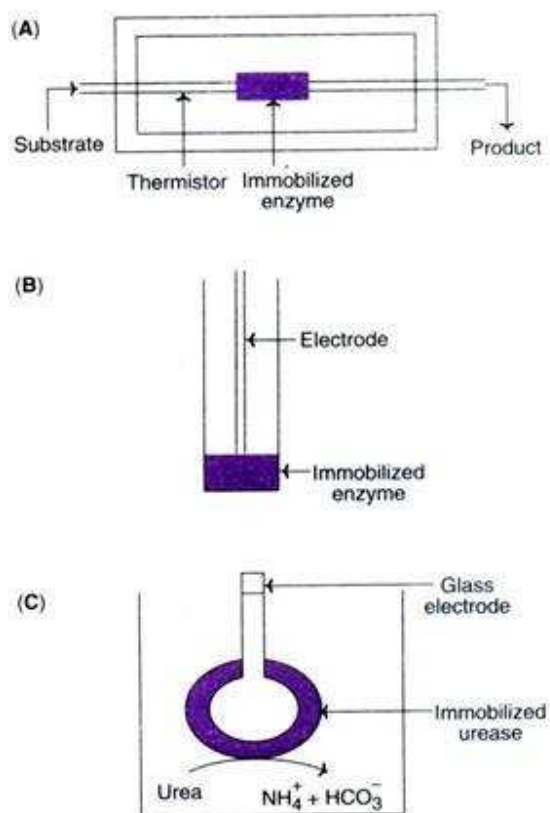


Fig. 21.12 : Immobilized enzymes or cells in analytical biochemistry (A) Enzyme thermistor, (B) Enzyme electrode, (C) Urease electrode.

In Affinity Chromatography and Purification:

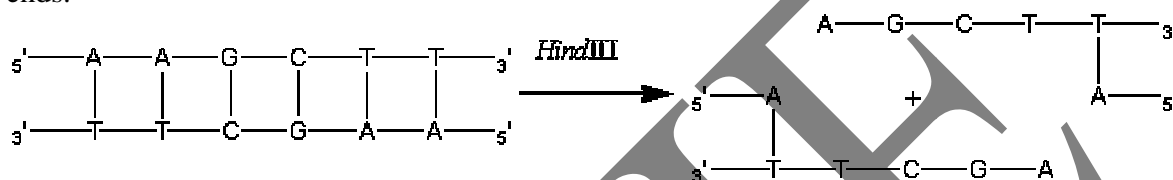
Immobilized enzymes can be used in affinity chromatography. Based on the property of affinity, it is possible to purify several compounds e.g. antigens, antibodies, cofactors.

Enzyme engineering

A most exciting development over the last few years is the application genetic engineering techniques to enzyme technology. A full description this burgeoning science is beyond the scope of this text but some suitable references are given at the end of this chapter. There are a number of properties which may be improved or altered by genetic engineering including the yield and kinetics of the enzyme, the ease of downstream processing and various safety aspects. Enzymes from dangerous or unapproved microorganisms and from slow growing or limited plant or animal tissue may be cloned into safe high-production microorganisms. In the future, enzymes may be redesigned to fit more appropriately into industrial processes; for example, making

glucose isomerase less susceptible to inhibition by the Ca^{2+} present in the starch saccharification processing stream.

The amount of enzyme produced by a microorganism may be increased by increasing the number of gene copies that code for it. This principle has been used to increase the activity of penicillin-G-amidase in *Escherichia coli*. The cellular DNA from a producing strain is selectively cleaved by the restriction endonuclease HindIII. This hydrolyses the DNA at relatively rare sites containing the 5'-AAGCTT-3' base sequence to give identical 'staggered' ends.



intact DNA → cleaved DNA

The total DNA is cleaved into about 10000 fragments, only one of which contains the required genetic information. These fragments are individually cloned into a cosmid vector and thereby returned to *E. coli*. These colonies containing the active gene are identified by their inhibition of a 6-amino-penicillanic acid-sensitive organism. Such colonies are isolated and the penicillin-G-amidase gene transferred on to pBR322 plasmids and re-cloned back into *E. coli*. The engineered cells, aided by the plasmid amplification at around 50 copies per cell, produce penicillin-G-amidase constitutively and in considerably higher quantities than does the fully induced parental strain. Such increased yields are economically relevant not just for the increased volumetric productivity but also because of reduced downstream processing costs, the resulting crude enzyme being that much purer.

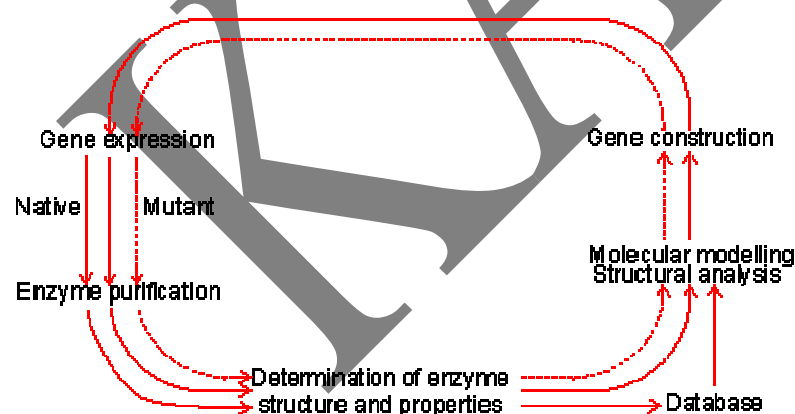


Figure 8.1. The protein engineering cycle.

The process starts with the isolation and characterisation of the required enzyme. This information is analysed together with the database of known and putative structural effects of amino acid substitutions to produce a possible improved structure. This factitious enzyme is constructed by site-directed mutagenesis, isolated and characterised. The results, successful or

unsuccessful, are added to the database, and the process repeated until the required result is obtained.

Another extremely promising area of genetic engineering is protein engineering. New enzyme structures may be designed and produced in order to improve on existing enzymes or create new activities. An outline of the process of protein engineering is shown in Figure 8.1. Such factitious enzymes are produced by site-directed mutagenesis (Figure 8.2). Unfortunately from a practical point of view, much of the research effort in protein engineering has gone into studies concerning the structure and activity of enzymes chosen for their theoretical importance or ease of preparation rather than industrial relevance. This emphasis is likely to change in the future.

As indicated by the method used for site-directed mutagenesis (Figure 8.2), the preferred pathway for creating new enzymes is by the stepwise substitution of only one or two amino acid residues out of the total protein structure. Although a large database of sequence-structure correlations is available, and growing rapidly together with the necessary software, it is presently insufficient accurately to predict three-dimensional changes as a result of such substitutions. The main problem is assessing the long-range effects, including solvent interactions, on the new structure. As the many reported results would attest, the science is at a stage where it can explain the structural consequences of amino acid substitutions after they have been determined but cannot accurately predict them. Protein engineering, therefore, is presently rather a hit or miss process which may be used with only little realistic likelihood of immediate success. Apparently quite small sequence changes may give rise to large conformational alterations and even affect the rate-determining step in the enzymic catalysis. However it is reasonable to suppose that, given a sufficiently detailed database plus suitable software, the relative probability of success will increase over the coming years and the products of protein engineering will make a major impact on enzyme technology.

Much protein engineering has been directed at subtilisin (from *Bacillus amyloliquefaciens*), the principal enzyme in the detergent enzyme preparation, Alcalase. This has been aimed at the improvement of its activity in detergents by stabilising it at even higher temperatures, pH and oxidant strength. Most of the attempted improvements have concerned alterations to:

1. the P_1 cleft, which holds the amino acid on the carbonyl side of the targeted peptide bond;
2. the oxyanion hole (principally Asn₁₅₅), which stabilises the tetrahedral intermediate;
3. the neighbourhood of the catalytic histidyl residue (His₆₄), which has a general base role; and
4. the methionine residue (Met₂₂₂) which causes subtilisin's lability to oxidation.

It has been found that the effect of a substitution in the P_1 cleft on the relative specific activity between substrates may be fairly accurately predicted even though predictions of the absolute effects of such changes are less successful. Many substitutions, particularly for the glycine residue at the bottom of the P_1 cleft (Gly₁₆₆), have been found to increase the specificity of the enzyme for particular peptide links whilst reducing it for others. These effects are achieved mainly by corresponding changes in the K_m rather than the V_{max} . Increases in relative specificity may be useful for some applications. They should not be thought of as the usual result of

engineering enzymes, however, as native subtilisin is unusual in being fairly non-specific in its actions, possessing a large hydrophobic binding site which may be made more specific relatively easily (e.g. by reducing its size). The inactivation of subtilisin in bleaching solutions coincides with the conversion of Met₂₂₂ to its sulfoxide, the consequential increase in volume occluding the oxyanion hole. Substitution of this methionine by serine or alanine produces mutants that are relatively stable, although possessing somewhat reduced activity.

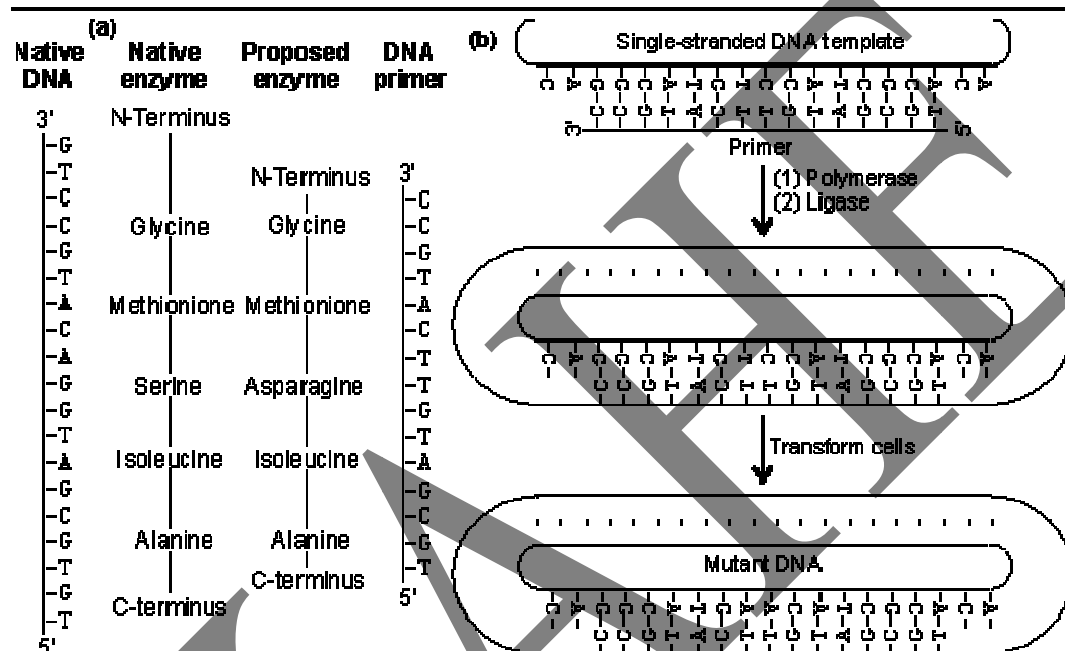


Figure 8.2. An outline of the process of site-directed mutagenesis, using a hypothetical example. (a) The primary structure of the enzyme is derived from the DNA sequence. A putative enzyme primary structure is proposed with an asparagine residue replacing the serine present in the native enzyme. A short piece of DNA (the primer), complementary to a section of the gene apart from the base mismatch, is synthesized. (b) The oligonucleotide primer is annealed to a single-stranded copy of the gene and is extended with enzymes and nucleotide triphosphates to give a double-stranded gene. On reproduction, the gene gives rise to both mutant and wild-type clones. The mutant DNA may be identified by hybridisation with radioactively labeled oligonucleotides of complementary structure.

Artificial enzymes

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

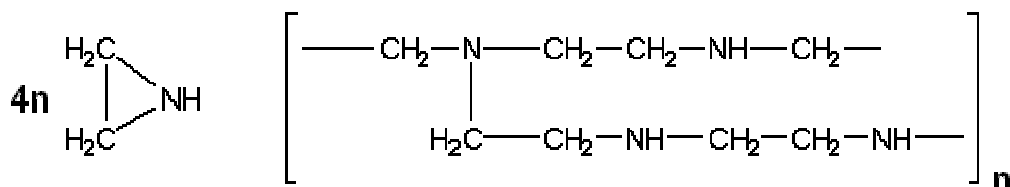
separately but it appears that, if the synzyme has a binding site for the reaction transition state, this often achieves both functions. Synzymes generally obey the saturation Michaelis-Menten kinetics. For a one-substrate reaction the reaction sequence is given by



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 $(\text{Ru}(\text{NH}_3)_5)^{3+}$ 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_m of 2 mM and V_{\max} 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 α -1, 4-linked D-glucose units joined head-to-tail in a ring (α -, β -, γ -, δ - and ϵ -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 β -cyclodextrin was covalently derivatised by an activated pyridoxal coenzyme. The resulting synzyme not only acted as 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:



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 α -chymotrypsin against 4-nitrophenyl 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 $(\text{R-PO}_2\text{-OR}')^-$ 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 $(\text{R-PO}_2\text{-OR}')^-$) of the antigens. The K_m values may be quite low, often in the micromolar region, whereas the V_{\max} values are low (below 1 s^{-1}), 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.

Synzymes

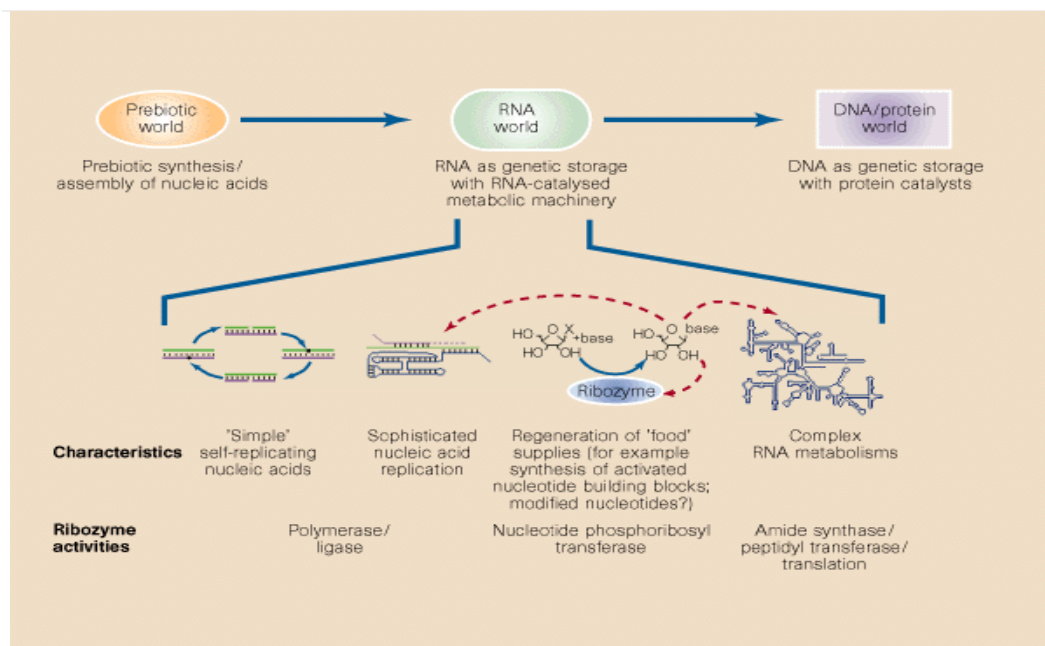
Synzymes are synthetic polymers with enzyme like function. Also called as artificial enzymes. A synzyme has a substrate binding site and active site. Both these sites are synthesized separately and assembled together to form a complete synzyme. Synzymes may be derivatives of certain proteins. Polylysine and polyglutamic acid etc can act as synzymes under natural conditions.

Abzymes

Abzymes are antibody molecule with catalytic activity. In biotechnology, an artificially created antibody that can be used like an enzyme to accelerate reactions. An abzyme (from antibody and enzyme), also called catmab (from catalytic monoclonal antibody), is a monoclonal antibody with catalytic activity. Abzymes are potential tools in biotechnology, e.g., to perform specific actions on DNA.

Ribozyme

Ribozymes are RNA molecules with catalytic activity. Crick and others firmly believed about the idea of a primitive RNA world or RNA as genetic material or master molecule before the takeover by DNA.



Essential quality of a genetic material:

- Information storage and replication

Even now, RNA is the genetic material in some viruses suggesting the capacity of RNA in storing information just like DNA. But the major question who did the function of proteins (the catalytic function)? The discovery of ribozymes further supports 'RNA world' that is RNA can do the function of protein or catalysis. The catalytic ability of RNA may be later passed on to the proteins. The reason for catalytic activity in RNA may be due to the presence of 2' OH group where in DNA it is 2'H.

The first ribozyme to be discovered was spliceosomes or self splicing intron in the ribosomal rRNA genes of the ciliated protozoan *Tetrahymena thermophila*. Later Sidney Altman discovered ribozymes in bacteria, Ribonuclease P an enzyme of bacteria consists of RNA and protein where RNA is found to have catalytic activity. Ribonuclease P is involved in sizing of large RNA precursors into smaller RNAs (RNA processing). Ribozymes can form three dimensional structures just like proteins and can bind specifically to substrates and cofactors

Based on function there are two types of ribozymes

1. Ribozymes that catalyse reactions on themselves or intra-molecular catalysis
2. Ribozymes that catalyse other molecules without undergoing any change or inter-molecular catalysis

Cellular reactions catalysed by ribozymes

- The maturation of hnRNAs or RNA processing (spliceosome)

CLASS: I M.Sc., BIOCHEMISTRY

COURSE NAME: ENZYMES AND MICROBIAL TECHNOLOGY

COURSE CODE: 19BCP102

**UNIT-III: IMMOBILIZATION OF ENZYMES
(BATCH-2019-2021)**

- Splicing of unwanted sections from primary RNA transcripts of ribosomal RNA synthesizing genes
- The formation of peptide bonds during synthesis of polypeptide chains (peptidyl transferase).

Applications:

- Ribozymes catalyse the degradation of other RNAs therefore can be used as potential pharmaceutical agents against viral RNAs
- As an anticancer agent to degrade RNAs from oncogenes

Disadvantage: Ribozymes are easily degraded inside the body by various enzymes. So ribozymes are encapsulated in liposomes and is introduced into the body.

Enzymes in organic solvents


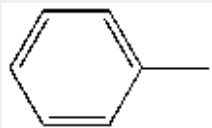
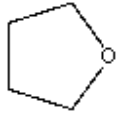
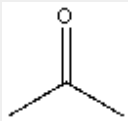
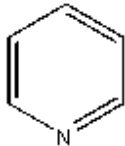
- First, water miscible solvents like ethanol and acetone were added. If the water concentration was high enough, activity remained.
- Biphasic mixtures were made in which an aqueous solution of an enzyme was emulsified in a water immiscible solvent like chloroform or ethylacetate. The substrate would partition into both phases, while the product hopefully would end up into the organic phase.
- Nearly nonaqueous solvents were used, with a few % water at less than the solubility limits of water.
- Finally, anhydrous organic solvents (0.01% water) were used. It is this later case that is most astonishing, since at first glance it is hard to believe that enzymatic activity was retained.

It is important to realize that in this last case, the enzyme is not in solution. It is rather in suspension and acts as a heterogeneous catalyst, much like palladium acts as a heterogeneous catalyst in the hydrogenation of alkenes. The suspension must be mixed vigorously and then sonicated to produce small suspended particles, so diffusion of reactants into the enzyme and out is not rate limiting. Let's explore the activity of chymotrypsin in a nonpolar solvent. Consider the following questions.

- Why aren't the enzymes inactive? Surely it must seem ridiculous that they aren't, since as we learned earlier, proteins are not that stable. A 100 amino acid protein on average is stabilized only about 10 kcal/mol over the denatured state, or the equivalent of a few H bonds. Surely the hydrophobic effect, one of the dominant contributors to protein folding and stability, would not stabilize the native structure of enzymes in nonpolar organic solvents, and the protein would denature. It doesn't however! Maybe the real question should be not whether water is necessary, but rather how much water is necessary. The enzyme can't "see" more than a monolayer or so of water around it. The data suggests that the nature of the organic solvent is very important. The most hydrophobic solvents are best in terms of their ability to maintain active enzymes! Chymotrypsin retains 104 more activity in octane than pyridine, which is more hydrophilic than octane. The more polar the solvent, the more it can strip bound water away from the protein. If

you add 1.5% water to acetone, the bound water increases from 1.2 to 2.4%, and the activity of chymotrypsin increases 1000 fold.

Chymotrypsin Activity in Organic Solvents

Solvent	Structure	kcat/K _m (M ⁻¹ min ⁻¹)	relative ratio kcat/K _m	H ₂ O bound to enzyme (% w/w)
Octane		63	15000x	2.5
Toluene		4.4	1000x	2.3
Tetrahydrofuran		0.27	175x	1.6
Acetone		0.022	5.5x	1.2
Pyridine		<0.004	1x (.004)	1.0

- How active are enzymes in nonpolar solvents? Enzymes are often studied in model transesterification reactions. Typical reaction conditions are enzyme at 1 mg/ml, with one substrate, an ester such as N-acteyl-L-Phe-ethyl ester, at 2-12 mM, and the other substrate, an alcohol, such as n-propanol (instead of being water as in a typical hydrolysis reaction) at 0.25-1.5 M. The more concentrated alcohol replaces the alcohol (ethanol) esterified in the ester. Michaelis-Menten kinetics are followed, with biomolecular rate constants of 10^{10} > than without the enzyme.

- How much water do the enzymes need? 1 molecule of chymotrypsin in octane has < 50 molecules of water associated and can demonstrate activity. To form a monolayer requires about 500 water molecules. Water can be added which presumably leads to more bound water and higher activity.
- How stable are the enzymes? Denaturation requires conformational flexibility, which apparently requires water. The half-life of chymotrypsin in water at 60°C is minutes, but in octane at 100°C it is hours. At 20°C, the half-life in water is a few days, but in octane it is > 6 months. Remember two things contribute to stability. The protein can denature at high temperatures. Also since chymotrypsin is a protease, it can cleave itself in an autolytic reaction.

Half-Life of Chymotrypsin Activity in Water and Octane

Solvent	60°C	100°C	20°C
water	Minutes	-	few days
octane	-	hours	> 6 months

- Is the enzyme specificity changed? The net binding energy is a function of the binding energy of the substrate - the binding energy of the water, since water must be displaced from the active site on binding. In an anhydrous solvent, specificity changes must be expected. For chymotrypsin, the driving force for binding of substrates in water is mostly hydrophobic. In water, the k_{cat}/K_m for the reaction of N-acetyl-L-Ser-esters is reduced 50,000x compared to the Phe ester. However, in octane, chymotrypsin is three times more active toward Ser esters than Phe esters.

Chymotrypsin Specificity Changes in Water and Octane

Substrate	k_{cat}/K_m	
	solvent: H ₂ O	solvent: Octane
N-acetyl-L-Ser-ester	1x	3x
N-acetyl-L-Phe-ester	50,000x	1x

Now consider competitive inhibitors. Napthalene binds 18 times more tightly than 1-napthoic acid, but in octane, the chymotrypsin binds napthoic acid 310 times as tightly. Likewise the ratio

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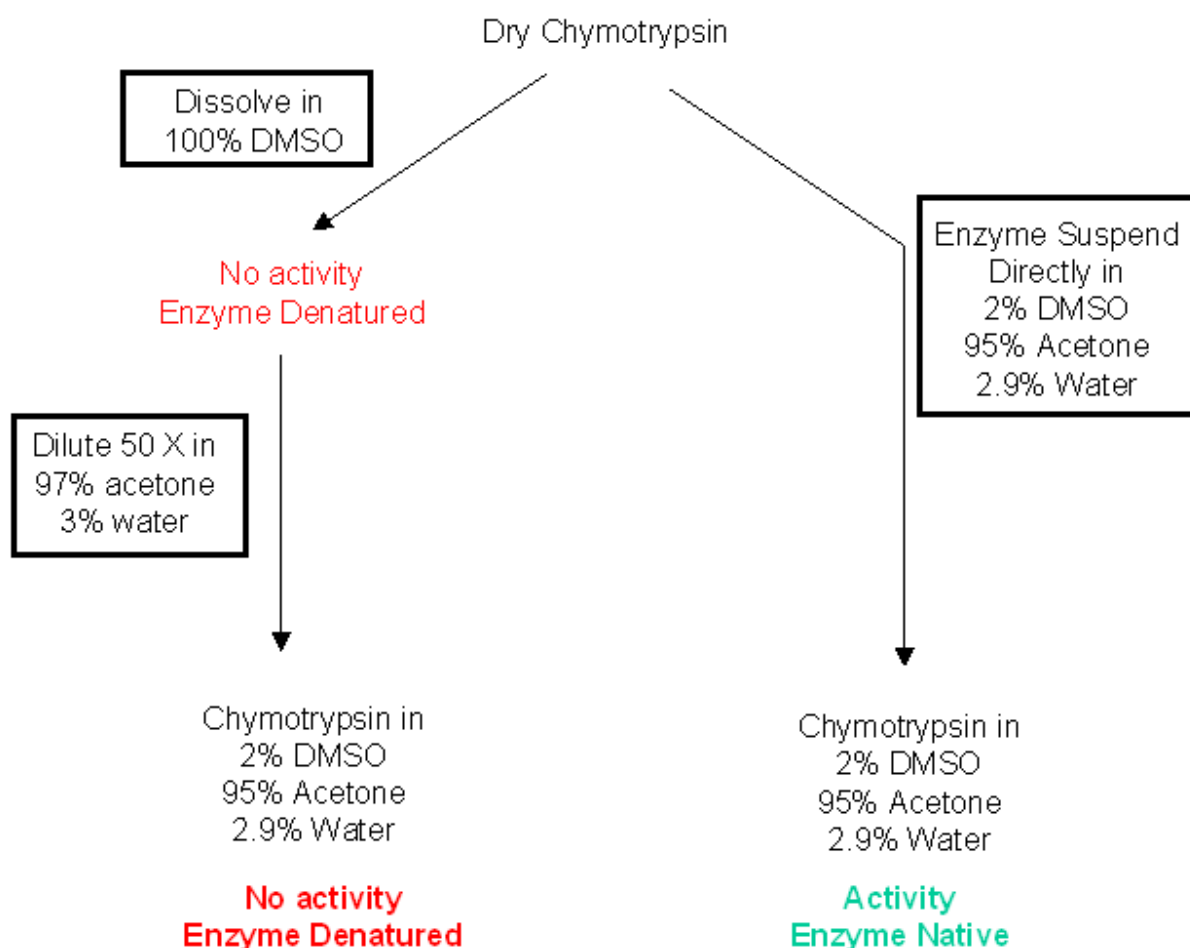
of [kcat/Km (L isomer)]/[kcat/Km (D isomer)] of N-acetyl-D- or N-acetyl-L-Ala-chloroethyl esters is 1000-10,000 in water, but less than 10 in octane.

Chymotrypsin Inhibition Constants in Water and Octane

Inhibitor	Inhibition Constant K_i (nM)	
	In water	In Octane
Benzene	21	1000
Benzoic acid	140	40
Toluene	12	1200
Phenylacetic acid	160	25
Naphthalene	0.4	1100
1-Naphthoic acid	7.2	3

- Can new reactions be carried out in nonpolar solvents? The quick answer is yes, since reactions in aqueous solutions can be unfavorable due to low K_{eq} 's, side reactions, or insolubility of reactants. Consider lipases which cleave fatty acid esters by hydrolysis in aqueous solutions. In nonaqueous solutions, reactions such as transesterification or ammonolysis can be performed. Enzymes are clearly active in organic solvents which appears to contradict our central concepts of protein stability. Two reasons could explain this stability.
- It is possible that from a thermodynamic view, the enzyme is stable in organic solvents. However, as was discussed above, this is inconceivable given the delicate balance of noncovalent and hydrophobic interactions required for protein stability.

2. The second reason must win the day: the protein is unable to unfold from a kinetic point of view. Conformational flexibility is required for denaturation. This must require water as the solvent. A specific example helps illustrate the effects of different solvents on chymotrypsin activity. Dry chymotrypsin can be dissolved in DMSO, a water miscible solvent. In this solvent it is completely and irreversibly denatured. If it is now diluted 50X with acetone with 3% water, no activity is observed. (In the final dilution, the concentrations of solvents are 98% acetone, 2.9% water, and 2% DMSO.) However, if dry chymotrypsin was added to a mixture of 98% acetone, 2.9% water, and 2% DMSO, the enzyme is very active. We end up with the same final solvent state, but in the first case the enzyme has no activity while in the second case it retains activity.



Dry enzymes added to a concentrated water-miscible organic solvent (like DMSO) will dissolve and surely denature, but will retain activity when added to a concentrated water-immiscible solvent (like octane), in which the enzyme will not dissolve but stay in suspension.

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It appears the enzymes have very restricted conformational mobility in non-polar solvents. By lyophilizing (freeze-drying) the enzyme against a specific ligand, a given conformation of a protein can be trapped or literally imprinted onto the enzyme. For example, if the enzyme is dialyzed against a competitive inhibitor (which can be extracted by the organic solvent), freeze-dried to remove water, and then added to a non-polar solvent, the enzyme activity of the "imprinted" enzyme in non-polar solvents is as much as 100x as great as when no inhibitor was present during the dialysis. If chymotrypsin is lyophilized from solutions of different pHs, the resulting curve of V/K_m for ester hydrolysis in octane is bell-shaped with the initial rise in activity reaching half-maximum activity at a pH of around 6.0 and a fall in activity reaching half-maximum at pH of approximately 9.

Use of enzymes in organic solvent allows new routes to organic synthesis. Enzymes, which are so useful in synthetic reactions, are:

- stereoselective - can differentiate between enantiomers and between prochiral substrates
- regioselective - can differentiate between identical functional groups in a single substrate
- chemoselective - can differentiate between different functional groups in a substrate (such as between a hydroxyl group and an amine for an acylation reaction)

Enzyme in anhydrous organic solvents are useful (from a synthetic point) not only since new types of reactions can be catalyzed (such as transesterification, ammonolysis, thiolysis) but also because the stereoselectivity, regioselectivity, and chemoselectivity of the enzyme often changes from activities of the enzyme in water.

Biosensor

A biosensor is an analytical device, used for the detection of an analyte, that combines a biological component with a physicochemical detector. The *sensitive biological element*, e.g. tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, etc., is a biologically derived material or biomimetic component that interacts, binds, or recognizes with the analyte under study. The biologically sensitive elements can also be created by biological engineering. The *transducer* or the *detector element*, which transforms one signal into another one, works in a physicochemical way: optical, piezoelectric, electrochemical, electrochemiluminescence etc., resulting from the interaction of the analyte with the biological element, to easily measure and quantify. The biosensor reader device with the associated electronics or signal processors that are primarily responsible for the display of the results in a user-friendly way. This sometimes accounts for the most expensive part of the sensor device, however it is possible to generate a user friendly display that includes transducer and sensitive element (holographic sensor). The readers are usually custom-designed and manufactured to suit the different working principles of biosensors.

Biosensor receptor

In a biosensor, the bioreceptor is designed to interact with the specific analyte of interest to produce an effect measurable by the transducer. High selectivity for the analyte among a matrix of other chemical or biological components is a key requirement of the bioreceptor. While the type of biomolecule used can vary widely, biosensors can be classified according to common

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types of bioreceptor interactions involving: antibody/antigen enzymes/ligands, nucleic acids/DNA, cellular structures/cells, or biomimetic materials.

Antibody/antigen interactions

An immunosensor utilizes the very specific binding affinity of antibodies for a specific compound or antigen. The specific nature of the antibody-antigen interaction is analogous to a lock and key fit in that the antigen will only bind to the antibody if it has the correct conformation. Binding events result in a physicochemical change that in combination with a tracer, such as a fluorescent molecules, enzymes, or radioisotopes, can generate a signal. There are limitations with using antibodies in sensors: 1. The antibody binding capacity is strongly dependent on assay conditions (e.g. pH and temperature) and 2. The antibody-antigen interaction is generally irreversible. However, it has been shown that binding can be disrupted by chaotropic reagents, organic solvents, or even ultrasonic radiation.

Artificial binding proteins

The use of antibodies as the bio-recognition component of biosensors has several drawbacks. They have high molecular weights and limited stability, contain essential disulfide bonds and are expensive to produce. In one approach to overcome these limitations, recombinant binding fragments (Fab, Fv or scFv) or domains (VH, VHH) of antibodies have been engineered. In another approach, small protein scaffolds with favorable biophysical properties have been engineered to generate artificial families of Antigen Binding Proteins (AgBP), capable of specific binding to different target proteins while retaining the favorable properties of the parent molecule. The elements of the family that specifically bind to a given target antigen, are often selected in vitro by display techniques: phage display, ribosome display, yeast display or mRNA display. The artificial binding proteins are much smaller than antibodies (usually less than 100 amino-acid residues), have a strong stability, lack disulfide bonds and can be expressed in high yield in reducing cellular environments like the bacterial cytoplasm, contrary to antibodies and their derivatives. They are thus especially suitable to create biosensors.

Enzymatic interactions

The specific binding capabilities and catalytic activity of enzymes make them popular bioreceptors. Analyte recognition is enabled through several possible mechanisms: 1) the enzyme converting the analyte into a product that is sensor-detectable, 2) detecting enzyme inhibition or activation by the analyte, or 3) monitoring modification of enzyme properties resulting from interaction with the analyte. The main reasons for the common use of enzymes in biosensors are: 1) ability to catalyze a large number of reactions; 2) potential to detect a group of analytes (substrates, products, inhibitors, and modulators of the catalytic activity); and 3) suitability with several different transduction methods for detecting the analyte. Notably, since enzymes are not consumed in reactions, the biosensor can easily be used continuously. The catalytic activity of enzymes also allows lower limits of detection compared to common binding techniques. However, the sensor's lifetime is limited by the stability of the enzyme.

Affinity binding receptors

Antibodies have a high binding constant in excess of 10^8 L/mol, which stands for a nearly irreversible association once the antigen-antibody couple has formed. For certain analyte

molecules like glucose affinity binding proteins exist that bind their ligand with a high specificity like an antibody, but with a much smaller binding constant on the order of 10^2 to 10^4 L/mol. The association between analyte and receptor then is of reversible nature and next to the couple between both also their free molecules occur in a measurable concentration. In case of glucose, for instance, concanavalin A may function as affinity receptor exhibiting a binding constant of 4×10^2 L/mol. The use of affinity binding receptors for purposes of biosensing has been proposed by Schultz and Sims in 1979 and was subsequently configured into a fluorescent assay for measuring glucose in the relevant physiological range between 4.4 and 6.1 mmol/L. The sensor principle has the advantage that it does not consume the analyte in a chemical reaction as occurs in enzymatic assays.

Nucleic acid interactions

Biosensors that employ nucleic acid interactions can be referred to as genosensors. The recognition process is based on the principle of complementary base pairing, adenine:thymine and cytosine:guanine in DNA. If the target nucleic acid sequence is known, complementary sequences can be synthesized, labeled, and then immobilized on the sensor. The hybridization probes can then base pair with the target sequences, generating an optical signal. The favored transduction principle employed in this type of sensor has been optical detection.

Epigenetics

It has been proposed that properly optimized integrated optical resonators can be exploited for detecting epigenetic modifications (e.g. DNA methylation, histone post-translational modifications) in body fluids from patients affected by cancer or other diseases. Photonic biosensors with ultra-sensitivity are nowadays being developed at a research level to easily detect cancerous cells within the patient's urine. Different research projects aim to develop new portable devices that use cheap, environmentally friendly, disposable cartridges that require only simple handling with no need of further processing, washing, or manipulation by expert technicians.

Organelles

Organelles form separate compartments inside cells and usually perform function independently. Different kinds of organelles have various metabolic pathways and contain enzymes to fulfill its function. Commonly used organelles include lysosome, chloroplast and mitochondria. The spatial-temporal distribution pattern of calcium is closely related to ubiquitous signaling pathway. Mitochondria actively participate in the metabolism of calcium ions to control the function and also modulate the calcium related signaling pathways. Experiments have proved that mitochondria have the ability to respond to high calcium concentration generated in the proximity by opening the calcium channel. In this way, mitochondria can be used to detect the calcium concentration in medium and the detection is very sensitive due to high spatial resolution. Another application of mitochondria is used for detection of water pollution. Detergent compounds' toxicity will damage the cell and subcellular structure including mitochondria. The detergents will cause a swelling effect which could be measured by an absorbance change. Experiment data shows the change rate is proportional to the detergent concentration, providing a high standard for detection accuracy.

Cells

Cells are often used in bioreceptors because they are sensitive to surrounding environment and they can respond to all kinds of stimulants. Cells tend to attach to the surface so they can be easily immobilized. Compared to organelles they remain active for longer period and the reproducibility makes them reusable. They are commonly used to detect global parameter like stress condition, toxicity and organic derivatives. They can also be used to monitor the treatment effect of drugs. One application is to use cells to determine herbicides which are main aquatic contaminant. Microalgae are entrapped on a quartz microfiber and the chlorophyll fluorescence modified by herbicides is collected at the tip of an optical fiber bundle and transmitted to a fluorimeter. The algae are continuously cultured to get optimized measurement. Results show that detection limits of certain herbicide can reach sub-ppb concentration level. Some cells can also be used to monitor the microbial corrosion. *Pseudomonas* sp. is isolated from corroded material surface and immobilized on acetylcellulose membrane. The respiration activity is determined by measuring oxygen consumption. There is linear relationship between the current generated and the concentration of sulfuric acid. The response time is related to the loading of cells and surrounding environments and can be controlled to no more than 5min.

Tissue

Tissues are used for biosensor for the abundance of enzymes existed. Advantages of tissues as biosensors include the following: easier to immobilize compared to cells and organelles

- the higher activity and stability from maintain enzymes in natural environment
- the availability and low price
- the avoidance of tedious work of extraction, centrifuge and purification of enzymes
- necessary cofactors for enzyme to function exists
- the diversity providing a wide range of choice concerning different objectives.

Glucose Oxidase and Biosensors

Glucose oxidase is a secreted enzyme produced predominantly by the fungi *Aspergillus* and *Penicillium* species. It catalyses the oxidation of the sugar β -D-glucose to form D-glucono-1,5-lactone and hydrogen peroxide:



Glucose Oxidase Reaction

Glucose oxidase can oxidise β -D-glucose using other oxidising substrates besides molecular oxygen, including quinines and one-electron acceptors. D-glucono-1,5-lactone can then hydrolyse spontaneously to produce gluconic acid.

Glucose oxidase is a dimeric protein composed of two identical subunits. Each subunit, or monomer, folds into two domains: one domain binds to the substrate, β -D-glucose, while the other domain binds non-covalently to a cofactor, flavin adenine dinucleotide (FAD), which it uses as a powerful oxidising agent. FAD is a common component in biological oxidation-

reduction (redox) reactions, in which there is a gain or loss of electrons from a molecule. In glucose oxidase, FAD acts as an electron acceptor, which causes it to be reduced to FADH₂; the FADH₂ is then oxidised by the final electron acceptor, molecular oxygen, with the oxygen being reduced to hydrogen peroxide (H₂O₂). The active site of glucose oxidase contains three important amino acids involved in catalysis: His516, and Glu412, which is hydrogen-bonded to His559.

Glucose oxidase usually occurs as a glycoprotein, with a mannose-type carbohydrate content of around 16%. However, different forms of the enzyme have been isolated, including a non-glycosylated enzyme from the fungus *Phanerochaete chrysosporium*. Glucose oxidase is secreted by the fungus, and is distributed between the extracellular fluid surrounding the mould, the cell wall, and in the slime mucilage. The synthesis of glucose oxidase can be induced by various substances, including molecular oxygen, which induces the transcription of the enzyme.

a Commercial Goldmine

Glucose oxidase may seem like an ordinary enzyme, but it has become commercially important in the last few years, gaining a multitude of different uses in the chemical, pharmaceutical, food, beverage, and other industries. In addition, gluconic acid, which is produced from the hydrolysis of D-glucono-1,5-lactone, has its own important industrial uses.

Rotation Function

Rotation function has intramolecular vectors that only depend on the molecule's orientation and not its position because even when the molecule is translated in the unit cell, all of the atoms are shifted by the same amount but the vectors between the atoms are the same. The Patterson map for the unknown protein structure is compared with the homologous known protein structure in different orientations.

Cholesterol oxidase

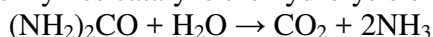
Cholesterol oxidase, cholesterol esterase and peroxidase have been co-immobilized onto electrochemically prepared polyaniline films. These polyaniline-enzyme films characterized using spectroscopic techniques, have been used to fabricate a cholesterol biosensor. This polyaniline-based cholesterol biosensor has a response time of about 240 s, an apparent K_m value as 75 mg dl⁻¹ and can be used to estimate cholesterol concentration up to 500 mg dl⁻¹. These polyaniline/cholesterol oxidase/cholesterol esterase films have a detection limit of 25 mg dl⁻¹ with sensitivity of 0.042 μA mg dl⁻¹. The enzyme films were found to be thermally stable up to 48 °C and have a shelf-life of about 6 weeks when stored at 4 °C. The values of the activation energy before and after the critical temperature were found to be 191 and 95.5 kJ mol⁻¹, respectively.

Urease

Ureases (EC 3.5.1.5), functionally, belong to the superfamily of amidohydrolases and phosphotriesterases.^[1] Ureases are found in numerous bacteria, fungi, algae, plants, and

some invertebrates, as well as in soils, as a soil enzyme. They are nickel-containing metalloenzymes of high molecular weight.

These enzymes catalyze the hydrolysis of urea into carbon dioxide and ammonia:



The hydrolysis of urea occurs in two stages. In the first stage, ammonia and carbamate are produced. The carbamate spontaneously and rapidly hydrolyzes to ammonia and carbonic acid. Urease activity increases the pH of its environment as it produces ammonia, which is basic.

Active site

The active site of all known ureases is located in the α (alpha) subunits. It is a bis- μ -hydroxo dimeric nickel center, with an interatomic distance of $\sim 3.5 \text{ \AA}$. Magnetic susceptibility experiments have indicated that, in jack bean urease, high spin octahedrally coordinated Ni(II) ions are weakly antiferromagnetically coupled. X-ray absorption spectroscopy (XAS) studies of *Canavalia ensiformis* (jack bean), *Klebsiella aerogenes* and *Sporosarcina pasteurii* (formerly known as *Bacillus pasteurii*) confirm 5–6 coordinate nickel ions with exclusively O/N ligands (two imidazoles per nickel).

The water molecules are located towards the opening of the active site and form a tetrahedral cluster that fills the cavity site through hydrogen bonds, and it's here where urea binds to the active site for the reaction, displacing the water molecules. The amino acid residues participate in the substrate binding, mainly through hydrogen bonding, stabilize the catalytic transition state and accelerate the reaction. Additionally, the amino acid residues involved in the architecture of the active site compose part of the mobile flap of the site, which is said to act as a gate for the substrate. Cysteine residues are common in the flap region of the enzymes, which have been determined not to be essential in catalysis, although involved in positioning other key residues in the active site appropriately. In the structure of *Sporosarcina pasteurii* urease the flap was found in the open conformation, while its closed conformation is apparently needed for the reaction. When compared, the α subunits of *Helicobacter pylori* urease and other bacterial ureases align with the jack bean ureases, suggesting that all ureases are evolutionary variants of one ancestral enzyme.

It is important to note that the coordination of urea to the active site of urease has never been observed in a resting state of the enzyme. .

Action in pathogenesis

Bacterial ureases are often the mode of pathogenesis for many medical conditions. They are associated with hepatic encephalopathy / Hepatic coma, infection stones, and peptic ulceration.

Infection stones

Infection induced urinary stones are a mixture of struvite ($\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$) and carbonate apatite [$\text{Ca}_{10}(\text{PO}_4)_6 \cdot \text{CO}_3$]. These polyvalent ions are soluble but become insoluble when ammonia is produced from microbial urease during urea hydrolysis, as this increases the surrounding environment's pH from roughly 6.5 to 9. The resultant alkalization results in stone crystallization. In humans the microbial urease, *Proteus mirabilis*, is the most common in infection induced urinary stones.

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Urease in hepatic encephalopathy / hepatic coma

Studies have shown that *Helicobacter pylori* along with cirrhosis of the liver cause hepatic encephalopathy and hepatic coma. *Helicobacter pylori* are microbial ureases found in the stomach. As ureases they hydrolyze urea to produce ammonia and carbonic acid. As the bacteria are localized to the stomach ammonia produced is readily taken up by the circulatory system from the gastric lumen. This results in elevated ammonia levels in the blood and is coined as hyperammonemia, eradication of *Helicobacter pylori* show marked decreases in ammonia levels.

Urease in peptic ulcers

Helicobacter pylori is also the cause of peptic ulcers with its manifestation in 55–68% reported cases. This was confirmed by decreased ulcer bleeding and ulcer reoccurrence after eradication of the pathogen. In the stomach there is an increase in pH of the mucosal lining as a result of urea hydrolysis, which prevents movement of hydrogen ions between gastric glands and gastric lumen. In addition, the high ammonia concentrations have an effect on intercellular tight junctions increasing permeability and also disrupting the gastric mucous membrane of the stomach.

Antibodies as biosensors

An immunosensor utilizes the very specific binding affinity of antibodies for a specific compound or antigen. The specific nature of the antibody-antigen interaction is analogous to a lock and key fit in that the antigen will only bind to the antibody if it has the correct conformation. Binding events result in a physicochemical change that in combination with a tracer, such as a fluorescent molecules, enzymes, or radioisotopes, can generate a signal. There are limitations with using antibodies in sensors:

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POSSIBLE QUESTIONS

Two marks

1. Define immobilized enzyme
2. Define artificial enzymes.
3. Define abzymes.
4. Define ribozymes.
5. Name three enzymes used as organic solvents
6. Define synzymes.

Essay type questions

1. What are the use of enzymes in clinical diagnosis and industry?
2. Write short notes for the following i) artificial enzymes.
 - i. ii) abzymes
 - ii. iii) ribozymes
3. Write short notes on covalent binding, entrapment and membrane coefficient of immobilization.
4. Give an account on enzymes in organic solvents.
5. What are the applications of enzymes employed in clinical diagnosis and industry.
6. Write short notes on abzymes and enzymes in organic solvents.
7. Discuss on the effect of immobilization on enzyme.
8. Write short notes on enzyme engineering.
9. Discuss on the methods of immobilization.
10. Write short notes on synzymes

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MULTIPLE CHOICE QUESTIONS
UNIT III

S.No	Questions	Option A	Option B	Option C	Option D	Answer
1	Proteases act on proteins to produce	Peptides	Amino acids	Glycoprotein	Glycosides	Amino acids
2	Fungal amylases are produced by	Aspergillus oryza	Bacillus substilin	Pseudomonas	Serratia	Aspergillus oryza
3	Lipases cleave triglycerides to produce glycerol and	Fatty acids	Phospholipids	Carbohydrates	Peptides	Fatty acids
4	The major use of L-Lactic acid in food is as	Hormones	colouring agent	Acidulant and preservative	Antibiotic	Acidulant and preservative
5	For the production of acetone – butanol the following microbe is used	Lactobacillus sps	Saccaromyces sps	Clostridium acetolytilium	Rhizopus sps	Clostridium acetolytilium
6	Backer's yeast is	Aspergillus niger	Saccharomyces cerevisiae	Clostridium sps	Lactobacillus sps	Clostridium sps
7	The amylase enzyme is used in _____ Industry	Baking	Brewing	Both	None	Both
8	Enzyme responsible for hydrolysis of triglycerides to free fatty acids & glycerol is	Protease	Pectinase	Lipase	Cellulose	Lipase
9	The enzyme useful in food industry is _____	Glucose isomerase	Pencillin acylase	Glucose oxidase	Glucokinase	Glucose isomerase
10	The best used organisms for α - amylase production are	Aspergillus and mucor	Streptomyces and achromobacter	Bacillus and streptomyces	Bacillus and aspergillus	Aspergillus and mucor
11	Modern detergents use the following enzyme as one of the ingredients	amylase	lipase	alkaline protease	neutral protease	lipase

12	The following is thrombolytic agent	Trypsin	Urokinase	Urease	Pepsin	Pepsin
13	Modern detergents use the following enzyme as one of the ingredients	amylase	lipase	alkaline protease	neutral protease	neutral protease
14	Serine proteases can be detected using	TPCK	PCMB	TLCK	DIPF	TLCK
15	The most studied enzyme by site-directed mutagenesis experiments is	subtilisin	Amylase	TG lipase	Triosephosphate isomerase	subtilisin
16	In the analysis of glucose, the enzyme immobilized is	glucose isomerase	glucose oxidase	gluco kinase	α -glucosidases	α -glucosidases
17	Ammonia levels can be measured using immobilised -----	Peroxide	Urease	Oxidase	Deminase	Peroxide
18	The members of ----- family form a source for industrial production of amylases	Trichoderma	Antherobacter	Clostridium	Aspergillus	Aspergillus
19	An enzyme indicator in ELISA system is	Uricase	Alkaline phosphatase	Arginase	Alcohol dehydrogenase	Alcohol dehydrogenase
20	“classification “ process during the commercial production of fruit juices requires	Papain	Pectinases	Bromelains	Pepsin	Pepsin
21	The protease used in detergent manufacture is	Acid protease	Neutral protease	Subtilisin	Papain	Neutral protease
22	One of the following enzymes is useful in the treatment of some types of cancer	Urokinase	Papain	Asparaginase	Superoxide dismutase	Urokinase

23	The site of enzyme modification by phosphorylation of the amino acid	cystine	phenylalanine	lysine	serine	lysine
24	Inactive zymogens are precursor of the following GI enzymes EXCEPT	ribonuclease	trypsin	carboxypeptidase	pepsin	ribonuclease
25	Isoenzymes are	enzyme that exist in more than aminoacid sequence in the same species	cannot be distinguished in a given except immunologically	by definition must have the same amino composition	have identical catalytic properties	enzyme that exist in more than aminoacid sequence in the same species
26	One IU of an enzyme is expressed as	m moles of substrate consumed/ml/min	μ moles of substrate consumed/ml/min	moles of substrate consumed/ml/min	μ moles of product formed /ml/min	μ moles of product formed /ml/min
27	The aminoacids that form the catalytic triad in chymotrypsin	Icr,asp,his	Asp,Lys,his	Thr,asp,his	Thr,glu,his	Asp,Lys,his
28	Papain is useful in	meat tenderization	thrombolysis	cheese-making	lather bating	meat tenderization
29	Alkaline serine proteases are enzymes related to	Starch industry	Dairy industry	Detergent industry	Wine industry	Detergent industry
30	Brewing industry mainly utilizes the organism	Saccharomyces cerevisceace	Saccharomyces ellipsoids	Staphylococcus	Candia pseudophopicates	Saccharomyces cerevisceace
31	Immobilised enzyme is one which has carrier which exhibit	With loss of catalytic activity	Without loss of catalytic activity	With loss of specificity	Without loss of specificity	With loss of catalytic activity
32	In anti biotic industry the conversion of penicillinG to 6 aminopenicillanic acid is done	Penicillinase	Pectinase	Penicillin acylase	Penicillin amidase	Penicillinase

33	In enzyme immobilization technique glutaraldehyde act as	cross linking agent	alkylating agent	solid support	condensing agent.	a
34	Isozymes of CPK are	1	2	3	4	3
35	The immobilized enzyme used in cheese making industry is	pectinase	rennet	sucrase	lipase	sucrase
36	Proteins hydrolysed into smaller peptide units & constitute large industrially enzyme.	Ligases	Hydrolases	Proteases	Pectinases	Ligases
37	The enzyme used in the confectionary for preparation of ice creams is	papain	Invertase	Alginate lyase	pectinase.	papain
38	carageenar is used in the immobilization of enzyme by	physical binding	cross-linking	entrapment	adsorption	adsorption
39	The tenderization of meat is carried out by	protease	lipase	rennet	pectinase	lipase
40	Which enzyme serve as a marker enzyme for outer membrane	Monoamine oxidase	Diamine oxidase	Benzyl amine oxidase	Oxidoreductase	Oxidoreductase
41	The coupling of specific electrode with immobilized enzyme is	Clark electrode	catalytic electrode	thermopile electrode	enzyme electrode	thermopile electrode
42	The protease used in detergent manufacture is	acid protease	pectinases	subtilisin	papain	subtilisin
43	One of the following enzymes is useful in the treatment of some types of cancers	urokinase	papain	asparaginase	super oxide dismutase	asparaginase
44	In the analysis of glucose, the enzyme immobilized is	glucose isomerase	glucose oxidase	glucokinase	α -glucosidase	glucose oxidase

45	“Clarification” process during the commercial production of fruit juices requires	Papain	Pectinases	bromelains	pepsin	Pectinases
46	Ammonia levels can be measured using immobilized.....	Peroxidase	urease	oxidase	deaminase	Peroxidase
47	An example of extra cellular enzymes	glucose-6-phosphatase	hexokinase	glucokinase	pancreatic amylase	pancreatic amylase
48	Pectinase is used in the process of	Coffee bean fermentation	Chillproofing of beer	Clarification of fruitjuices	Candy preparation	Clarification of fruitjuices
49	The microbial proteases which one of interest for application in the food industry are a	Endo peptidase	Exopeptidone	Intracellular enzyme	Glycoprotein	Endo peptidase
50	The enzyme most widely used as a diagnostic agent is_____	Acid aspartokinase	phosphatase	Alkaline phosphatase	Urease	Alkaline phosphatase
51	Which enzyme is tested to assess the effectiveness of pasteurization in milk?	Protease	Pectinase	Phosphatase	Hydrolase	Protease
52	The commercial production of bacterial Amylase is effected by_____	Pseudomonas putid	Bacillus subtilis	Aeromonas dydrophilla	Salmonella typhi	Bacillus subtilis
53	The enzyme most widely used as a diagnostic agent is_____	aspartokinase	Acid phosphatase	Alkaline phosphatase	Urease	Alkaline phosphatase
54	The enzyme which is also called maltase is _____	Alpha glucosidase	Beta glucosidase	Glucose oxidase	Invertase	Alpha glucosidase
55	The limitation with immobilized enzyme is_____	The increased stability of the enzyme	The loss of activity after certain period	The enzymes can be reused	All options	The loss of activity after certain period

56	Which of the following microbial enzymes are used in the clarification of fruit juices	Amylase	Lipase	Pectinase	All options	Pectinase
57	Most Commonly used enzymes in Leather industry	Proteases	Lipases	Proteases and Lipases	None	Proteases and Lipases
58	Which enzyme is used in manufacture of syrups.	pectinase	glucose oxidase	streptokinase	amylase	amylase
59	Bacterial proteases are used on a large scale in	Washing powder	Leather processing	Batch process	Clarification of fruit juices	Washing powder
60enzyme is extracted by disruption of Yeast cell wall in the enzyme immobilization	amylase	cellulose	invertase	papain	invertase

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**UNIT-IV: MICROBIAL GROWTH
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UNIT IV
SYLLABUS

Microbial Growth: Balanced and Unbalanced microbial growth; Measurement of growth; Principles of microbial growth and culture systems-batch culture, fed batch culture, semi-continuous culture and continuous culture. Isolation and screening of industrially important microbes. Important strains for better yield. Design of a fermenter. Types of bioreactor-Continuous stirred tank, Bubble column, Airlift, Fluidized bed, Packed bed and Photobioreactor.

Solid substrate fermentation and Media fermentation. Examples of bioprocess for the production of biomass. Microbial metabolic products-primary and secondary metabolites.

Balanced and Unbalanced microbial growth

The growth of a culture is related to the composition of the medium. If all the essential components are available, the growth is balanced. If, however, one or several essential components are missing the growth is terminated due to unbalanced growth, which often leads to death of the culture.

If two different energy sources are available in the growth medium, the growth curve normally shows two exponential phases - diauxi.

Measurement of growth

The growth of microorganisms is a highly complex and coordinated process, ultimately expressed by increase in cell number or cell mass. The process of growth depends on the availability of requisite nutrients and their transport into the cells, and the environmental factors such as aeration, O₂ supply, temperature and pH.

Doubling time refers to the time period required for doubling the weight of the biomass while generation time represents the period for doubling the cell numbers. Doubling times normally increase with increasing cell size and complicity as given below.

Bacteria 0.30 – 1 hour

Yeasts 1 – 2 hours

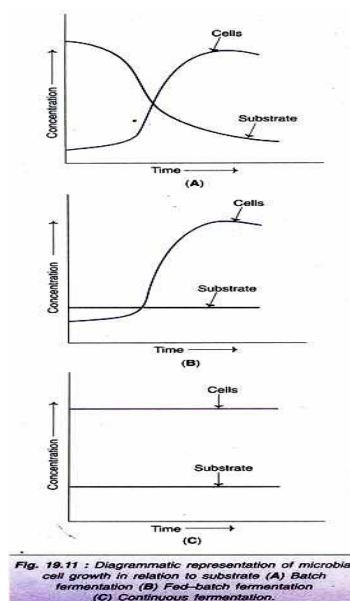
Animal cells 25 -48 hours

Plant cells 20 -70 hours

In general, when all other conditions are kept ideal, growth of the microorganisms is dependent on the substrate (nutrient) supply. The microorganisms can be grown in batch, fed-batch, semi-continuous or continuous culture systems in a bioreactor.

A diagrammatic representation of microbial cell growth in relation to substrate is depicted in Fig. 19.11. In batch fermentation, the growth medium containing the substrates is inoculated with microorganisms, and the fermentation proceeds without the addition of fresh growth medium.

In fed-batch fermentation, substrates are added at short time intervals during fermentation. In batch and fed-batch fermentation, the growth of the cells is quite comparable. And in both cases, growth medium is not removed until the end of fermentation process.



In case of continuous fermentation, as the fermentation proceeds, fresh growth medium is added continuously. Simultaneously, an equal volume of spent medium containing suspended microorganisms is removed. This enables the cells to grow optimally and continuously (Fig. 19.11C).

Principles of microbial growth and culture systems

Batch Culture

A batch fermentation is regarded as a closed system. The sterile nutrient culture medium in the bioreactor is inoculated with microorganisms. The incubation is carried out under optimal physiological conditions (pH, temperature, O₂ supply, agitation etc.). It may be necessary to add acid or alkali to maintain pH, and anti-foam agents to minimise foam. Under optimal conditions for growth, the following six typical phases of growth are observed in batch fermentation (Fig. 19.12).

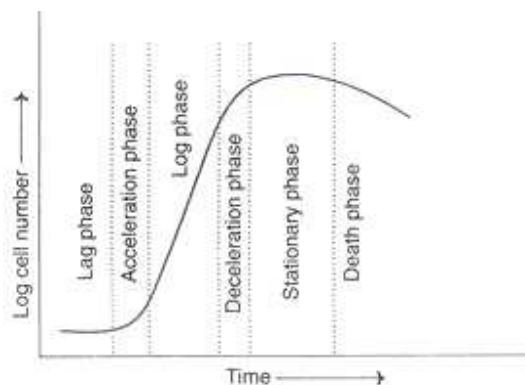


Fig. 19.12 : Pattern of microbial cell growth in batch culture or batch fermentation.

1. Lag phase
2. Acceleration phase
3. Logarithmic (log) phase (exponential phase)
4. Deceleration phase
5. Stationary phase
6. Death phase.

1. Lag phase:

The initial brief period of culturing after inoculation is referred to as lag phase. During the lag phase, the microorganisms adapt to the new environment—available nutrients, pH etc. There is no increase in the cell number, although the cellular weight may slightly increase.

The length of the lag phase is variable and is mostly determined by the new set of physiological conditions, and the phase at which the microorganisms were existing when inoculated. For instance, lag phase may not occur if the culture inoculated is at exponential phase (i.e., log phase), and growth may start immediately.

2. Acceleration phase:

This is a brief transient period during which cells start growing slowly. In fact, acceleration phase connects the lag phase and log phase.

3. Log phase:

The most active growth of microorganisms and multiplication occur during log phase. The cells undergo several doublings and the cell mass increases. When the number of cells or biomass is plotted against time on a semi logarithmic graph, a straight line is obtained, hence the term log phase.

Growth rate of microbes in log phase is independent of substrate (nutrient supply) concentration as long as excess substrate is present, and there are no growth inhibitors in the medium. In general, the specific growth rate of microorganisms for simpler substrates is greater than for long chain molecules. This is explained on the basis of extra energy needed to split long chain substrates.

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Two log phases are observed when a complex nutrient medium with two substrates is used in fermentation, and this phenomenon is referred to as diauxy. This happens since one of the substrates is preferentially metabolised first which represses the breakdown of second substrate. After the first substrate is completely degraded second lag phase occurs, during which period, the enzymes for the breakdown of second substrate are synthesized. Now a second log phase occurs.

4. Deceleration phase:

As the growth rate of microorganisms during log phase decreases, they enter the deceleration phase. This phase is usually very short-lived and may not be observable.

5. Stationary phase:

As the substrate in the growth medium gets depleted, and the metabolic end products that are formed inhibit the growth, the cells enter the stationary phase. The microbial growth may either slow down or completely stop. The biomass may remain almost constant during stationary phase. This phase, however, is frequently associated with dramatic changes in the metabolism of the cells which may produce compounds (secondary metabolites) of biotechnological importance e.g. production of antibiotics.

6. Death phase:

This phase is associated with cessation of metabolic activity and depletion of energy reserves. The cells die at an exponential rate (a straight line may be obtained when the number of surviving cells are plotted against time on a semi logarithmic plot). In the commercial and industrial fermentations, the growth of the microorganisms is halted at the end of the log phase or just before the death phase begins, and the cells are harvested.

Fed-Batch Culture

Fed-batch fermentation is an improvement of batch fermentation wherein the substrate is added in increments at different times throughout the course of fermentation (Note: In batch culture method, substrate is added only at the beginning of the fermentation). Periodical substrate addition prolongs log and stationary phases which results in an increased biomass. Consequently, production of metabolites (e.g. antibiotics) during stationary phase is very much increased.

As it is difficult to directly measure substrate concentration in fed-batch fermentation, other indicators that correlate with substrate consumption are used. The formation of organic acids, production of CO₂ and changes in pH may be measured, and accordingly substrate addition carried out. In general, fed-batch fermentation requires more careful monitoring than batch fermentation, and is therefore not a preferred method by industrial biotechnologists.

Fed-batch fermentation for the production of recombinant proteins:

In recent years, fed-batch fermentation has become popular, due to very high yield, for the production of recombinant proteins. Depending on the microorganism and the nature of recombinant protein, the fed-batch fermentation can increase the product yield from 25% to 1000% compared to batch fermentation. Careful monitoring of the fermentation reaction and appropriate addition of substrates (carbon and nitrogen sources, and trace metals) substantially increases the product yield.

Limitations:

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The major limitation of fed batch fermentation is that the microorganisms in the stationary phase produce proteolytic enzymes or proteases. These enzymes attack the recombinant proteins that are being produced. By carefully monitoring the fermentation, the log phase can be prolonged and the onset of stationary phase is delayed. By this way, the formation of proteases can be minimized.

Fed-batch cultures for higher organisms:

Fed-batch cultures are successfully employed for mammalian and insect cells. This is very advantageous for the production of human therapeutic proteins with good yield.

Semi-Continuous Culture

Some of the products of fermentation are growth-linked, and such products are formed at the end of the log phase e.g. ethanol production. In semi-continuous fermentation, a portion of the culture medium is removed from the bioreactor and replaced by fresh medium (identical in nutrients, pH, temperature etc.).

This process of culture medium change can be repeated at appropriate intervals. In the semi-continuous fermentation, the lag phase and other nonproductive phases are very much shortened. The product output is much higher compared to batch culture systems. Semi-continuous fermentation technique has been successfully used in the industrial production of alcohol. There are however, certain disadvantages of semi-continuous fermentation. These include the technical difficulties of handling bioreactors, long culture periods that may lead to contamination, mutation and mechanical breakdown.

Continuous Culture

Continuous fermentation is an open system. It involves the removal of culture medium continuously and replacement of this with a fresh sterile medium in a bioreactor. Both addition and removal are done at the same rate so that the working volume remains constant.

Further, to maintain a steady state condition in continuous process, it is advisable that the cell loss as a result of outflow is balanced by growth of the organisms. The two common types of continuous fermentation and bioreactors are described below (Fig. 19.13).

Homogeneously mixed bioreactors:

In this type, the culture solution is homogeneously mixed, and the bioreactors are of two types

Chemostat bioreactors:

The concentration of any one of the substrates (carbohydrate, nitrogen source, salts, O₂) is adjusted to control the cell growth and maintain a steady state.

Turbidostat bioreactors:

In this case, turbidity measurement is used to monitor the biomass concentration. The rate of addition of nutrient solution can be appropriately adjusted to maintain a constant cell growth.

Plug flow bioreactors:

In plug flow bioreactors, the culture solution flows through a tubular reaction vessel without back mixing. The composition of the medium, the quantity of cells, O₂ supply and product

formation vary at different locations in the bioreactor. Microorganisms along with nutrient medium are continuously added at the entrance of the bioreactor.

Industrial applications of continuous fermentation:

Continuous fermentation processes have been used for the production of antibiotics, organic solvents, single-cell protein, beer and ethanol, besides waste-water treatment.

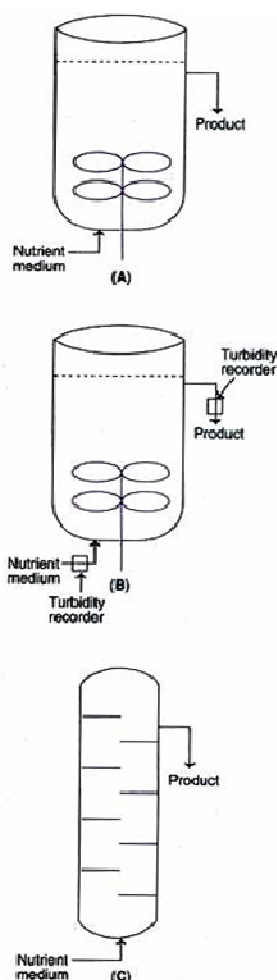


Fig. 19.13 : Continuous fermentation bioreactors
(A) Chemostat bioreactor (B) Turbidostat bioreactor
(C) Plug flow bioreactor.

Advantages of continuous fermentation:

1. The size of the bioreactor and other equipment used in continuous fermentation are relatively smaller compared to batch fermentation for the production of the same quantity of product.
2. The yield of the product is more consistent since the physiological state of the cells is uniform.

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3. The 'down time' between two successive fermentations for cleaning and preparing the bioreactor for reuse is avoided in continuous fermentation.

4. Continuous fermentation can be run in a cost-effective manner.

Disadvantages of continuous fermentation:

Despite many advantages of continuous fermentation (described above), it is not very widely used in industries. Some of the drawbacks are listed.

1. Continuous fermentation may run continuously for a period of 500 to 1,000 hours. Maintenance of sterile conditions for such a long period is difficult.

2. The recombinant cells with plasmid constructs cannot function continuously and therefore the product yield decreases.

3. It is not easy to maintain the same quality of the culture medium for all the additions. Nutrient variations will alter the growth and physiology of the cells, and consequently the product yield.

In addition to the disadvantages listed above, industrial biotechnologists are rather reluctant to switch over to continuous fermentation from the batch fermentation. However, it is expected that continuous fermentation will also become, popular in due course.

Growth Kinetics of Microorganisms:

The different types of fermentation processes- batch, fed-batch, semi-continuous and continuous are described above. The kinetics of microbial growth with special reference to log phase of batch fermentation are briefly discussed here.

After completion of lag phase, the cell enters log phase which is characterized by exponential growth (See Fig. 19.12). If the initial number of cells is N_0 , then

After 1st generation, the cell number will be $N_0 \times 2^1$.

After 2nd generation, the cell number will be $N_0 \times 2^2$.

After 3rd generation, $N_0 \times 2^3$ and so on. Thus, the number of cells after a given time (N_t) will be as follows:

$$N_t = N_0 \times 2^n$$

where n is the number of generations.

The term doubling time (t_d) or mean generation time (MGT) refers to the time taken for doubling the cell number or biomass. The specific growth rate constant expressed by μ , is the direct measure of rate of growth of the organism. If N is the number of cells at a given time, then the increase in the number of cells (growth rate) with time is given by the formula.

$$dN/dt = \mu N \quad (1)$$

If X is the biomass concentration at a given time, then the increase in the biomass (growth rate) with time is given by.

$$dX/dt = \mu X \quad (2)$$

In general, the specific growth rate (μ) is a function of the concentration of limiting substrate (S), the maximum specific growth rate (μ_{max}) and a substrate specific constant (K_s). Their relationship was expressed by Monod by the following equation

$$\mu = \mu_{max} \frac{S}{K_s + S} \quad (3)$$

Both S and K_s are expressed as concentrations e.g., in moles or grams per liter.

The growth rate (μ) of an organism is not fixed but it is variable depending on the environmental conditions such as concentration of substrate and temperature. At a low concentration, the substrate is the limiting factor for growth (Fig. 19.14A). The Fig. 19.14B represents the growth rate for a given substrate concentration (by plotting against S).

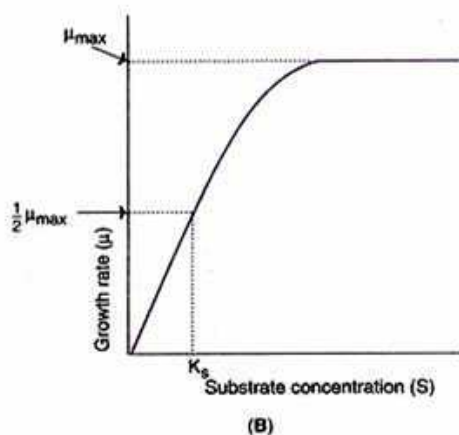
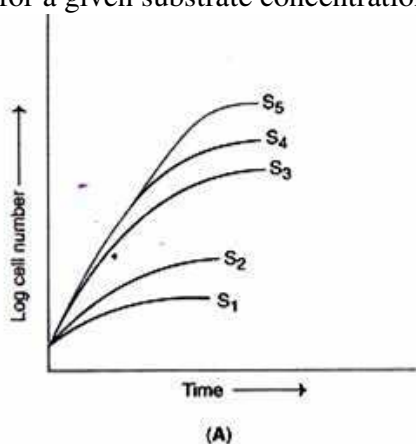


Fig. 19.14 : Growth curves for unicellular organism in batch culture (A) With increasing concentrations of a substrate ($S_1 < S_2 < S_3 < S_4 < S_5$) (B) The effect of a given substrate concentration on growth rate (K_s = substrate concentration to produce half-maximal growth rate)

In batch culture, the substrate is initially present at a higher concentration i.e. $(S) > K_s$, hence the equation (3) is approximately 1.

$$S / K_s + S = 1$$

Thus, $\mu = \mu_{\max}$.

When the substrate concentration is low, as usually occurs at the end of growth phase, then,

$$S / K_s + S < 1$$

Hence $\mu < \mu_{\max}$.

Isolation and screening of industrially important microbes

Isolation of Microorganisms:

There are over a million species of microorganisms widely distributed in nature. Less than 1% of the world's microorganisms have been studied. In fact, only a few hundred species are important for industrial use. A selected list of organisms along with their products is given in Table 19.3.

TABLE 19.3 A selected list of important microorganisms and their products	
<i>Microorganism</i>	<i>Product</i>
Algae	
<i>Chlorella sorokiniana</i>	Single-cell protein
<i>Spirulina maxima</i>	Single-cell protein
Bacteria	
<i>Acetobacter aceti</i>	Acetic acid
<i>Acetobacter woodii</i>	Acetic acid
<i>Bacillus subtilis</i>	Bacitracin
<i>B. brevis</i>	Gramicidin
<i>B. thuringiensis</i>	Endotoxin
<i>Clostridium acetium</i>	Acetic acid
<i>Methylophilus methylotrophus</i>	Glutamic acid
<i>Pseudomonas denitrificans</i>	Vitamin B ₁₂
Actinomycetes	
<i>Streptomyces aureofaciens</i>	Tetracycline
<i>S. griseus</i>	Streptomycin
<i>S. tridiae</i>	Neomycin
<i>Nocardia mediterranei</i>	Rifamycin
<i>Micromonospora purpurea</i>	Gentamycin
Fungi	
<i>Aspergillus niger</i>	Citric acid
<i>A. oryzae</i>	Amylase, cellulase, single-cell protein
<i>Candida lipolytica</i>	Lipase
<i>C. utilis</i>	Single-cell protein
<i>Penicillium chrysogenum</i>	Penicillin
<i>Saccharomyces cerevisiae</i>	Ethanol, wine, single-cell protein
<i>S. lipolytica</i>	Citric acid, single-cell protein
<i>Rhizopus nigricans</i>	Steroids
<i>Gibberella fujikuroi</i>	Gibberellin
<i>Trichoderma viride</i>	Cellulase

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The good sources for the isolation of microorganisms are soils, lakes and river muds. It is estimated that a gram of soil contains 10^6 — 10^8 bacteria, 10^4 — 10^6 actinomycete spores and 10^2 — 10^4 fungal spores.

The common techniques employed for the isolation of microorganisms are given below:

1. Direct sponge of the soil
2. Soil dilution
3. Gradient plate method (Pour plate and streak plate technique)
4. Aerosol dilution
5. Flotation
6. Centrifugation.

For full details on the isolation procedures, the reader must refer books on microbiology. The actual technique for the isolation of microorganisms depends on the source and the physiological properties of microorganisms.

The general scheme adopted for isolating microorganisms from soil or water source is given below:

- i. The sample (soil or water) is diluted with sterile water to which an emulsifying agent (Tween) is added.
- ii. Sample is thoroughly mixed and allowed to stand at room temperature.
- iii. Supernatant is diluted, 10^{-1} to 10^{-10}
- iv. Various culture media are inoculated with diluted samples and incubated.
- v. Colonies from the plates are isolated and identified.
- vi. The required pure strains are maintained and preserved.

Enrichment Methods for Isolation of Microorganisms:

The culture conditions can be appropriately modified to isolate certain types of microorganisms. The types of organisms that can be isolated by use of enrichment methods is given in Table 19.4. For instance, thermophiles can be isolated by using high temperature while acidophilus can be isolated in acidic pH. Enrichment methods are certainly useful for quick isolation of specific types of organisms.

Strains of Microorganisms from Unusual Environments:

Biotechnologists often prefer to isolate microorganisms from very extreme and unusual environments. This is done with a hope that such strains may be capable of producing new products of industrial importance. The unusual environments such as cold habitats, high altitudes, deserts, deep sea and petroleum fields are constantly being tried for this purpose. The enrichment methods described above (Table 19.4) will be very useful for isolating unusual strains.

TABLE 19.4 Types of microorganisms that can be isolated by enrichment methods

Type of organisms	Enrichment method
Thermophiles	High temperature (42–100°C)
Psychrotrophs	Low temperature (5–15°C)
Acidophiles	Low pH (2–4)
Halophiles	High NaCl concentration
Anaerobes	N ₂ atmosphere
Actinoplanes	Pollen grains
Myxobacteria	Wood bark

Screening of Metabolites for Isolation of Microorganisms:

The microorganisms can be tested directly for the product formation, and isolated. In fact, the water or soil samples can be directly used or suitably diluted for metabolite screening. Agar plates can be used for screening metabolites formed from the microorganisms. For instance, if the required product is an antibiotic, then the test system consists of the strains of organisms which inhibit the zones, on the agar plates.

The inhibitory activity indicates the possible presence of some antibiotic being produced by the microorganisms. Another example is the isolation of microorganisms producing amylases. When grown on agar plates containing starch, and then stained with iodine, amylase-producing organisms can be identified and isolated.

Screening for New Metabolites, and Isolation of Microorganisms:

Industrial microbiologists continue their search for newer metabolites produced by microorganisms. Research work is particularly directed for identifying chemotherapeutically important products for the treatment of tumors, bacterial diseases (newer antibiotics against resistant strains) and viral diseases, besides several other substances (e.g. hormones, enzyme inhibitors). In addition, isolation of microorganisms for improvement of food industry, and for efficient degradation of the environmental pollutants and hazardous chemicals also assumes significance.

Important strains for better yield

Preservation of Microorganisms:

There are distinct methods for preservation of microorganisms. The most important being storage by refrigeration, freezing and lyophilization.

Strain Improvement of Micro-organisms Used in fermentation.

In industries the micro-organism are selected by using various screening procedure. The strain which is selected on industrial scale for commercial production of a product should be able to produce high yield of product constantly. This constant high yield of product makes the fermentation economic as well as face the competition with other industries. For obtaining high

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yield of product the industries carry out strain improvement as well as strain selection programs continuously. During the strain improvement program various parameters are adjusted to increase product yield.

The parameters like pH, temperature, media components, aeration, agitation, inoculum levels are adjusted and variation in this levels are tested for increasing yield of product. But however this change in parameters doesn't give large yield and effective results by microbial strains used. The fermentation organism which is being used in fermentation can give constantly increased high yield of product by use of strain improvement program and selection of the most efficient strain for production of product on industrial scale.

The strain improvement of micro-organism used in fermentation process is done by altering the genetic make up of strain and selecting the most efficient strain from various improved strain and result in increased yield of product. After the strain improvement program the selected strain should be genetically stable. The selected strain should produce desirable product in large amount and undesired product in less quantity. Before carrying out the mutation program the selected strain should be efficient under all optimum fermentation condition. The selected strain is exposed to various strain improvement programs and the strain giving high yield of product is selected among all tested strains. The strain that is selected should be able to produce high amount of yield as compared to the original unaltered parent strain.

The genetic make-up of selected strain can be changed by using following methods:-

1. Genetic recombination or gene transfer.
2. Mutation
3. Genetic engineering

1] Genetic recombination or gene transfer

The genetic recombination mechanism exist in different strains of bacteria and some actinomycetes. In genetic recombination mechanism transfer of a gene from one type of strain to other type of strain of micro-organism takes place. This gene transfer takes place by conjugation, transformation or transduction. Gene transfer can be carried out by protoplast fusion of two different efficient strains and new improved strain can be developed.

The fungal strain undergo para sexual cycle and mitotic cell division which is very important and of great value in strain improvement. Genetic Breeding is also possible in different genera of yeast. Thus by using genetic recombination and gene transfer technique it is possible to combine the desired characters from two different strains of same species by interstrain breeding and obtain a efficient strain that is able to produce high yield of product on industrial scale.

2] Mutation

Mutation can be defined as change in genetic structure of micro-organism. The ability of micro-organism to produce a desired product can be enhanced by a mutation process. In mutation process the most stable and efficient strains are exposed to mutagenesis by using different mutagenic agents. The mutagenic agents like ionization, ultraviolet radiation, acids, and alkalies are used in mutation. Result of mutation obtain should be increased in yield of desired product and decreased yield of undesired product. The mutagenic agents are used in such concentration that maximum number of cells die due to mutagenic agents and only the that are capable to carry

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out mutation and have the capacity to tolerate the levels of mutagenic agents are able to survive. From the survived cells the cells which are undergone mutation and have the capacity to produce high yield of fermented product is selected.

The selection of a mutated cell is a very difficult task. Whenever the microbial cells are subjected to mutation there are two possibilities and that are the mutation occurred may be major mutation or minor mutation. The strain undergone desired mutation should be carefully selected, isolated and maintained properly. The selected mutant strains with altered morphological and biochemical characters are now the strain that produce high yield of desired product. The improved selected strain should be tested with laboratory scale experiments followed by pilot plant experiments and then further used on commercial scale. The finalized strains are selected, purified and maintained. All the test and experiments should be carried out with high accuracy as these procedures are time consuming and expensive. Further when these strains are used on industrial scale a proper record of yield produce should be maintained as minor changes should be noted.

3] Genetic engineering

Genetic engineering technique is the most successful technique used for strain improvement programs on industrial scale. It is also called as Recombinant DNA technology and in this process there is alteration of genetic characters of cells and hence result in change in phenotypic character of cell. This In this technique a desired type of gene is introduced from one microbial cell to other microbial cells by using cloning vectors, like plasmids, phages or cloning vectors. A specific and desired character of a microbial cells can be introduced in the selected strain to improve the yield of product.

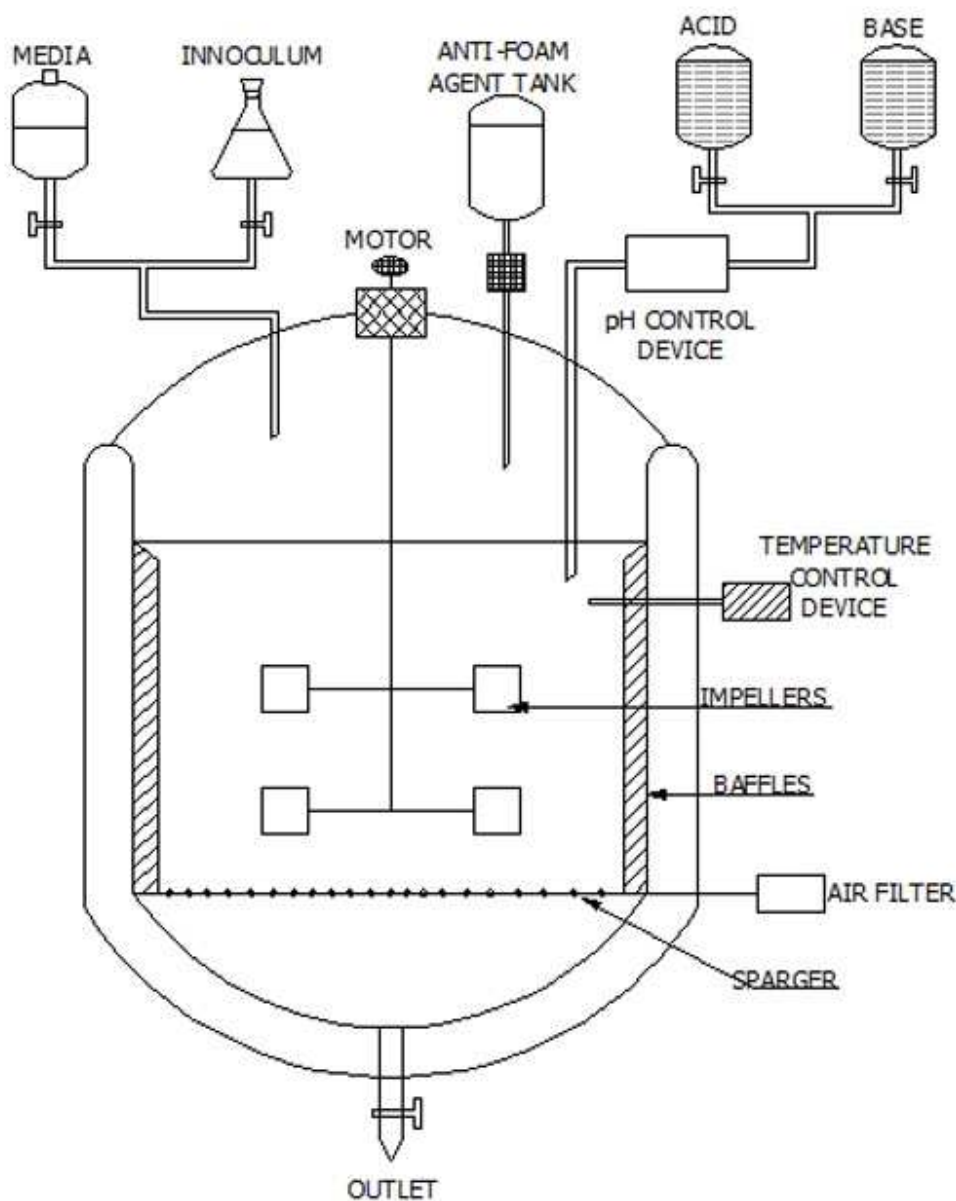
Design of a Fermenter

Fermentation

Fermentation can be defined as a metabolic process in which cheap raw materials such as sugar or carbohydrates are converted into economically important products like acids, gases and alcohols by micro-organism. This process is carried out in a equipment called as fermenter.

A Fermenter can be defined as a vessel in which sterile nutrient media and pure culture of micro-organism are mixed and fermentation process is carried out under aseptic and optimum condition.

Fermenter provides a sterile environment and optimum condition that are important for growth of micro-organisms and synthesis of desired product.



A fermenter should be constructed in such a way that it can make provisions for the below activities:

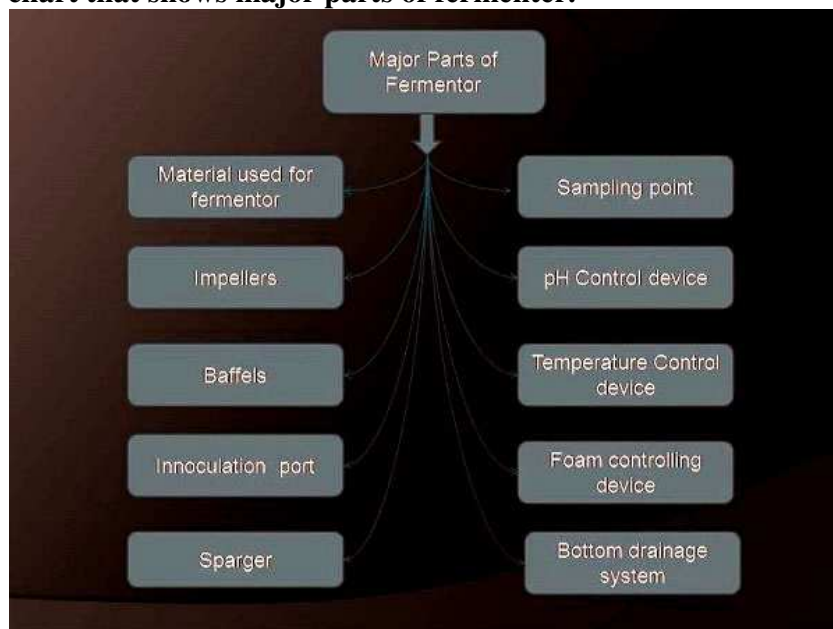
- Sterilization
- Temperature control
- pH control
- Foam control

- Aeration and agitation
- Sampling point
- Inoculation points for micro-organisms, media and supplements
- Drainage point for drainage of fermented media
- Harvesting of product
- Cleaning
- Facility of providing hot, cold water and sterile compressed air.

Here one by one we will be studying major parts of fermenter and their function.

1. Material used for fermenter
2. Impellers
3. Baffles
4. Inoculation port
5. Sparger
6. Sampling point
7. pH control device
8. Temperature control system
9. Foam control device
10. Bottom drainage system

Here is a flow chart that shows major parts of fermenter.



1. Material used for fermenter

The material used for designing of a fermenter should have some important functions.

- It should not be corrosive
- It should not add any toxic substances to the fermentation media.
- It should tolerate steam sterilization process.

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- It should be able to tolerate high pressure and resist pH changes.

The fermenter material used is also decided on type of fermentation process. For example, in case of Beer, Wine, Lactic acid fermentation, the fermenter tanks are made up of wooden material. Whereas material such as iron, copper, glass and stainless steel can be used in some cases. Most of the time, 304 and 316 stainless steel is used for designing of a fermenter and these fermenters are mostly coated with epoxy or glass lining.

A fermenter should provide the facility to control and monitor various parameters for a successful fermentation process.

2. Impellers

- Impellers are an agitation device. They are mounted on the shaft and introduced in the fermenter through its lid.
- They are made up of impeller blades and the position may vary according to its need.
- These impellers or blades are attach to a motor on lid.
- The important function of an impeller is to mix micro-organisms, media and oxygen uniformly.
- Impeller blades reduce the size of air bubbles and distributes these air bubbles uniformly into the fermentation media.
- Impellers also helps in breaking foam bubbles in the head space of fermenter. This foam formed during fermentation process can cause contamination problem and this problem is avoided by the use of impellers.

3. Baffles

- Baffles are mounted on the walls of a fermenter.
- The important function of baffles is to break the vortex formed during agitation process by the impellers.
- If this vortex is not broken, the fermentation media may spill out of fermenter and this may result in contamination as well as can lead to different problems. So it is important to break the vortex formed by using a barrier.
- Baffles acts as a barrier which break the vortex.

4. Inoculation Port

- Inoculation port is a device from which fermentation media, inoculum and substrate are added in the fermentation tank.
- Care should be taken that the port provides aseptic transfer.
- The inoculation port should be easy to sterilize.

5. Spargers

- A Sparger is an aeration system through which sterile air is introduced in the fermentation tank.
- Spargers are located at the bottom of the fermentation tank.
- Glass wool filters are used in a sparger for sterilization of air and other gases.
- The sparger pipes contain small holes of about 5-10 mm. Through these small holes pressurized air is released in the aqueous fermentation media.

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- The air released is in the form of tiny air bubbles. These air bubbles help in mixing of media.

6. Sampling point

- Sampling point is used for time to time withdrawal of samples to monitor fermentation process and quality control.
- This sampling point should provide aseptic withdrawal of sample.

7. pH Control device

- The pH controlling device checks the pH of media at specific intervals of time and adjusts the pH to its optimum level by addition of acids or alkalis.
- Maintaining pH to its optimum level is very important for growth of micro-organism to obtain a desired product.

8. Temperature control

- Temperature control device generally contains a thermometer and cooling coils or jackets around fermenter.
- During the fermentation process, various reactions take place in the fermenter. Heat is generated and released in the fermentation media. This increase in temperature is detrimental to the growth of micro-organisms, which may slow down the fermentation process.
- So, it is necessary to control this rise in temperature. This is done by passing cool water through the coils or jackets present around fermenter.

9. Foam controlling device

- A Foam controlling device is placed on the top of fermenter with an inlet into fermenter. This device contains a small tank containing anti-foaming agent.
- Foam is generated during fermentation. It is necessary to remove or neutralize this foam with the help of anti-foaming agents, lest the media may spill out of fermenter and lead into contamination and a mess.

10. Bottom drainage system

- It is an aseptic outlet present at the bottom of fermenter for removal of fermented media and products formed.

Types of Bioreactor

1. Continuous Stirred Tank Bioreactors:

A continuous stirred tank bioreactor consists of a cylindrical vessel with motor driven central shaft that supports one or more agitators (impellers). The shaft is fitted at the bottom of the bioreactor (Fig. 19.1 A). The number of impellers is variable and depends on the size of the bioreactor i.e., height to diameter ratio, referred to as aspect ratio.

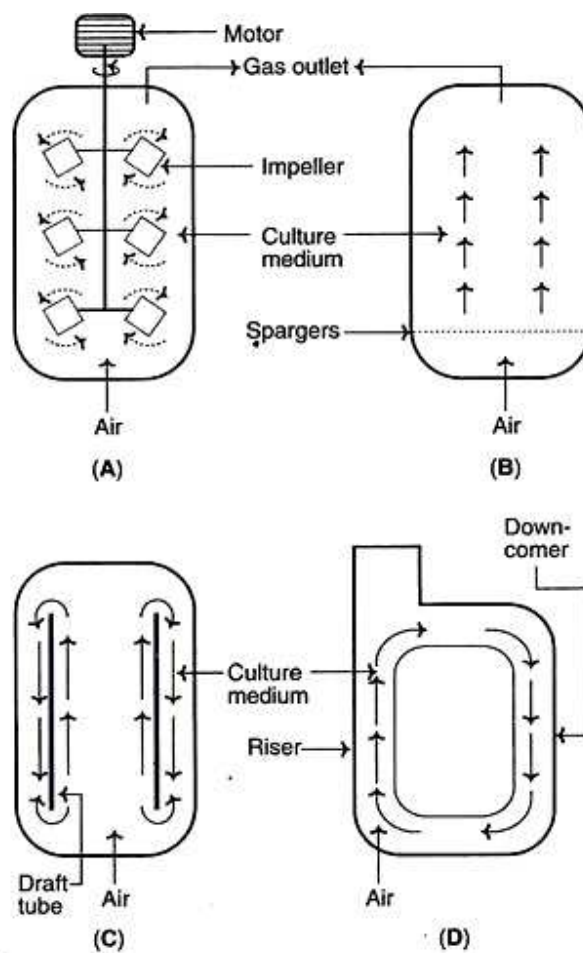


Fig. 19.1 : Types of bioreactors (A) Continuous stirred tank bioreactor (B) Bubble column bioreactor (C) Internal-loop airlift bioreactor (D) External-loop airlift bioreactor.

The aspect ratio of a stirred tank bioreactor is usually between 3-5. However, for animal cell culture applications, the aspect ratio is less than 2. The diameter of the impeller is usually $\frac{1}{3}$ rd of the vessel diameter. The distance between two impellers is approximately 1.2 impeller diameter. Different types of impellers (Ruston disc, concave bladed, marine propeller etc.) are in use.

In stirred tank bioreactors or in short stirred tank reactors (STRs), the air is added to the culture medium under pressure through a device called sparger. The sparger may be a ring with many holes or a tube with a single orifice. The sparger along with impellers (agitators) enables better gas distribution system throughout the vessel.

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The bubbles generated by sparger are broken down to smaller ones by impellers and dispersed throughout the medium. This enables the creation of a uniform and homogeneous environment throughout the bioreactor.

Advantages of STRs:

There are many advantages of STRs over other types. These include the efficient gas transfer to growing cells, good mixing of the contents and flexible operating conditions, besides the commercial availability of the bioreactors.

2. Bubble Column Bioreactors:

In the bubble column bioreactor, the air or gas is introduced at the base of the column through perforated pipes or plates, or metal micro porous spargers (Fig. 19.1B). The flow rate of the air/gas influences the performance factors —O₂ transfer, mixing. The bubble column bioreactors may be fitted with perforated plates to improve performance. The vessel used for bubble column bioreactors is usually cylindrical with an aspect ratio of 4-6 (i.e., height to diameter ratio).

3. Airlift Bioreactors:

In the airlift bioreactors, the medium of the vessel is divided into two interconnected zones by means of a baffle or draft tube. In one of the two zones referred to a riser, the air/gas is pumped. The other zone that receives no gas is the down comer. The dispersion flows up the riser zone while the down flow occurs in the down comer. There are two types of airlift bioreactors.

Internal-loop airlift bioreactor (Fig. 11.1C) has a single container with a central draft tube that creates interior liquid circulation channels. These bioreactors are simple in design, with volume and circulation at a fixed rate for fermentation.

External loop airlift bioreactor (Fig. 19.1D) possesses an external loop so that the liquid circulates through separate independent channels. These reactors can be suitably modified to suit the requirements of different fermentations. In general, the airlift bioreactors are more efficient than bubble columns, particularly for more denser suspensions of microorganisms. This is mainly because in these bioreactors, the mixing of the contents is better compared to bubble columns.

Airlift bioreactors are commonly employed for aerobic bioprocessing technology. They ensure a controlled liquid flow in a recycle system by pumping. Due to high efficiency, airlift bioreactors are sometimes preferred e.g., methanol production, waste water treatment, single-cell protein production. In general, the performance of the airlift bioreactors is dependent on the pumping (injection) of air and the liquid circulation.

Two-stage airlift bioreactors:

Two-stage airlift bioreactors are used for the temperature dependent formation of products. Growing cells from one bioreactor (maintained at temperature 30°C) are pumped into another bioreactor (at temperature 42°C). There is a necessity for the two-stage airlift bioreactor, since it is very difficult to raise the temperature quickly from 30°C to 42°C in the same vessel. Each one of the bioreactors is fitted with valves and they are connected by a transfer tube and pump (Fig.

19.2A). The cells are grown in the first bioreactor and the bioprocess proper takes place in the second reactor.

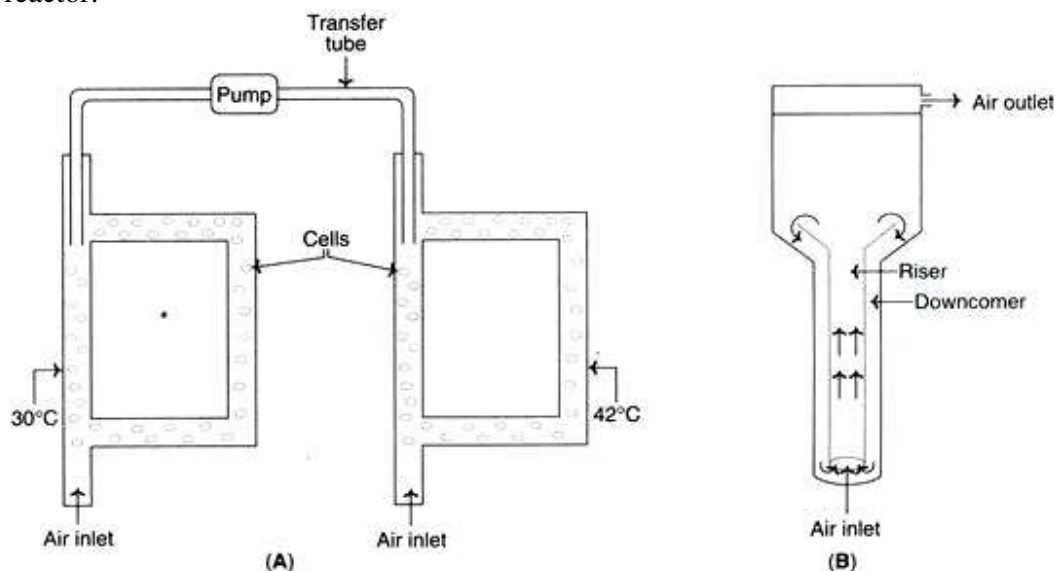


Fig. 19.2 : Types of bioreactors (A) Two-stage airlift bioreactor (B) Tower bioreactor.

Tower bioreactors:

A pressure-cycle fermenter with large dimensions constitutes a tower bioreactor (Fig. 19.2B). A high hydrostatic pressure generated at the bottom of the reactor increases the solubility of O₂ in the medium. At the top of the riser, (with expanded top) reduces pressure and facilitates expulsion of CO₂. The medium flows back in the down comer and completes the cycle. The advantage with tower bioreactor is that it has high aeration capacities without having moving parts.

4. Fluidized Bed Bioreactors:

Fluidized bed bioreactor is comparable to bubble column bioreactor except the top position is expanded to reduce the velocity of the fluid. The design of the fluidized bioreactors (expanded top and narrow reaction column) is such that the solids are retained in the reactor while the liquid flows out (Fig. 19.3A). These bioreactors are suitable for use to carry out reactions involving fluid suspended biocatalysts such as immobilized enzymes, immobilized cells, and microbial flocs.

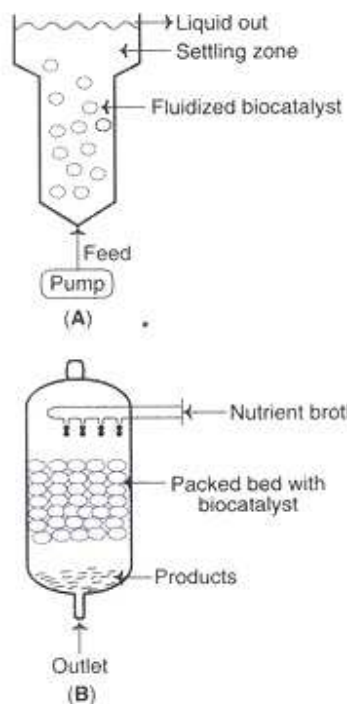


Fig. 19.3 : Types of bioreactors (A) Fluidized bed bioreactor (B) Packed bed bioreactor.

For an efficient operation of fluidized beds, gas is spared to create a suitable gas-liquid-solid fluid bed. It is also necessary to ensure that the suspended solid particles are not too light or too dense (too light ones may float whereas too dense ones may settle at the bottom), and they are in a good suspended state. Recycling of the liquid is important to maintain continuous contact between the reaction contents and biocatalysts. This enables good efficiency of bioprocessing.

5. Packed Bed Bioreactors:

A bed of solid particles, with biocatalysts on or within the matrix of solids, packed in a column constitutes a packed bed bioreactor (Fig. 19.3B). The solids used may be porous or non-porous gels, and they may be compressible or rigid in nature. A nutrient broth flows continuously over the immobilised biocatalyst. The products obtained in the packed bed bioreactor are released into the fluid and removed. While the flow of the fluid can be upward or downward, down flow under gravity is preferred.

The concentration of the nutrients (and therefore the products formed) can be increased by increasing the flow rate of the nutrient broth. Because of poor mixing, it is rather difficult to control the pH of packed bed bioreactors by the addition of acid or alkali. However, these bioreactors are preferred for bioprocessing technology involving product-inhibited reactions. The packed bed bioreactors do not allow accumulation of the products to any significant extent.

6. Photo-Bioreactors:

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These are the bioreactors specialized for fermentation that can be carried out either by exposing to sunlight or artificial illumination. Since artificial illumination is expensive, only the outdoor photo-bioreactors are preferred. Certain important compounds are produced by employing photo-bioreactors e.g., p-carotene, asthaxanthin.

The different types of photo-bioreactors are depicted in Fig. 19.4. They are made up of glass or more commonly transparent plastic. The array of tubes or flat panels constitute light receiving systems (solar receivers). The culture can be circulated through the solar receivers by methods such as using centrifugal pumps or airlift pumps. It is essential that the cells are in continuous circulation without forming sediments. Further adequate penetration of sunlight should be maintained. The tubes should also be cooled to prevent rise in temperature.

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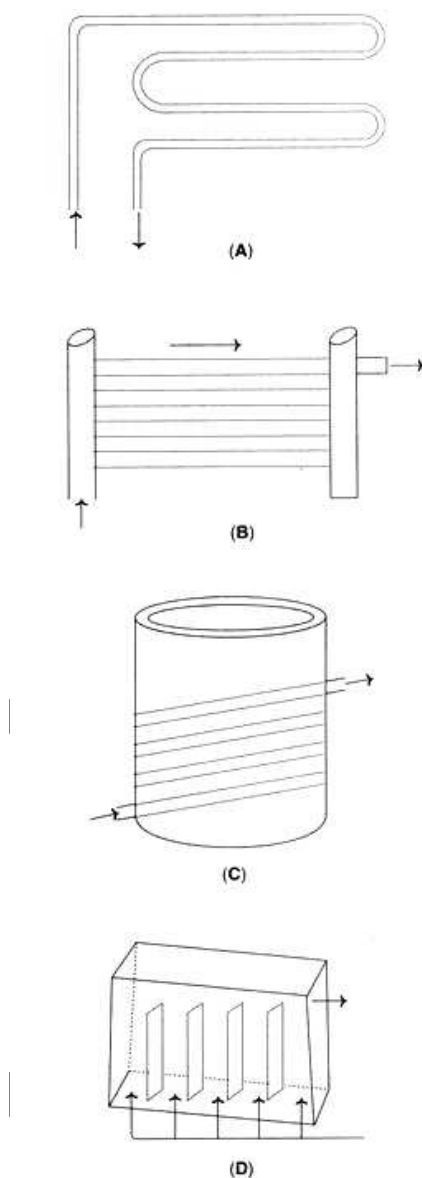


Fig. 19.4 : Types of photobioreactors (A) Continuous run tubular loop (B) Multiple parallel tube (C) Helical wound tubular loop (D) Flat panel configuration.

Photo-bioreactors are usually operated in a continuous mode at a temperature in the range of 25-40°C. Microalgae and cyanobacteria are normally used. The organisms grow during day light while the products are produced during night.

Solid substrate fermentation

There are many biotechnological processes that involve the growth of organisms on solid substrates in the absence or near absence of free water (Table 2.1). Solid substrate fermentation (SSF) deals with substrates that are solid and contain low moisture levels. The most regularly used solid substrates are cereal grains (rice, wheat, barley and corn), legume seeds, wheat bran, lignocellulose materials such as straws, sawdust or wood shavings, and a wide range of plant and animal materials. Most of these compounds are polymeric molecules – insoluble or sparingly soluble in water – but most are cheap and easily obtainable and represent a concentrated source of nutrients for microbial growth.

SSF can be defined in terms of the following properties:

- A solid porous matrix which can be biodegradable or not, but with a large surface area per unit volume, in the range of 10^3 to 10^6 m²/cm³, for a ready microbial growth on the solid/gas interface.
- The matrix should absorb water amounting to one or several times its dry weight with a relatively high water activity on the solid/gas interface in order to allow high rates of biochemical processes.
- Air mixture of oxygen with other gases and aerosols should flow under a relatively low pressure and mix the fermenting mash.
- The solid/gas interface should be a good habitat for the fast development of specific cultures of moulds, yeasts or bacteria, either in pure or mixed cultures.
- The mechanical properties of the solid matrix should stand compression or gentle stirring, as required for a given fermentation process. This requires small granular or fibrous particles, which do not tend to break or stick to each other.
- The solid matrix should not be contaminated by inhibitors of microbial activities and should be able to absorb or contain available microbial foodstuffs such as carbohydrates (cellulose, starch, sugars) nitrogen sources (ammonia, urea, peptides) and mineral salts.

Table 2.1: Examples of SSF

EXAMPLE	SUBSTRATE	MICROORGANISM(S)
Mushroom production	Straw, manure	<i>Agaricus bisporus</i> , <i>Lentinus edodes</i> , <i>Volvarellia volvacea</i>
Sauerkraut	Cabbage	Lactic acid bacteria
Soy sauce	Soya beans and wheat	<i>Aspergillus oryzae</i>
Tempeh	Soya beans	<i>Rhizopus oligosporus</i>
Ontjom	Peanut press cake	<i>Neurospora sitophila</i>
Cheeses	Milk curd	<i>Penicillium roquefortii</i>
Leaching of metals	Low-grade ores	<i>Thiobacillus</i> sp.
Organic acids	Cane sugar, molasses	<i>Aspergillus niger</i>
Enzymes	Wheat, bran etc.	<i>Aspergillus niger</i>
Composting	Mixed organic material	Fungi, bacteria, actinomycetes
Sewage treatment	Components of sewage	Bacteria, fungi, protozoa

The microbiological components of SSF can occur as single pure cultures, mixed identifiable

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cultures or totally mixed indigenous microorganisms. Some SSF processes e.g., tempeh and onjom production, requires selective growth of organisms such as moulds that need low moisture levels to carry out fermentation with the help of extracellular enzymes secreted by fermenting microorganisms. However, bacteria and yeasts, which require higher moisture content for efficient fermentation can also be used for SSF, but with a lower yield.

SSF are normally multistep processes involving the following steps:

- Pre-treatment of substrate raw materials either by mechanical, chemical or biochemical processing to enhance the availability of the bound nutrients and also to reduce the size of the components, e.g., pulverizing straw and shredding vegetable materials to optimize the physical aspects of the process. However, the cost of pre-treatment must be balanced with eventual product value.
- Hydrolysis of primarily polymeric substrates, e.g., polysaccharides and proteins.
- Utilization (fermentation) of hydrolysis products.
- Separation and purification of end products.

The low moisture content of SSF enables a smaller reactor volume per substrate mass than LSF and also simplifies product recovery. However, serious problems arise with respect to mixing, heat exchange, oxygen transfer, moisture control and gradients of pH, nutrient and product as a consequence of the heterogeneity of the culture. The latter characteristic of SSF renders the measurement and control of the above mentioned parameters difficult, laborious and often inaccurate, thereby limiting the industrial potential of this technology. Due to these problems, the micro-organisms that have been selected for SSF are the more tolerant to a wide range of cultivation conditions.

Environmental parameters that influence SSF

Water activity: Water lost during fermentation through the processes of evaporation and metabolic activity is normally replaced by humidification or periodic addition of water. When moisture levels drop too low, the substrate becomes less accessible and when moisture levels are too high, the porosity of the substrate is reduced resulting in lower diffusion rates and decreased gas exchange. This would result in a decreased rate of substrate degradation and may lead to an increased risk of contamination.

Temperature: Is controlled by aeration and/or agitation of the substrate. Heat generation in this system is more problematic than in liquid fermentations and has a big impact on relative humidity.

Aeration: Depends on the microorganisms in the fermentation. Most systems are aerobic. Aeration rates are also closely related to the need to dissipate heat, CO₂ and other volatile substances that may be inhibitory. The rate of oxygen transfer in SSF is related to the size of the particles which determines void space. Oxygen transfer in the void space is related to moisture level as the oxygen dissolves in the film of moisture around the substrate particles.

Bioreactors used in SSF

Most SSF processes are batch fermentations. Some processes do not require vessels and require spreading of substrate on a suitable floor surface. Bioreactor designs (Fig. 2.6) for SSF are inherently simpler than for liquid cultivations. They are classified into fermentations without agitation (tray systems and air flow systems), with occasional agitation and with continuous agitation (slow rotating drums).

Rotating drum: usually a cylindrical drum mounted on its side onto rollers that support and rotate the vessel. They are equipped with an inlet and outlet for circulation of humidified air and often contain baffles or sections to agitate the contents. They are used in enzyme and microbial biomass production. Their main disadvantage is that the drum is not filled to capacity (only 30% capacity or mixing is inefficient). Rotary movements of the drum need to be controlled so as to minimize damage to the mycelial growth due to shear.

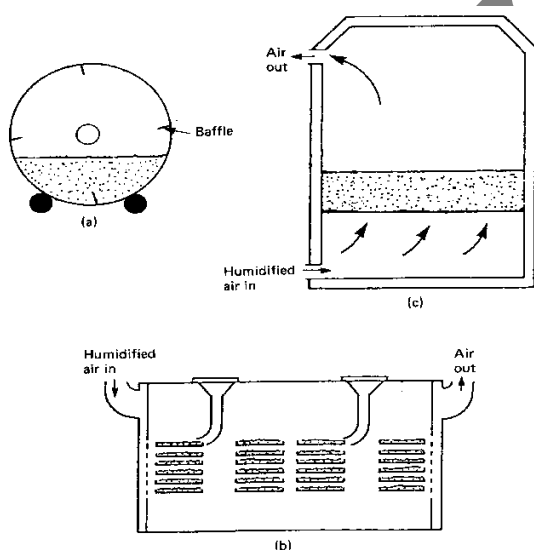


Fig.2.6: Solid substrate fermenter designs. (a) Rotating drum, (b) tray system, (c) forced air-flow system (Ward, 1992).

Tray fermenters: used extensively for the production of fermented oriental foods and enzymes. Trays hold layers of substrate 2.5-5 cm deep and are stacked in chambers usually force aerated with humidified air. In a forced-air cultivation chamber, bed temperature is monitored and appropriate temperature adjustments are made to the recycling air flow. There is less shear in these systems.

Bed systems: used in commercial koji production. Consist of a bed of substrate (1 m deep) through which humidified air is forced from below, continuously.

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Column bioreactors: a column, usually glass or plastic that contain loosely packed solid substrate. Temperature control is achieved by a jacket surrounding the column. These systems are used for organic acids, ethanol and biomass production.

Fluidized bed reactors: used for biomass production for animal feeds. To prevent adhesion and aggregation in these systems, they are continuously agitated with forced air.

Advantages of SSF

- Low moisture content of the substrates allow for minimal contamination.
- Can be carried out with small volumes of substrate, lowering the operational cost of the reactors.
- More energy economical.
- Product separation is easy and less cumbersome.
- Low cost media.
- No problems with foaming.
- Low waste water output.

Disadvantages of SSF

- Media are heterogenous, hence the mash is not properly mixed.
- Substrate moisture level is difficult to control.
- Reactor parameters such as pH, temperature and dissolved oxygen need precise control.
- Continuous mixing or agitation of the medium is required to overcome control parameters.
- Continuous agitation of the medium often damages the mycelia, retarding their growth and resulting in poor growth of the organisms.

Media fermentation

Design of Media Fermentation

In a fermentation process, the choice of the most optimum micro-organisms and fermentation media is very important for high yield of product. The quality of fermentation media is important as it provides nutrients and energy for growth of micro-organisms. This medium provides substrate for product synthesis in a fermenter.

Media Fermentation consists of major and minor components.

- Major components include Carbon and Nitrogen source.
- Minor components include inorganic salts, vitamins, growth factors, anti-foaming agents, buffers, dissolved oxygen, other dissolved gases, growth inhibitors and enzymes.

Nutrients required for fermentation media also depend upon the type of fermentation organisms as well as the type of fermentation process to be used. Poor choice of fermentation media might result in poor yield of output. Types of nutrients present in the fermentation media always determine the yield of the product.

There are two uses of fermentation media

1. Growth media

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2. Fermentation media

Growth medium contains low amounts of nutrients. It is useful in creating raw material for further fermentation processes.

Fermentation media contains high amounts of nutrients. It is used in creating final products using fermentation.

For example, growth of yeast requires 1% carbon. But during fermentation of alcohol, yeast requires 12 to 13 % carbon in the medium.

What is the role of Fermentation Media?

During the fermentation process, media contains high amounts of nutrients, micro-organism and optimum conditions. When these micro-organisms are incubated at the desired optimum conditions, they enjoy luxurious metabolism. Here, the fermentation organisms become hyperactive due to presence of high quantities of nutrients, thus it results in consumption of excess nutrients and partial degradation of fermentation media. The waste effluents excreted by the microbes could be the desired output product of the fermentation process.

The amount of substrate given to microbes should not reach inhibitory concentration levels because excess substrate inhibits vital enzymes and may results in death of cells. Also, water present in cytoplasm is important for metabolism process. If excess sugar or salt is available in the fermentation media, it would tie up cytoplasm water and may result in lack of water for metabolism and cause death of microbes, thus affecting fermentation output.

Excess substrate may increase osmotic pressure and effect enzyme activities in a cell. Microbes excrete this excess substrate in the form of partially digested fermentation media. It is converted to an insoluble inert compound in the form of reserve food material and this reserve food material is harmless to cells.

There are two types of fermentation media used in industries.

1. Synthetic media
2. Crude media

Let us discuss these types of media.

1. Synthetic media

Synthetic media is useful in the field of research as each and every component is chemically known and the exact composition of nutrients is predetermined. So, in case of synthetic media, variation in levels and concentration of nutrients can be controlled. Here, by experimentation with synthetic media, the effect of nutrients on growth and yield of product can be analysed. We can redesign the synthetic media as per our needs. It is very useful in controlling the growth and yield of product in a lab environment. We can also use it to determine the metabolic pathway used in the synthesis of products.

With the help of radio-isotope labeling technique, we can determine the main ingredients that gets used up to create the final desired product. In this way, we can know the exact proportions of ingredients required for our process. We can optimize this process by using alternative sources of carbon or nitrogen, and creating a fermentation media which is the most optimum for our

needs. The use of Synthetic media allows us to experiment with various sources of fermentation media in the lab as the results are accurately reproducible for a given composition.

An advantage of a well designed synthetic media is that it lacks sources of protein and peptides. Hence, there is no foam formation, and chances of contamination are very less. Product recovery is easier because synthetic media contains pure components.

Although there is a big list of advantages of synthetic media, there are some disadvantages. A major disadvantage is the cost of media. The most important aspect of fermentation is that it should be economic and profitable. Synthetic media is never used on industrial scale because it is expensive. This process is only suitable for experimentation in a lab on a small scale.

2. Crude Media

Crude media is generally used on an industrial scale for fermentation process. Crude media contains a rough composition of media required for fermentation. It gives high yield of product and contains undefined sources of ingredients. Crude media contains high level of nutrients, vitamins, proteins, growth factors, anti-foaming agents and precursors. It is important to ensure that crude media should not contain toxic substances that could effect the growth of bacteria and yield of product.

Ingredients of Crude Media

1) Inorganic nutrients

Crude media contains inorganic salts containing cations and anion along with a carbon source. Sometimes, fermentation micro-organisms have a specific requirement of ions like magnesium ions, phosphates or sulphates. These requirements are fulfilled by addition of these ions to balance the crude media.

2) Carbon source

Simple to complex carbohydrates can be added to media as a source of carbon. We can add different sugars like mannitol, sorbitol, organic acids, fatty acids, proteins, peptides we can choose any of these as a source of carbon. The selection of carbon source depends upon the availability as well as the cost of raw material. In most of the fermentation media, crude source of carbon is added.

1. **Simple carbohydrates** – simple sugars are semi purified polysaccharides and sugar alcohol are added. Sources of simple carbohydrates are Black strap molasses, Corn molasses, Beet molasses, sulphite waste liquor, Hydrol (corn sugar molasses), Cannery waste.
2. **Complex carbohydrates** – Source of complex carbohydrates are Starch, Corn, Rice, Rye, Milo, wheat potatoes etc. Source of starch cellulose are corn cobs, straws, wood waste, saw meal etc.

3) Nitrogen source

Salts of urea, ammonia, and nitrate can be used as a nitrogen source. When fermentation organisms are non-proteolytic in nature, pure form of urea, ammonia and nitrate are used as a source of nitrogen. When fermentation organisms are proteolytic in nature, animal and plant raw material is used; like distillery dried solubles, Casein, Cereal grains, peptones, yeast extract, hydrolysate, and soybean meal etc.

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4) Growth factors

Crude media constituents provides enough amount of growth factors so no extra addition of growth factor is required. If there is a lack of any kind of vitamins or nutrients, growth factors can be added to media. Examples are yeast extract, and beef extract.

5) Precursors

Precursors are generally present in the media as crude constituents. Precursors are added in the fermentation media at time of fermentation as it get incorporated in the molecules of product without bringing any kind of change to the final product. This helps in improving yield and quality of product. Sometimes, precursors are added in pure form depending upon the need of product. For example, Cobalt chloride is added less than 10 ppm in fermentation of vitamin B12.

6) Buffers

Buffers are used to control drastic changes of pH. Sometimes, media components may act as buffers. For example, protein, peptides, aminoacids act as good buffers at neutral pH. Sometimes inorganic buffers like K_2HPO_4 , KH_2PO_4 , and $CaCO_3$ etc, can be added as required. Generally, during the fermentation process, pH changes to acidic or alkaline pH. The cheapest and easily available buffer is $CaCO_3$.

Microbial metabolic products-primary and secondary metabolites

Primary Metabolites:

Primary metabolism, also referred to as trophophase, is characterized by balanced growth of microorganisms. It occurs when all the nutrients needed by the organisms are provided in the medium. Primary metabolism is essential for the very existence and reproduction of cells. In the trophophase, the cells possess optimal concentrations of almost all the macromolecules (proteins, DNA, RNA etc.).

It is during the period of trophophase, an exponential growth of microorganisms occurs. Several metabolic products, collectively referred to as primary metabolites, are produced in trophophase (i.e., during the period of growth).

The primary metabolites are divided into two groups:

1. Primary essential metabolites:

These are the compounds produced in adequate quantizes to sustain cell growth e.g. vitamins, amino acids, nucleosides. The native microorganisms usually do not overproduce essential primary metabolites, since it is a wasteful exercise. However, for industrial overproduction, the regulatory mechanisms are suitably manipulated.

2. Primary metabolic end products:

These are the normal and traditional end products of fermentation process of primary metabolism. The end products may or may not have any significant function to perform in the microorganisms, although they have many other industrial applications e.g. ethanol, acetone, lactic acid. Carbon dioxide is a metabolic end product of *Saccharomyces cerevisiae*. This CO_2 is essential for leavening of dough in baking industry.

Limitations in growth:

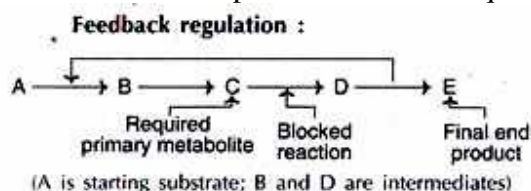
Due to insufficient/ limited supply of any nutrient (substrate or even O_2), the growth rate of microorganisms slows down. However, the metabolism does not stop. It continues as long as the cell lives, but the formation of products differs.

Overproduction of primary metabolites:

Excessive production of primary metabolites is very important for their large scale use for a variety of purposes.

Overproduction of several metabolites has been successfully accomplished by eliminating the feedback inhibition as briefly described below:

1. By using auxotrophic mutants with a block in one of the steps in the biosynthetic pathway concerned with the formation of primary metabolite (this should be an intermediate and not the final end product). In this manner, the end product (E) formation is blocked, hence no feedback inhibition. But overproduction of the required metabolite (C) occurs as illustrated below.



In the above example, an un-branched pathway is shown. This type of manipulation for overproduction of metabolites can be done for branched metabolic pathways also.

2. Mutant microorganisms with anti-metabolite resistance which exhibit a defective metabolic regulation can also overproduce primary metabolites.

Secondary Metabolites:

As the exponential growth of the microorganisms ceases (i.e. as the trophophase ends), they enter idiophase. Idiophase is characterized by secondary metabolism wherein the formation of certain metabolites, referred to as secondary metabolites (idiolites) occurs.

These metabolites, although not required by the microorganisms, are produced in abundance. The secondary metabolites however, are industrially very important, and are the most exploited in biotechnology e.g., antibiotics, steroids, alkaloids, gibberellins, toxins.

Characteristics of secondary metabolites:

1. Secondary metabolites are specifically produced by selected few microorganisms.
2. They are not essential for the growth and reproduction of organisms from which they are produced.
3. Environmental factors influence the production of secondary metabolites.
4. Some microorganisms produce secondary metabolites as a group of compounds (usually structurally related) instead of a single one e.g. about 35 anthracyclines are produced by a single strain of *Streptomyces*.
5. The biosynthetic pathways for most secondary metabolites are not clearly established.
6. The regulation of the formation of secondary metabolites is more complex and differs from that of primary metabolites.

Functions of secondary metabolites:

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Secondary metabolites are not essential for growth and multiplication of cells. Their occurrence and structures vary widely. Several hypotheses have been put forth to explain the role of secondary metabolites, two of them are given below.

1. The secondary metabolites may perform certain (unknown) functions that are beneficial for the cells to survive.
2. The secondary metabolites have absolutely no function. Their production alone is important for the cell, whatever may be the product (which is considered to be useless).

Overproduction of secondary metabolites:

As already stated, the production of secondary metabolites is more complex than primary metabolites. However, the regulatory manipulations employed for excess production of primary metabolites can also be used for the secondary metabolites as well.

Several genes are involved in the production of secondary metabolites. Thus, around 300 genes participate in the biosynthesis of chlortetracycline while 2000 genes are directly or indirectly involved in the production of neomycin. With such complex systems, the metabolic regulation is equally complex to achieve overproduction of secondary metabolites. Some regulatory mechanisms are briefly discussed hereunder.

Induction:

Addition of methionine induces certain enzymes and enhances the production of cephalosporin. Tryptophan regulates ergot alkaloid biosynthesis.

End product regulation:

Some of the secondary metabolites inhibit their own biosynthesis, a phenomenon referred to as end product regulation e.g. penicillin, streptomycin, puromycin, chloramphenicol. It is possible to isolate mutants that are less sensitive to end product inhibition, and in this manner the secondary metabolite production can be increased.

Catabolite regulation:

In this regulation process, a key enzyme involved in a catabolic pathway is inactivated, inhibited or repressed by adding a commonly used substrate. Catabolic repression can be achieved by using carbon or nitrogen sources. The mechanism of action of catabolite regulation is not very clearly understood.

The most commonly used carbon source is glucose. It is found to inhibit the production of several antibiotics e.g. penicillin, streptomycin, bacitracin, chloramphenicol, puromycin. The nitrogen sources such as ammonia also act as catabolite regulators (i.e. inhibitors) for the overproduction of certain antibiotics.

Phosphate regulation:

Inorganic phosphate (Pi) is required for the growth and multiplication of prokaryotes and eukaryotes. Increasing Pi concentration (up to 1 mM) is associated with an increased production of secondary metabolites e.g. antibiotics (streptomycin, tetracycline), alkaloids, gibberellins. However, very high Pi concentration is inhibitory, the mechanism of action is not very clear.

Auto regulation:

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In some microorganisms (particularly actinomycetes), there occurs a self regulation for the production of secondary metabolites. A compound designated as factor A which is analogous to a hormone is believed to be closely involved in auto regulation for the production streptomycin by *Streptomyces griseus*. More such factors from other organisms have also been identified.

Bioconversions:

Microorganisms are also used for chemical transformation of unusual substrates to desired products. This process, also referred to as biotransformation, is very important in producing several compounds e.g. conversion of ethanol to acetic acid (in vinegar), sorbitol to sorbose, synthesis of steroid hormones and certain amino acids.

In bioconversion, microorganisms convert a compound to a structurally related product in one or a few enzymatic reactions. The bioconversions can be carried out with resting cells, spores or even killed cells. Non-growing cells are preferred for bioconversions, since high substrate concentration can be used, besides washing the cells easily (to make them free from contamination).

Sometimes, mixed cultures are used for bioconversions to carry out different reactions. In recent years, the yield of bioconversion is increased by using immobilized cells at a lower cost

Examples of bioprocess for the production of biomass

The capacity to do work is referred to as energy. Energy may be considered as a form of matter which is inter-convertible. The modern man is mostly dependent on three sources for his energy needs—coal, natural gas and oils, collectively referred to as fossil fuels or fossil energy sources.

The fear of depletion of global fossil fuels has forced man to look for suitable alternative energy sources such as solar, hydro, tidal and wind power, and more recently nuclear energy. In addition to these, advances in biotechnology have helped to fruitfully utilize the energy from biological systems.

Biomass:

Biomass is the total cellular and organic mass, produced by the living organisms. It is the primary product of photosynthesis and is a good source of energy i.e. bioenergy. Broadly speaking, biomass represents all forms of matter derived from biological activities. These include plants and agricultural products, microorganisms, animal wastes and manure.

The term biomass is also used to collectively describe the waste materials produced in food and agricultural industries. Besides being a good source of energy, biomass is important for the production of several commercially important products. Thus, biomass is appropriately regarded as a renewable source of energy which can be directly converted to energy or energy carrier compounds by various means.

In most developed countries, biomass is utilized for the production of industrial and commercial products (ethanol, oils, methane, and single – cell protein). In contrast, in the developing countries (India, Latin America, Africa), a major part of the biomass is directly used as a source of energy (as firewood).

It is estimated that the annual net yield of plant biomass is around 175 billion tons of dry matter (125 billion tons on land and 50 billion tons on oceans). Forests significantly contribute to the

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production of land based biomass (around 45%). Agricultural crops on the cultivated land account for about 6% of the plant biomass.

The agricultural biomass products (cereals, pulses, oils, animal feed etc.) adequately meet requirements of foods for humans and animals, besides other basic needs (fuels, chemicals etc.). From the chemical point of view, about 50% of the land produced biomass is in the complex form of lignocellulose.

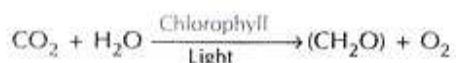
Fossil fuels-derivatives of biomass:

The modern society is dependent on the nonrenewable sources of energy namely oil, gas and coal. These fossil fuels are actually derivatives of ancient biomass. It took millions of years for the fossil fuels to be deposited beneath the earth and oceans.

However, in just within a century of exploration, the major fuel reserves (particularly gas and oil) are depleted, and at the present rate, they are not likely to last long. As such, there exists an energy crisis throughout the world. Consequently, researchers continue their search for alternate and renewable sources of energy.

Photosynthesis-the ultimate source of energy:

Photosynthetic organisms are the ultimate sources for trapping the solar energy. In the presence of photosynthetic pigment chlorophyll, carbon dioxide is converted into complex carbohydrates with the evolution of oxygen.



In the reactions that follow later, solar energy is trapped into molecules such as fat and proteins, besides other complex carbohydrates (cellulose, hemicellulose, and lignin). Photosynthetic organisms are the true solar energy converters. It is estimated that at present more than 10 times more energy is generated by photosynthesis annually than consumed by the world's population.

Unfortunately, the role of photosynthesis is not well recognized to solve the present day problem of energy crisis. This, despite the fact that it is only the photo synthetically produced biomass that is available today in the form of fossil fuels. The biomass produced by photosynthesis can be appropriately utilized for the production of fuels (alcohol, methane) and various other commercial products.

Chemical nature of biomass:

The plant biomass is mainly composed of cellulose, hemicellulose, lignin, starch, proteins, water soluble (sugars, amino acids) and fat soluble (oils, pigments) compounds. In fact, majority of these constituents are present in the plant cell walls.

There is a wide variation in the chemical composition of the biomass, depending on the source. For instance, the biomass obtained from sugar cane and beet sugar is rich in sugars while the biomass of potato and topioca is rich in starch. On the other hand, cotton has high content of cellulose. The chemical nature of biomass derived from industrial and municipal wastes is highly variable which mostly depends on the sources that contribute to the biomass.

Sources and Utilization of Biomass:

The major sources of biomass are natural vegetation, energy crops, and agricultural, industrial and urban organic wastes (Fig. 31.1). Their production in turn is dependent on the solar energy.

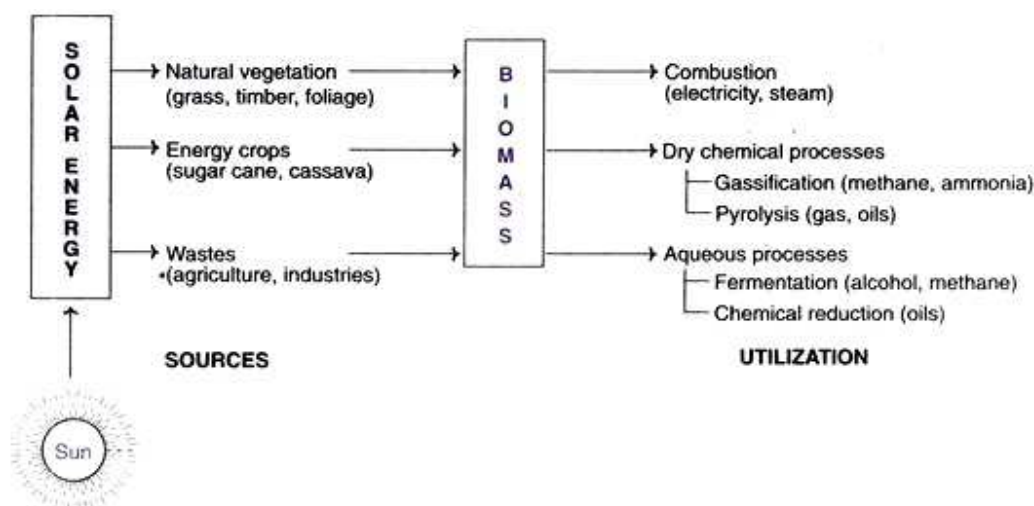


Fig. 31.1 : An overview of the sources and utilization of biomass.

The natural vegetation (growing natural forests and aquatic weeds) significantly contributes to biomass. Wood-rich plants are grown in many countries (particularly developing countries) to generate fire for cooking and other purposes. In recent years, well planned and organized plantations are carried out in some countries to produce biomass to meet energy demands. For instance, sugar cane and cassava plantations in Brazil and Australia are used for ethanol production. Plants rich in lignocellulose are grown in America and Sweden which are useful for the production of liquid fuels (ethanol, methanol).

Agricultural, industrial and municipal wastes were earlier considered as useless and discarded. But in the recent past, many countries have developed methods for converting these wastes into biofuels and commercially important products. The successfully used agricultural wastes include straw, bagasse, bran, cotton wastes. Among the industrial wastes, molasses, whey, distillery wastes and sewage are the important ones. The biomass is utilized for the production of biofuels and various other compounds. The technique mainly depends on the chemical nature and moisture content of biomass.

Combustion:

Low moisture containing biomass (wood, straw, bran) can be directly burnt by a process referred to as combustion to generate electricity.

Dry chemical processes:

The biomass with little moisture content can be subjected to various dry chemical processes- pyrolysis, gasification to produce methanol, oil and ammonia biomass.

Aqueous processes:

The biomass with high water content is used in aqueous processes such as fermentation to produce ethanol, oils and methane. An overview of the sources and utilization of biomass is depicted in Fig. 31.1.

Production of Alcohol from Biomass:

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Alcohol, chemically ethanol (C_2H_5OH) has been produced by fermentation for thousands of years. Although the developed countries these days prefer to manufacture ethanol by chemical means, the developing countries continue to produce it by microbial fermentation. Alcohol is the liquid fuel which is mostly produced from the biomass. The raw materials (biomass) used for alcohol production include starchy materials (wheat, rice, maize, potato) and cellulosic materials (wood, agricultural wastes).

Energy-Rich Crops:

Some of the plants are very efficient in converting CO_2 into biomass and such plants are collectively referred to as energy-rich crops.

Sugar and starch crops:

Certain plants like sugar cane, sugar beet, cereals and tuber crops produce high quantities of starch and fermentation sugars. These crops supply energy-rich foods and feeds. Such plants are useful for production of biofuels, particularly ethanol often referred to as bioethanol.

Wood-rich plants:

Some plants grow very fast and they serve as good suppliers of wood. E.g. Eucalyptus, Butea, Melia, Casurina. These plants are important sources of firewood. It is estimated that approximately 50% of the total wood harvested annually is utilized for the purpose of firewood. Wood is also useful for the supply of pulp for paper manufacture.

Petroleum plants:

There are certain plants which can accumulate high molecular weight hydrocarbons. They are referred to as petro-crops or gasoline plantations. The products of these hydrocarbon-rich plants can serve as good substitutes of conventional petroleum and petroleum products.

The rubber plant (Hevea rubber), grown in South-East Asia is the principal source of rubber. Rubber is collected in the form of latex from the stems of trees. This plant meets about one third of the total world's demand of rubber.

However, the rubber produced from petroleum is preferred for use in automobiles and planes, due to low-cost and high elasticity. Besides Hevea rubber, there are some other plants for the production of natural rubber e.g. *Parthenium argentatum* (guayule), *Taraxacum koksaghyz* (Russian dandelion) grown in Mexico and some parts of USA.

Euphorbia lathyrus and *E. terucalli* contain high contents of terpenoids (complex hydrocarbons) that can be directly converted to gasoline/petrol. It is estimated that *E. terucelli* can yield about 5-10 barrels of oil/acre/year.

Aak plant (*Calotropis procera*) secretes latex which is very rich in hydrocarbons. These hydrocarbons, and the yield are comparable to *Euphorbia lathyrus*, and they also serve as good substitutes of petroleum.

For obvious reasons, the cultivation of petroleum plants is encouraged throughout the world.

Besides its utility for the generation biofuels (alcohol, methane), biomass is also used for the production of butanol, acetone, single-cell protein and many other products. As such, the contribution of biomass to the world's requirements of energy is very low. It is around 5% in the U.S.A., and may be a little higher in the developing countries. However, being a renewable

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source of energy, biomass will have immense value in future. This is particularly true as the world's non-renewable fuels (gas and oil) get depleted.

There is a growing realization on the fuel value of biomass. In the coming years, biomass production and utilization strategies will be fully exploited. In addition, further improvements in the biotechnological processes for better management and utilization of industrial, agricultural and domestic wastes will also solve the problem of world energy crisis.

POSSIBLE QUESTIONS

Two marks

1. What are the phases involved in microbial growth?
2. Define fermentation.
3. What are the techniques employed in isolation of industrially important microbes?
4. Define biomass.
5. Define solid state fermentation.
6. Define media state fermentation.
7. What are the ways employed to measure the microbial growth?

Essay type questions

1. Write short notes on balanced and unbalanced microbial growth.
2. Explain the design of a fermenter in detail.
3. Write short notes on measurement of microbial growth.
4. Explain about solid substrate fermentation and media fermentation.
5. Explain about the isolation and screening of industrially important microbes.
6. What are the examples of bioprocess for the production of biomass?
7. Discuss on isolation and screening of industrially important microbes.
8. Write short notes on solid substrate fermentation and media fermentation.
9. Describe the measurement of microbial growth.
10. Write short notes on microbial metabolic pathways.

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MULTIPLE CHOICE QUESTIONS
UNIT IV

S.No	Questions	Option A	Option B	Option C	Option D	Answer
1	Isolation of a micro – organism with potential industrial application is achieved in	Primary Screening	Secondary Screening	Plate count method	Fermentation	Secondary Screening
2	Enrichment culture is the technique resulting in the	Increase of the desired organism	Increase of the mixed population	Increase of unwanted organism	Above all	Increase of the desired organism
3	Protein is separated from the fermented broth by	Precipitation	Adsorption	Dialysis	Reverse osmosis	Precipitation
4	The air and gas is pumped in which zone of air lift bioreactor	Riser	Down comer	Both	None	Riser
5	Purification step is generally used in	Upstream processing	Downstream processing	Fed batch culture	Continuous flow culture	Downstream processing
6	In two stage air lift bioreactors the temperature needed in two steps is	20°C & 35°C	30° & 42°C	45°&60°C	10°&20°C	30° & 42°C
7	Cryo preservation done at what temperature	- 18°C	-180°C	-196°C	-4°C	-196°C
8	Fermentation that add substrate at regular interval	continous culture	Batch culture	fed batch culture	semi continuos culture	fed batch culture
9	The basic nutritional requirements of micro organisms during fermentation process---	C and Nitrogen	Sulphur	Zinc	Molybdenum	C and Nitrogen
10	Fermentation Means_____	Chemical transformation of organic compounds carried out by m.o and their enzymes	Chemical transformation carried out by enzymes	Chemical transformation carried out only by micro organism	None	Chemical transformation of organic compounds carried out by m.o and their enzymes

11	The plant biomass is composed of	cellulose	starch	lignin	all of the above	starch
12	The secondary metabolites	Play no role in the growth of microorganisms	Are alkaloids, antibiotics etc	Play a significant role in the growth of micro organisms	Known as idiolites	Known as idiolites
13	Give an example of butyl alcohol producing bacteria	Streptococcus	Salmonella	Clostridium	Acetobacter	Acetobacter
14	Which of the following organic acid was first manufactured industrially by fermentation	Butyric acid	Citric acid	Lactic acid	Formic acid	Lactic acid
15	In alcoholic fermentation the pyruvate is converted into----- ----	Ethanol and CO ₂	Oxygen and methanol	Methanol and oxygen	CO ₂ and glucose	Ethanol and CO ₂
16	The following are one carbon compounds except	Formic acid	Methanol	Carbon monoxide	Ethanol	Ethanol
17	Micro organism capable of growing one carbon substrate are called	Methylotroph	Organotroph	lithotroph	phototroph	Methylotroph
18	Following are methanol producing organisms except	Methymonos	Methylobacter	Methylococcus	Methylo bacilli	Methylo bacilli
19	The following are two carbon compounds except	Ethanol	Acetate	Glyoxylate	Carbon monoxide	Carbon monoxide
20	Which one of the following is not a two carbon compounds	Ethanol	Acetate	Glyoxylate	Dimethylamine	Glyoxylate
21	Clostridium acetobutylicum produce	Lactic acid	Acetone-butanol	Acetone	Formic acid	Acetone-butanol
22	The end product of anaerobic fermentation of glucose is	Lactic acid	Pyruvic acid	Citric Acid	Formic acid	Lactic acid
23	Technique that find out the antibiotic producing micro organism is	Spread plate	pour plate	Streak plate	Crowded plate	Crowded plate
24	The fermenter that is used both in batch and continuous model is	Deep- Shaft Reactor	Airlift fermenter	Bubble column fermenter	None of the above	Airlift fermenter

25	The following are true with regard to solid or semi-solid culture	agar with nutrient media gives solid or semisolid culture	Solid culture is generally avoided for microbial products	Solid culture is used for the production of amylase from <i>Aspergillus Niger</i>	all options	all options
26	Ribulose-1-Phosphate 36. Organism involved in butyric acid fermentation	<i>Pseudomonas</i>	<i>Bacillus popilliac</i>	<i>Candida albicans</i>	<i>Clostridium aceto butilicum</i>	<i>Clostridium aceto butilicum</i>
27	Technique used to find growth factor producers is	Autography	autoradiography	auxanography	auxotrophy	auxanography
28	In a fermenter the function of impeller is to	Stirr the Contents	Warm the contents	Filter the Contents	Cool the contents	Stirr the Contents
29	Which one of the following is not a usual strain improvement procedure	Genetic recombination	Natural Mutant selection	Mutation induction	Medium enrichment	Medium enrichment
30	A Fermenter vessel	Is made up of steel	Known as bio reactor	Is Designed to carryout biological reactions Under controlled conditions	Do not require sterilization	Is Designed to carryout biological reactions Under controlled conditions
31	Amino acids are examples of.....	secondary metabolites	both primary and secondary	primary metabolites	all of the above	primary metabolites
32	The most commonly used carbon source for the production of secondary metabolites	glucose	sucrose	lactose	fructose	glucose
33	Secondary metabolism is also referred as	Idiophase	trophophase	exponential phase	regulatory phase	Idiophase
34	Which one of the following is a secondary metabolite?	amino acids	steroids	antibiotics	vitamins	antibiotics
35	Alkaloids are involved in....	primary metabolites	hormones	secondary metabolites	all options	secondary metabolites
36	Which of the following is a primary metabolite?	Vitamins	steroids	antibiotics	gibberellins	Vitamins

37	Primary metabolites are produced in.....	exponential phase	idiophase	trophophase	regulatory phase	trophophase
38	Biogas production from biomass is an process	aerobic	photolytic	hydrolytic	anaerobic	anaerobic
39	The liquid fuel which is produced from the biomass	oils	alcohol	petroleum	water	water
40	Biogas is a mixture of.....	O ₂ , CH ₄ , H ₂ S, Na	CO ₂ , CH ₄ , H ₂ S, N	H ₂ O, CO ₂ , CH ₄ , N	CH ₄ , CO ₂ , N	CH ₄ , CO ₂ , N
41	The first stage in the screening for microorganisms of potential industrial application is their_____	Isolation	Extraction	Purification	Filtration	Isolation
42	Micro organisms are transferred from one medium to another by_____	Fermentation	putrifaction	Subculturing	Staining	Subculturing
43	Chemical mutagens and UV Light are used for_____	Culture improvement	Non-growth of culture	Non – Contamination	Culture retardation	Culture improvement
44	Microorganisms for industrial application are grown in_____	Culture tubes	Shake flasks	Stirred fermenter	all options	all options
45	Fermentation vessel	Designed to carryout biological reaction under controlled condition	Is always without oxygen	Does not require sterilization	Is always with oxygen	Designed to carryout biological reaction under controlled condition
46	In fermentor ,air passes into	vertex	Walls	Sparger	Filter	Sparger
47	Baffles in a Fermenter	Control Foam	Control Temperature	Improve the efficiency of O ₂ transfer	Filter microorganisms	Improve the efficiency of O ₂ transfer
48	In a Fermenter soya bean oil is used as a	Antifoam agent	Heating agent	Cooling agent	Pressure maintaining agent	Antifoam agent
49	Aeration and agitation of a liquid medium can cause the production of	Antifoam	Foam	Fermentation	Innoculation	Foam

50	A sparger may be defined as device for introducing ----- into the fermentation	Air	Liquid	Inoculum	Anti foaming agents	Air
51	Growth of microorganisms on moist solid material in absence of free water is	media fermentation	solid substrate fermentation	batch fermentation	continuous fermentation	solid substrate fermentation
52	When ___ becomes excessive there is a danger that filters wet resulting in contamination?	media	foaming	aeration	all the above	foaming
53	The media used in industrial fermentation is generally rich in	proteins	carbohydrates	oil	fat	proteins
54	Continuous and adequate mixing of the microbial culture ensures optical supply of	nutrients	oxygen	both nutrients and oxygen	none	both nutrients and oxygen
55	A pressure cycle fermenter with large dimensions constitute a	airlift bioreactors	tower bioreactors	packed bed bioreactors	photo bio reactor	tower bioreactors
56	Which screening technique is employed in detecting and isolating antibiotic producers	enrichment culture technique	the crowded plate technique	membrane filtration of sample	none	the crowded plate technique
57	Variation in viscosity of compounds may include in fermentation media is defined	Fast metabolism	Rheology	Antifoams	None	None
58	The following are two carbon compounds except	Ethanol	Acetate	Glyoxylate	Carbon monoxide	Carbon monoxide
59	Immobilized enzymes:	Potential of activity	Presentation of activity	Preparation of activity	All options	Presentation of activity
60	Substrate concentration at which an enzyme attains half its maximum velocity is	Threshold value	Michaelis-Menton constant	Concentration level	None	Michaelis-Menton constant

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COURSE CODE: 19BCP102 UNIT-V: PRODUCTION OF FERMENTED PRODUCTS AND DOWNSTREAM PROCESSING
(BATCH-2019-2021)

UNIT V
SYLLABUS

Production of fermented products and downstream processing: Production of alcohol and alcoholic beverages. Microbial production of Organic acids: Source, recovery and uses of Citric acid, Lactic acid, Acetic acid and L-ascorbic acid. Production of antibiotics: Penicillin and Tetracyclin. Bioinsecticides: Production of Bacterial and fungal polysaccharides, commercial production of Xanthan gum and pullulan. Production of edible mushroom and SCP. Biofertilizers (*Phosphobacterium* and *Rhizobium sp.*,- Basics only).

Production of alcohol and alcoholic beverages

The production of alcohol beverages is a process that involves the active participation of microorganisms, most often yeasts. Humans have been producing alcoholic beverages for thousands of years. The production of alcohol in these drinks is based primarily on yeast fermentation. Yeasts are eukaryotic microorganisms that ferment variety of sugars from different sources into the final products of carbon dioxide and alcohol.

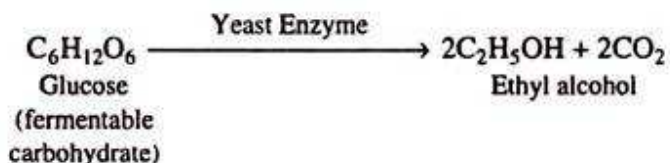
Ethyl alcohol is the most common solvent and raw material. It is next to water and is used in laboratory and chemical industry. Much of this alcohol is obtained synthetically from ethylene. However, its production from microbial fermentation using variety of cheap sugary substrates is still commercially important.

It is imperative that the microorganisms used must have a high tolerance for alcohol, must grow vigorously and produce a large quantity of alcohol. Yeasts, particularly *Saccharomyces cerevisiae*, represent the best known microorganisms used in the production of ethyl alcohol.

Some of the inexpensive substrates used in alcohol industry are molasses from cane sugar or waste sulphite liquor from paper industries. Starch yielding grains (corn), potatoes, grapes may also be used as substrate if their prices permit. Some countries used sugar-beet for the purpose.

Reaction:

The chemical reaction that results in the microbial fermentation of carbohydrate into alcohol can be represented as follows:



1. Commercial Production using Molasses as Raw Material:

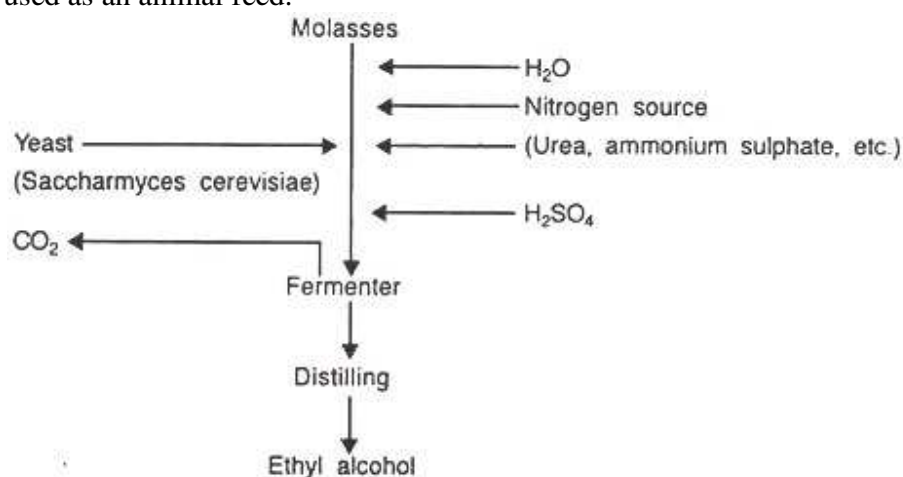
Molasses contain about 50% fermentable carbohydrates (sugars). Big deep tanks of steel or stainless steel are used as containers in the industrial production method.

Molasses is diluted to a suitable sugar concentration (15-16%); a small quantity of nitrogen source (e.g., ammonium phosphate, urea, ammonium sulphate) and sulphuric acid (H₂SO₄) is

added in it. pH of this medium is maintained at about 5.0 and an actively growing *Saccharomyces cerevisiae* culture is added in it.

The fermentation starts and is allowed to proceed for about 24-40 hours at about 25-30°C temperature. The fermented medium is then distilled and passes through rectifying columns to obtain ethyl alcohol. The yield of ethyl alcohol ranges about 50% of the fermentable sugar concentration present in the medium.

The large amount of CO₂ which is produced during the fermentation process as a result of decarboxylation is recovered and compressed to its solid state. The yeast recovered is usually used as an animal feed.



Steps in the manufacture of ethyl alcohol using molasses as fermentation substrate

2. Commercial Production Using Starch as Raw Material:

When starches such as corn are used as the raw material they have first to be hydrolysed to release simple fermentable sugars. The hydrolysis can be accomplished with enzymes from barley malt or moulds (e.g., *Aspergillus oryzae*) or by heat-treatment of acidified material. After the simple fermentable sugars are obtained, the fermentation process proceeds similarly to that of molasses.

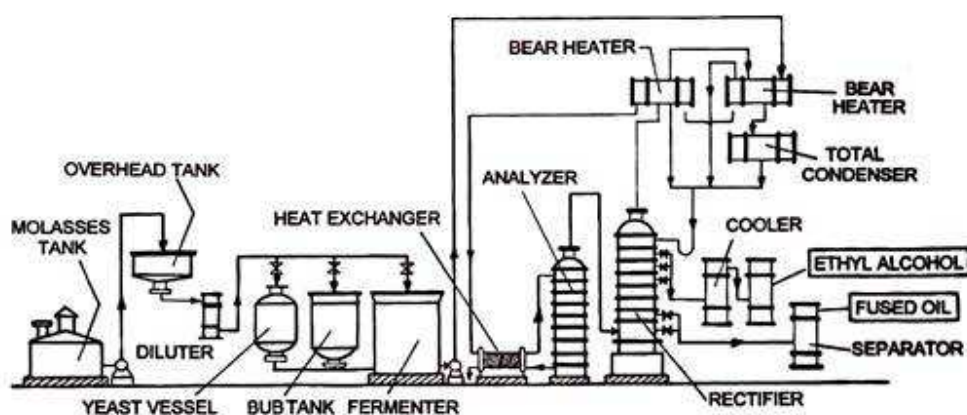
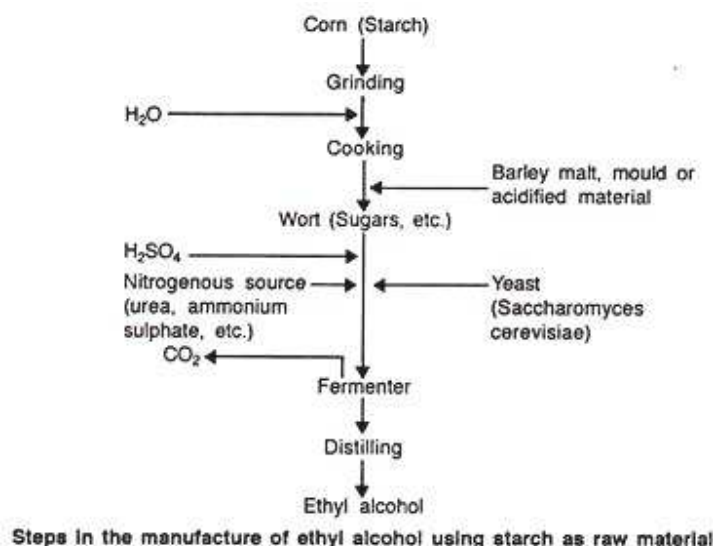


FIG. 40.1. Commercial production of alcohol from molasses

Microbial production of one of the organic feed stocks from plant substances such as molasses is presently used for ethanol production. This alcohol was produced by fermentation in the early days but for many years by chemical means through the catalytic hydration of ethylene. In modern era, attention has been paid to the production of ethanol for chemical and fuel purposes by microbial fermentation. Ethanol is now-a-days produced by using sugar beet, potatoes, corn, cassava, and sugar cane (Fig. 20.6).

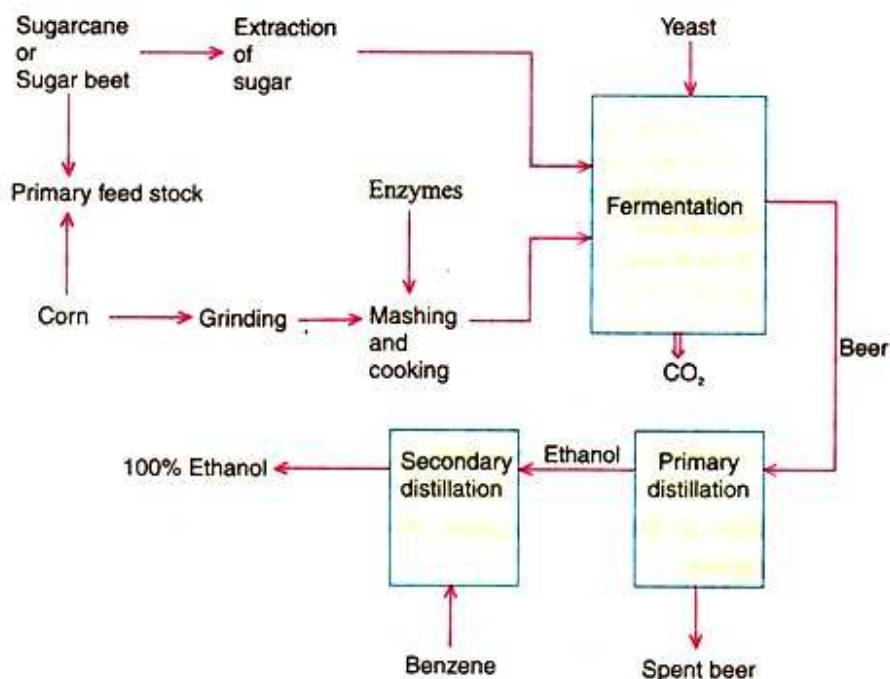


Fig. 20.6: Ethanol production from molasses.

Both yeasts (*Saccharomyces cerevisiae*, *S. uvarum*, *S. carlsbergensis*, *Candida brassicae*, *C. utilis*, *Kluyveromyces fragilis*, *K. lactis*) and bacteria (*Zymomonas mobilis*) have been employed for ethanol production in industries.

The commercial production is carried out with *Saccharomyces cerevisiae*. On the other hand, *uvarum* has also largely been used. The *Candida utilis* is used for the fermentation of waste sulphite liquor since it also ferments pentoses.

Recently, experimentation with *Schizosaccharomyces* has shown promising results. When whey from milk is used, strain of *K. fragilis* is recommended for the production of ethanol. It is also found that *Fusarium*, *Bacillus* and *Pachysolen tannophilus* (yeast) can transform pentose sugars to ethanol.

Theoretically, it is interesting to note that fermentation process retains most of the energy of the sugar in the form of ethanol. The heat of combustion of solid sucrose is 5.647 MJ mol⁻¹, the heat of combustion of glucose is 2.816 MJ mol⁻¹ but the heat release is 1.371 MJ mol⁻¹.

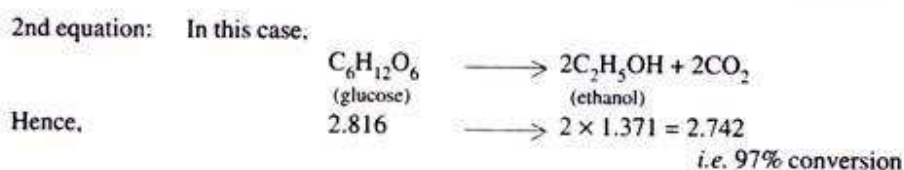
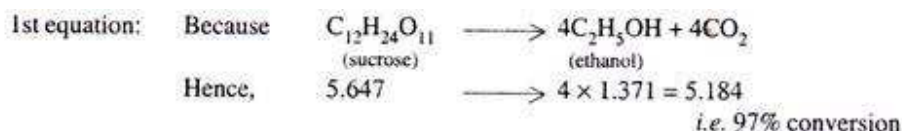
The equations are given below:

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Thus the above reactions show that 97% sugar transforms into ethanol. But in practice, the fermentation yield of ethanol from sugar is about 46% or one hundred grams of pure glucose will yield 48.4 grams of ethanol, 46.6 g of CO₂, 3.3 grams of glycerol and 1.2 g of yeast. The biosynthesis of ethanol is given in Fig. 20.6.

It is noteworthy that the ethanol at high concentration inhibits the yeast. Hence, the concentration of ethanol reduces the yeast growth rate which affect the biosynthesis of ethanol.

It can produce about 10-12 % ethanol but the demerit of yeast is that it has limitation of converting whole biomass derived by their ability to convert xylulose into ethanol. The *Zymomonas* has a merit over yeast that it has osmotic tolerance to higher sugar concentration. It is relatively having high tolerance to ethanol and have more specific growth rate.

1. Preparation of Medium:

Three types of substrates are used for ethanol production:

- Starch containing substrate,
- Juice from sugarcane or molasses or sugar beet,
- Waste products from wood or processed wood. Production of ethanol from whey is not viable.

If yeast strains are to be used, the starch must be hydrolysed as yeast does not contain amylases. After hydrolysis, it is supplemented with celluloses of microbial origin so as to obtain reducing sugars. About 1 ton of starch required 1 litre of amylases and 3.5 litre of glucoamylases. Following steps are involved in conversion of starch into ethanol (Fig. 20.7).

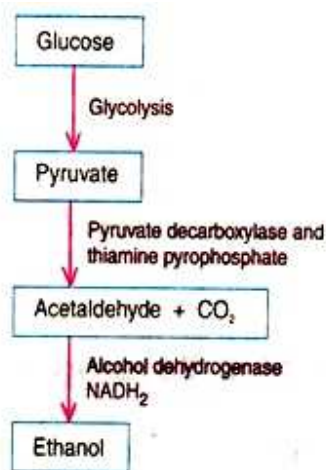


Fig. 20.7 : Biosynthesis of ethanol.

On the other hand, if molasses are used for ethanol production, the bagasse can also give ethanol after fermentation. Several other non-conventional sources of energy such as aquatic plant biomass, wood after hydrolysis with celluloses gives ethanol. Sulphite waste-liquor, a waste left after production of paper, also contains hexose as well as pentose sugar. The former can be microbially easily converted.

2. Fermentation:

Ethanol is produced by continuous fermentation. Hence, large fermenters are used for continuous manufacturing of ethanol. The process varies from one country to another. India, Brazil, Germany, Denmark have their own technology for ethanol production.

The fermentation conditions are almost similar (pH 5, temperature 35°C) but the cultures and culture conditions are different. The fermentation is normally carried out for several days but within 12h starts production. After the fermentation is over, the cells are separated to get biomass of yeast cells which are used as single cell protein (SCP) for animal's feed.

The culture medium or supernatant is processed for recovery of ethanol (Fig. 20.6). Ethanol is also produced by batch fermentation as no significant difference is found both in batch and continuous fermentation.

Although as stated earlier within 12h *Saccharomyces cerevisiae* starts producing ethanol at the rate of 10% (v/v) with 10-20g cells dry weight/lit. The reduction in fermentation time is accomplished use of cell recycling continuously in fermentation.

3. Recovery:

Ethanol can be recovered upto 95 percent by successive distillations. To obtain 100 percent, it requires to form an azeotropic mixture containing 5 percent water. Thus 5 percent water is removed from azeotropic mixture of ethanol, water and benzene after distillation. In this procedure, benzene water ethanol and then ethanol-benzene azeotropic mixture are removed so that absolute alcohol is obtained.

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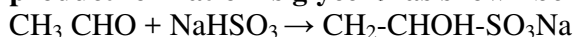
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Neuberg's Fermentation:

Yeasts utilize pyruvate during fermentation resulting in the formation of an intermediary product acetaldehyde.

This is trapped by hydrogen sulfite to yield the acetaldehyde in precipitated form and fluid product formation is glycerol as shown below:



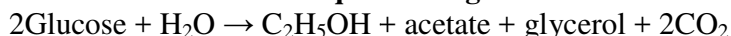
Now in place of acetaldehyde, dihydroxyacetone phosphate acts as a hydrogen acceptor which is reduced to glycerol-3-phosphate.

After removal of phosphate i.e. dephosphorylation, it gives glycerol as given below:



Neuberg's fermentation process is categorized as reward and third fermentation.

The first fermentation equation is given below:



Wine production

Wine is made from grapes or other fruit. The grapes are first cleaned of leaves and stems and the fruit is crushed into must that is ready for fermentation. The yeasts used for the fermentation grow a film on the fruit or in the environment. These wild strains play an important role in the final properties of the drink. However, cultivated strains of *Saccharomyces cerevisiae* are often added to improve the consistency of the final product. There are hundreds of commercially available yeast strains for wine fermentation.

In the fermentation process, energy that is converted to heat is produced as well. It is important to keep the temperature in the fermentation vessel lower than 40°C to keep the yeasts alive. To improve yeast growth, additional nutrients, like diammonium phosphate, are sometimes added in the fermentation step.

When making red wine, there is an additional fermentation step after alcoholic fermentation. Malic acid, naturally present in grape juice, can be converted to lactic acid by lactic acid bacteria naturally found in wineries or added artificially.

Beer production

Beer is the most consumed alcoholic beverage in the world. It is made most often of malted barley and malted wheat. Sometimes a mixture of starch sources can be used, such as rice. Unmalted maize can be added to the barley or wheat to lower cost. Potatoes, millet and other foods high in starch are used in different places in the world as the primary carbohydrate source. The process of making beer is called brewing. It includes breaking the starch in the grains into a sugary liquid, called wort, and fermenting the sugars in the wort into alcohol and carbon dioxide by yeasts. Two main species are used in the fermentation process: *Saccharomyces cerevisiae* (top-fermenting, since it forms foam on top of the wort) and *Saccharomyces uvarum* (bottom-fermenting). Top-fermenting yeasts are used to produce ale, while bottom-fermenting produce lagers. The temperature used for top-fermenting (15-24°C) leads to the

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production of a lot of esters and flavor products that give beer a fruity taste. Hops are added to introduce a bitter taste and to serve as a preservative.

Brewer's yeasts are very rich in essential minerals and B vitamins, with the exception of vitamin B12. Beer brewing in modern days is performed by added pure cultures of the desired yeast species to the wort. Additional yeasts species that are used in making beer are *Dekkera/Brettanomyces*. After the fermentation is finished, the beer is cleared of the yeasts by precipitation or with the use of clearing additives.

Other types of alcohol beverages are made by the fermentation activity of microorganisms as well. A few examples are sake (uses the fungus *Aspergillus oryzae* to facilitate starch fermentation from rice), brandy, whiskey (both are distilled alcohol), and other alcohol beverages with higher percentage of alcohol compared to wine and beer.

Vinegar

Vinegar is a food product made by acetic acid bacteria that can ferment the alcohol in alcoholic liquids to acetic acid.

Vinegar has been used for cooking and in the household and different industries due to its mildly acidic nature for many centuries. It is one of the foods together with beer, wine, bread and fermented dairy products, that is the result of fermentation by microorganisms and has been around for thousands of years. It is a mixture of acetic acid (most often 5%) and water.

The fermentation is performed usually by acetic acid bacteria, from the genus *Acetobacter*, from the alcohol in variety of sources (e.g., apple cider, wine, potatoes, fermented grain). *Acetobacter* bacteria are Gram negative aerobic rods. They are naturally present in environments where alcohol is being produced and can be isolated from damaged fruit, apple cider, etc. In these liquids, the bacteria form a film on the surface, since they are aerobic and need good oxygen supply. This film, called mother of vinegar, can be used as a starter culture of acetic fermentation in fresh alcohol liquids. Mother of vinegar can also be found in unpasteurized store brand vinegar. Acetic acid bacteria are transmitted in nature by vectors like fruit flies and Vinegar eels.

Mother of vinegar: Mother of vinegar is used as a starter culture for vinegar production. It is made of a specific cellulose and acetic acid bacteria

This acetic acid fermentation needs oxygenation. If left at room temperature alcohol containing solution with *Acetobacter* will be converted to vinegar in months. The industrial process can be completed within hours since air is bubbled and mixed through the solution.

Vinegar can also be an undesired product in wine production. If the temperature in the fermentation vessel is too high, the *Acetobacter* will outgrow the yeasts and the produced alcohol will be converted to vinegar.

There are bacteria that can convert sugars straight to acetic acid in anaerobic fermentation. Such species include *Clostridium* and *Acetobacterium* but they cannot tolerate acetic acid of concentrations higher than a few percent. The product made from these bacteria must be concentrated while oxidative fermentation by *Acetobacter* can produce up to 20% acetic acid.

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Vinegar is a food product made all over the world from many different carbohydrate sources where alcohol fermentation has been performed. Some of them are more commonly used, such as apple cider and grapes, while others such as coconut water, dates, kiwifruit are used in specific regions of the world. Vinegar is used not only in food preparation but also as a cleaning agent due to its acidic nature and strong antibacterial properties. It can be used to lower the glycemic index of foods if consumed together with them. It has also been shown to reduce the risk of fatal ischemic heart disease when consumed frequently with oil in salad dressings.

A. Undistilled Beverages:

(i) Beer Fermentation:

Beer is an undistilled product of grain-mash fermentation brought about by yeast. Beer fermentation involves the conversion of starchy raw materials into sugars and then into alcohol. It is generally prepared from malted barley but other starchy grains, e.g., maize, rice are also used as raw materials. Yeasts especially *Saccharomyces cerevisiae* or *S. carlsbergensis* are used in the fermentation process.

1. Commercial Production:

Five major steps are involved in the manufacture of beer (Fig. 40.2).

They are:

- (i) Malting,
- (ii) Mashing,
- (iii) Fermenting,
- (iv) Maturing, and
- (v) Finishing.

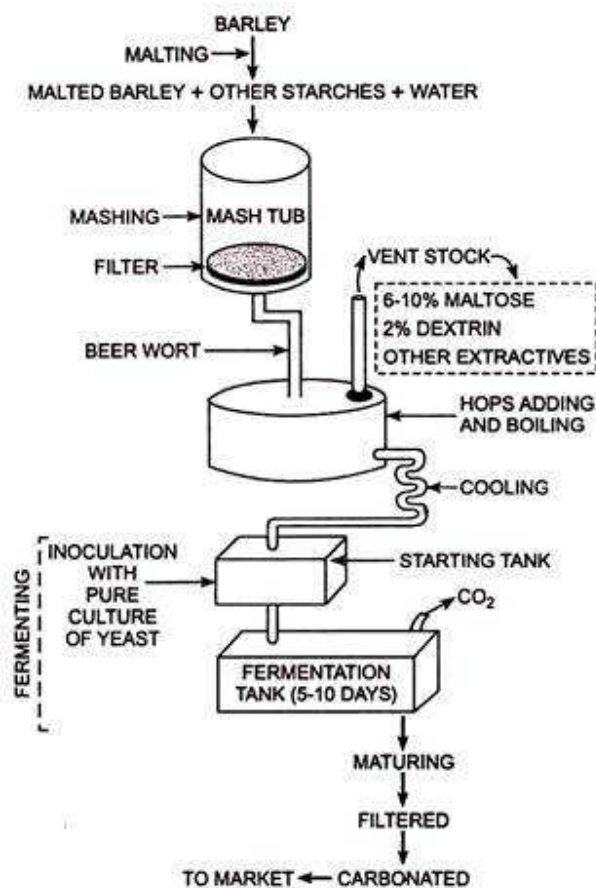


FIG. 40.2. Commercial production of beer.

(i) Malting:

The starchy grains are first malted. The malt is prepared by first soaking the grains in water and then allowing it to germinate at 17°C. During germination, large amount of amylases, an enzyme, are produced which are subsequently involved in hydrolysing the starch into fermentable sugars. After the germination is completed, the grains are dried at 65°C.

(ii) Mashing:

After malting, the pure barley malt is invariably mixed with other grains such as corn, rye, sorghum, wheat, etc. It is called 'ground malt'. The latter is first mixed with warm water at about 70°C and 5.0 pH. Mashing brings about partial hydrolysis resulting in the digestion of starch and protein; the partially hydrolysed solution of mash is filtered and this filtrate is called 'beer wort'. Beer wort serves as a rich nutrient medium for the microorganisms. Beer wort is now boiled with hops which are the papery scales of the female flowers of the hop vine, *Humulus lupulus*. The hops are added for flavour, aroma and mild antibacterial activity to prevent the growth of spoilage bacteria.

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(ii) Fermenting:

The beer wort is now inoculated with a pure culture of *Saccharomyces cerevisiae* or *S. carlsbergensis* and allowed to ferment at low temperature (5°C-14°C) for longer period (5-10 days).

(iv) Maturing:

The fermented beer wort is refrigerated at 0°C for several months (usually 6-8) to remove the harsh flavour and other undesirable characteristics.

(v) Finishing:

This is the final step in which the matured beer is carbonated, filtered and finally bottled, canned or barreled. Bottled or canned beer is usually pasteurized at 60°C for about 20 minutes to prevent microbial spoilage.

2. Types of Beer:

(i) Lager beer:

It means literally, the stored beer. Lager beer is produced by 'bottom' fermentation and is rather high in alcohol (3.93%) and extract with a relatively low proportion of hops.

(ii) Bock beer:

It is a heavy beer, dark in colour and high in alcohol (4.69%). It is brewed for consumption in early spring.

(iii) Ale beer:

Ale beer is pale in colour, tart in taste, high in alcohol (4.75%) and contains more hops. This beer is produced by 'top' fermentation.

(iv) Porter beer:

It is a dark ale but sweeter than the usual ale in taste. It is brewed from dark or black malt to produce a wort of high extract. The flavour of hops is less distinct than that of normal ale.

(v) Weiss beer:

This beer is produced mainly from wheat as a result of 'top' fermentation. It is rather light (2.75% alcohol), possesses a distinct flavour of malt and hop, is tart and contains a large quantity of natural fermentation gas. Weiss beer is somewhat turbid in appearance.

(vi) Stout beer:

This beer is stronger porter beer possessing high alcohol concentration. It is dark in colour and possesses a sweet taste and strong flavour of malt. The flavour of hops is more pronounced than that of the porter beer.

(vii) Cereal beverage:

This beer contains less than 0.5% alcohol. It is sometimes referred to as "near beer".

(ii) Wine Fermentation:

Wine is an undistilled product of fruit juice fermentation brought about by yeast. Wine is produced by the normal alcoholic fermentation of fruit juices, especially the grape juice. The microorganisms used in the wine fermentation are the various strains of *Saccharomyces cerevisiae* such as *S. cerevisiae* var. *ellipsoidens*.

1. Commercial Production:

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Five major steps are involved in commercial production of wines.

They are:

- (i) Crushing,
- (ii) Fermenting,
- (iii) Tanking,
- (iv) Maturing, and
- (v) Finishing.

(i) Crushing:

Grapes are harvested and ripened to a stage they contain highest sugar percentage. These fruits are crushed in a wine press and the crushed fruits with juice are called “must”. The must is generally treated with SO₂ to prevent microbial spoilage.

(ii) Fermenting:

The “must” is now inoculated with the starter culture of selected strain of the yeast and is aerated slightly to promote vigorous yeast growth. Once the fermentation starts, the rapid production of CO₂ maintains anaerobic condition. The temperature is kept usually at 25-30°C during fermentation period ranging from 5-11 days in order to inhibit multiplication of wild yeast and undesirable bacteria that live high temperature.

(iii) Tanking:

When most of the sugar is fermented the juice is separated from solid parts of fruits by allowing it to pass into tanks. These tanks, provided with valves to let the CO₂ escape, are completely filled with juice. The anaerobic condition for alcoholic fermentation is allowed to continue for about 12 days to increase the percentage of alcoholic concentration.

(iv) Maturing:

The wine is then allowed to mature in wooden tanks for 2 to 5 or more years. During maturing period the wine clears and develops the desired flavour due to formation of volatile ester.

(v) Finishing:

The wine may be finally cleared with the addition of gelatin, casein or Spanish clay. The cleared wine is filtered, bottled and pasteurized to prevent microbial spoilage.

2. Types of Wine:

Variety of grape, strain of yeast, nature of fermentation, etc. result in various varieties of wines. The latter differ from each other in so many attributes that it becomes difficult to classify them properly.

However, some important varieties of wine are as follows:

(i) Red wines:

The wines red in colour are put under this category. In preparing them the grapes are crushed and stemmed but their skins and seeds are left in the ‘must’. The alcoholic content percentage of these wines ranges from 11 to 12.

Examples:

Rose wines, Burgundy wines, Claret wines, Vinorosso wines, etc.

(ii) Dry wines:

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Those wines which fall under this category contain too little sugar to be detected by taste, i.e., the sugars are almost completely fermented. Alcohol percentage of dry wines ranges from 19-20.

Examples:

Post wines, Takay wines, Muscatel wines, etc.

(iii) Sweet wines:

Those wines are “sweet wines” that have good quantities of unfermented sugars.

(iv) Sparkling wines:

These are the wines that are made effervescent by secondary fermentation in closed containers and contain CO₂. They too possess 11-12% alcohol.

Examples:

Sparkling Burgundy, Champagne, etc.

(v) Still wines:

The wines that do not contain CO₂ are called “still wines”. They contain 12-15% alcohol.

Examples:

Sherries, Vermouth, etc.

(vi) Fortified wines:

These are the wines which contain added alcohol in the form of ‘brandy’.

(iii) Other Undistilled Alcoholic Beverages:

Fermented undistilled alcoholic beverages are consumed all over the world. In some countries the use of particular undistilled beverages has been passed down from ancient times.

Some important ones are as follows:

(i) Kuass:

It is Russian and is prepared by mixing equal parts of barley malt, rye malt and rye flours adding boiling water and then inoculating with yeast for fermentation. Peppermint is added to the fermented product for flavour.

(ii) Sake:

It is Japanese and is prepared by fermenting rice. It is yellow in colour and contains 14-24% of alcohol. Hops are used in its preparation.

(iii) Pulque:

It is produced in Mexico and is prepared by allowing the sweet juice of agave to undergo fermentation. Fermentation is usually completed in one day.

(iv) Taette:

It is Scandinavian and is prepared from milk. It has a pleasant acid taste. Yeasts cause characteristic changes in flavour.

(v) Chicha:

It is a common fermented beverage of Peru and Bolivia and is prepared from maize by yeast-fermentation.

(vi) Hard cidar:

Hard cidar is produced by the fresh apple juice fermentation or, sometimes, pear juice fermentation for 24 hours.

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(vii) Pombe:

It is an alcoholic beverage prepared by allowing millet grain to sprout and undergo conversion of starch to sugars and by permitting a spontaneous fermentation of the starch water.

(viii) Ginger beer:

The beverage is characterized by its distinct acid nature, the ginger flavour, and the presence of a small amount of alcohol.

(ix) Palm wine:

Palm wine or 'Toddy' (tori) is prepared from the fermented juice obtained from the inflorescence of many palms. The important species of palms which yield toddy and *Phoenix dactylofera*, *Borassus flavellifer*, *Cocos nucifera*, etc. When toddy is distilled, it yields 'arrack'.

(x) Root beer:

It contains an infusion of various roots, barks and herbs with the addition of sugar and yeast. The herbs generally used are ginger, sarsaparilla and wintergreen. Fermentation sets and the beverage becomes charged with CO₂.

(xi) Mead:

This undistilled fermented alcoholic beverage is of great antiquity. It is obtained from honey and water and possesses a wine like flavour.

B. Distilled Beverages:

(i) Whisky:

Whisky is distilled from fermented grain-mash containing upto 50% ethyl alcohol.

Following are some varieties of whisky:

(i) Malt whisky:

This whisky is manufactured by microbial fermentation and subsequent distillation of malted barley (barley grains are soaked in water and then germinated at 17°C).

(ii) Grain whisky:

This whisky is manufactured by the microbial fermentation and subsequent distillation of a mixture of malted and un-malted barley with un-malted maize.

(iii) Scotch whisky:

Malt whisky is matured (aged) in oak casks at least 3 years and then is blended with grain whisky. This new product is scotch whisky.

(iv) Bourbon whisky:

This whisky is manufactured by the microbial fermentation of a grain-mash in which corn is predominant (at least 51 %).

(v) Irish whisky:

This whisky is prepared from a grain-mash in which rye is predominant.

(vi) Arrak (Far East) and Sake (Japan) whisky:

This whisky is prepared from microbial fermentation of rice-grains. Since the rice is starchy, its starch is hydrolysed by enzyme amylases derived from *Aspergillus oryzae* before fermentation.

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(ii) Rum:

Rum is any alcoholic distillate from the fermented juice of sugarcane, sugarcane syrup, sugarcane molasses or other sugarcane by-product and contains about 40% alcohol. Blackstrap molasses containing 12-14% fermentable sugar are fermented by *Saccharomyces cerevisiae* or other yeasts at pH 4.0-4.7 and initial temperature of 27°C which rises finally to 35.5°C. Fermentation is completed within 3 to 7 days.

Rum is usually aged (matured) in charred white-oak barrels and possesses a characteristic flavour, aroma and colour. Rum may be used in the preparation of ice cream, candies, in curing of tobacco and, sometimes, as medicine.

(iii) Gin:

Gin is the liquor which is prepared by distillation from a fermented mash of malt or raw grain. The finest gin is distilled from the malt of barley and rye. It requires several distillations. The flavour of gin and its medicinal value are due to oil of juniper.

(iv) Brandy:

Brandy is the distillation product of fermented grape juice (wine) and contains about 40-50% alcohol. The best brandy is made in France in the Charente district. The finest grade of brandy are made from the white wines. The brown colour of brandy develops when it is stored in wooden casks. Sometimes, the brandy is coloured with caramel. It contains about 60-70% alcohol.

(v) Vodka:

It is Russian alcoholic beverage (distilled) produced by the yeast fermentation of potatoes. It is allowed to age for long periods in wooden casks; flavour and aroma are introduced into the purified alcohol during the blending process.

Microbial production of Organic acids: Source, recovery and uses of Citric acid, Lactic acid, Acetic acid and L-ascorbic acid

1. Citric Acid:

Citric acid was first discovered as a constituent of lemon. Today, we know citric acid as an intermediate of ubiquitous Krebs cycle (citric acid cycle), and therefore, it is present in every living organism. In the early days, citric acid was isolated from lemons (that contain 7-9% citric acid), and today about 99% of the world's citric acid comes from microbial fermentation.

Applications of Citric Acid:

1. Citric acid, due to its pleasant taste and palatability, is used as a flavoring agent in foods and beverages e.g., jams, jellies, candies, desserts, frozen fruits, soft drinks, wine. Besides brightening the colour, citric acid acts as an antioxidant and preserves the flavors of foods.

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2. It is used in the chemical industry as an antifoam agent, and for the treatment of textiles. In metal industry, pure metals are complexed with citrate and produced as metal citrates.
3. In pharmaceutical industry, as trisodium citrate, it is used as a blood preservative. Citric acid is also used for preservation of ointments and cosmetic preparations. As iron citrate, it serves as a good source of iron.
4. Citric acid can be utilized as an agent for stabilization of fats, oils or ascorbic acid. It forms a complex with metal ions (iron, copper) and prevents metal catalysed reactions. Citric acid is also used as a stabilizer of emulsions in the preparation of cheese.
5. In detergent/cleaning industry, citric acid has slowly replaced polyphosphates.

Microbial Strains for Citric Acid Production:

Many microorganisms can produce citric acid. The fungus *Aspergillus Niger* is most commonly used for industrial production of citric acid. The other organisms (although less important) include *A. clavatus*, *A. wentii*, *Penicillium luteum*, *Candida catenula*, *C. guilliermondii* and *Corynebacterium sp.*

For improved industrial production of citric acid, mutant strains of *A. Niger* have been developed. The strains that can tolerate high sugar concentration and low pH with reduced synthesis of undesirable byproducts (oxalic acid, isocitric acid and gluconic acid) are industrially important.

Microbial Biosynthesis of Citric Acid:

Citric acid is a primary metabolic product (of primary metabolism) formed in the tricarboxylic acid (Krebs) cycle. Glucose is the predominant carbon source for citric acid production. The biosynthetic pathway for citric acid production involves glycolysis wherein glucose is converted to two molecules of pyruvate. Pyruvate in turn forms acetyl CoA and oxaloacetate which condense to finally give citrate. The major steps in the biosynthesis of citric acid are depicted in Fig. 24.1.



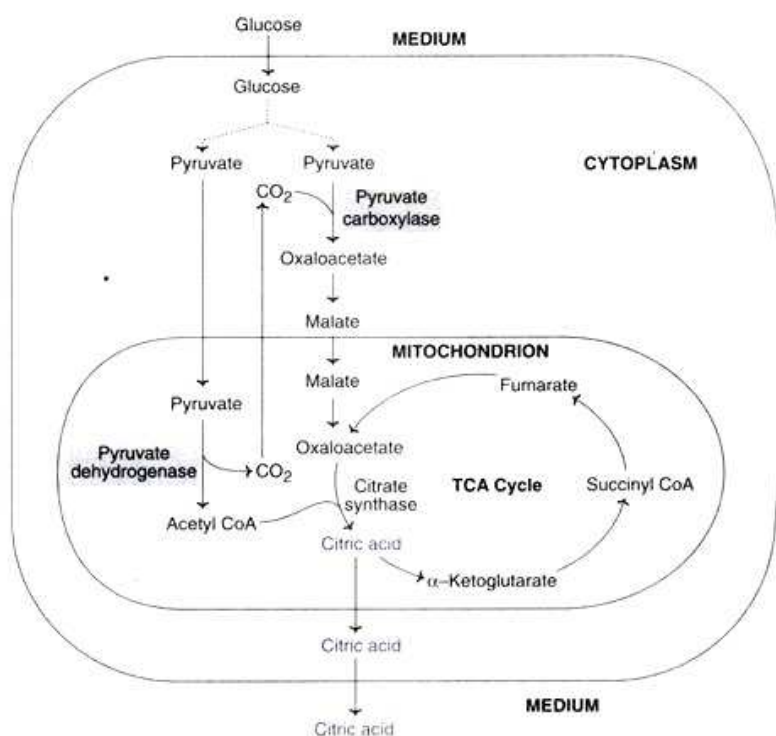


Fig. 24.1 : An outline of metabolic pathway for the biosynthesis of citric acid (TCA cycle-Tricarboxylic acid cycle).

Enzymatic regulation of citric acid production:

During the synthesis of citric acid, there is a tenfold increase in the activity of the enzyme citrate synthase while the activities of other enzymes (aconitase, isocitrate dehydrogenase) that degrade citric acid are reduced. However, recent evidence does not support the theory that reduction in the operation of tricarboxylic acid (i.e. degradation of citric acid) contributes to accumulation of citric acid.

Increased citric acid is more likely due to enhanced biosynthesis rather than inhibited degradation. Further, there are anaplerotic reactions that replenish the TCA cycle intermediates to keep the cycle continuously in operation. Pyruvate carboxylase that converts pyruvate to oxaloacetate is also a key enzyme in citric acid production.

Yield of citric acid:

Theoretically, the yield of citric acid for the most commonly used substrate sucrose has been calculated. It is worked out that from 100 g sucrose, 112 g of anhydrous citric acid or 123 g of

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citric acid — 1 hydrate can be formed. However, due to oxidation of sugar to CO₂ during trophophase, the yield of citric acid is lower than the calculated.

Factors in the Regulation of Citric Acid Production:

Strict maintenance of controlled nutrient conditions is very crucial for maximal production of citric acid. The optimal conditions that have been worked out for *A. Niger* for the production of citric acid are briefly described (Table 24.1).

TABLE 24.1 Optimal parameters/conditions for citric acid production	
<i>Condition/parameter</i>	<i>Optimum</i>
Sugar concentration	10–25%
Trace metal concentration	
Manganese	<10 ⁻⁸ M
Zinc	<10 ⁻⁷ M
Iron	<10 ⁻⁴ M
pH	1.5–2.5
Dissolved O ₂ tension	150 mbr
Ammonium salts concentration	>0.2%
Time	150–250 hours

Carbohydrate source:

A wide range of raw materials can be used for the supply of carbohydrates. These include molasses (sugar cane or sugar beet), starch (from potatoes), date syrup, cotton wastes, banana extract, sweet potato pulp, and brewery waste and pineapple waste water.

A high yield of citric acid production occurs if the sugars that are rapidly metabolised are used e.g. sucrose, glucose, maltose. At present, cane molasses and beet molasses are commonly used. The variations in the composition of molasses (seasonal and production level), have to be carefully considered for optimizing citric acid production.

The concentration of carbohydrate significantly influences citric acid production. Ideally, the sugar concentration should be 12-25%. At a concentration less than 5% sucrose, citric acid formation is negligible, and increases as the concentration is raised to 10% and then stabilizes (Fig. 24.2). It is believed that a high sugar concentration induces increased glucose uptake and consequently enhanced citric acid production.

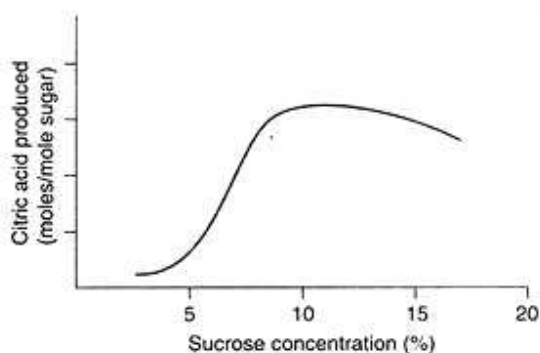


Fig. 24.2 : Effect of sugar concentration on citric acid production.

Trace metals:

Certain trace elements (Fe, Cu, Zn, Mn, Mg, Co) are essential for the growth of *A. Niger*. Some of the trace metals particularly Mn^{2+} , Fe^{3+} and Zn^{2+} increase the yield of citric acid. The effect of manganese ions has been investigated to some extent. These ions promote glycolysis and reduce respiration; both these processes promote citric acid production.

As regards iron, it is a cofactor for the enzyme aconitase (of TCA cycle). It is estimated that an Fe concentration of 0.05-0.5 ppm is ideal for optimal citric acid production. At higher Fe concentration, the yield is lower which can be reversed to some extent by adding copper.

pH:

The pH of the medium influences the yield of citric acid, and it is maximal when pH is below 2.5. At this pH, the production of oxalic acid and gluconic acid is suppressed. Further, at low pH, transport of citric acid is much higher. If the pH is above 4, gluconic acid accumulates at the expense of citric acid. And when the pH goes beyond 6, oxalic acid accumulates. Another advantage with low pH is that the risk of contamination is very minimal, since many organisms cannot grow at this pH.

Dissolved O_2 :

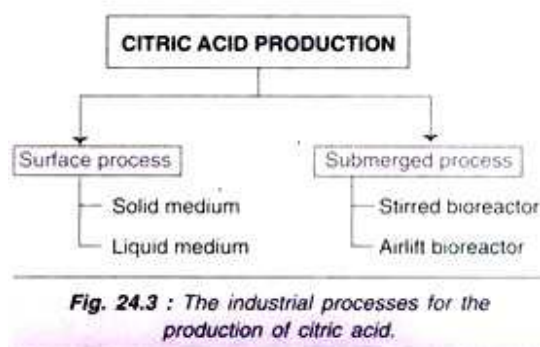
The yield of citric acid production substantially increases when the dissolved O_2 tension is higher. This can be achieved by strong aeration or by sparging with pure O_2 . It has been observed that sudden interruptions in O_2 supply (as occurs during power breakdowns) cause drastic reduction in citric acid production without harming the growth of the organism.

Nitrogen source:

Ammonium salts, nitrates and urea are the nitrogen sources used in the media for citric acid production. All the three compounds are equally good sources, as long as they do not adversely affect the pH of the medium. If molasses are used for nutrient supply, addition of extra nitrogen source is not required. However, some workers have shown that exogenous addition of ammonium ions stimulates citric acid production.

Production Processes for Citric Acid:

There are two processes by which citric acid can be industrially produced — the surface process and submerged process (Fig. 24.3).



The surface process:

This is characterized by growing the microorganisms as a layer or a film on a surface in contact with the nutrient medium, which may be solid or liquid in nature. Thus, the surface process has supported-growth systems.

The submerged process:

In this case, the organisms are immersed in or dispersed throughout the nutrient medium. There are two types of submerged fermenters (bioreactors) stirred bioreactors and airlift bioreactors.

Surface Processes:

Solid surface fermentation:

Surface processes using solid substrates are particularly carried out in less developed areas of some Asian countries. The solid substrates such as wheat bran or pulp from sweet potato starch are used, as culture media. The pH of the medium is adjusted to 4-5, and then sterilized. Now the inoculum in the form of spores of *A. niger* is spread as layers (3-6 cm thickness) and incubated at 28°C.

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The growth of the organisms can be accelerated by the addition of α -amylase. Solid-state fermentation takes about 80 to 100 hours for maximal production of citric acid. At the end of the process, citric acid can be extracted into hot water and isolated.

Liquid surface fermentation:

Surface fermentation using liquid as nutrient medium is the oldest method for citric acid production. It is still in use due to a simple technology, low energy costs and higher reproducibility. Further, the interference of trace metals and dissolved O_2 tension are minimal. The labour costs are however, higher since the manpower requirements are more for cleaning the systems. About 20% of the citric acid in the world is produced by surface processes.

The nutrient supply for surface fermentation normally comes from beet molasses. The fermentation is usually carried out in aluminium trays filled with sterile nutrient medium. The inoculum in the form of spores is sprayed over the medium. A sterile air is passed for supplying O_2 as well as cooling. The temperature is maintained around 30°C during fermentation.

As the spores germinate (that occurs within 24 hours of inoculation), a layer of mycelium is formed over the medium. The pH of the nutrient medium falls to less than 2, as the mycelium grows in size and forms a thick layer on the surface of the nutrient solution. The fermentation is stopped after 7-15 days.

The mycelium and nutrient solution are separated. The mycelium is mechanically pressed and thoroughly washed to obtain maximum amount of citric acid. The nutrient solution is subjected to processing for the recovery of citric acid. The final yield of citric acid is in the range of 0.7-0.9 of per gram of sugar.

Submerged Processes:

Around 80% of the world's supply of citric acid is produced by submerged processes. This is the most preferred method due to its high efficiency and easy automation. The disadvantages of submerged fermentation are — adverse influence of trace metals and other impurities, variations in O_2 tension, and advanced control technology that requires highly trained personnel.

Two types of bioreactors are in use— stirred tanks and aerated towers. The vessels of the bioreactors are made up of high-quality stainless steel. The sparging of air occurs from the base of the fermenter.

The success and yield of citric acid production mainly depend on the structure of mycelium. The mycelium with forked and bulbous hyphae and branches which aggregate into pellets is ideal for

citric acid formation. On the other hand, no citric acid production occurs if the mycelium is loose and filamentous with limited branches. An adequate supply of O_2 (20-25% of saturation value) is required for good production of citric acid. The ideal aeration rate is in the range of 0.2-1 vvm (volume/ volume/ minute).

The submerged fermenters have the problem of foam formation which may occupy about 1 /3rd of the bioreactor. Antifoam agents (e.g. lard oil) and mechanical antifoam devices are used to prevent foaming. Nutrient concentration is very important in the industrial production of citric acid. A diagrammatic representation of sucrose, citric acid and biomass concentration with respect to cultivation time is shown in Fig. 24.4. It is estimated that under optimal conditions, in about 250-280 hours, 100- 110 g/l of citric acid is obtained from 140 g/l of sucrose with a biomass (dry weight) of 8-12 g/l.

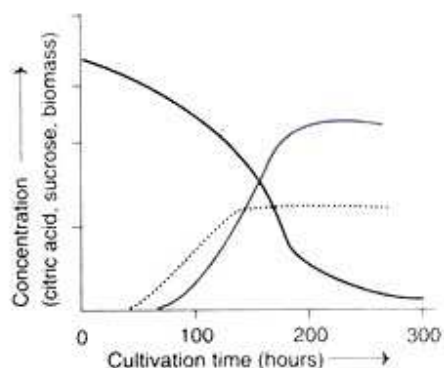


Fig. 24.4 : A diagrammatic representation of citric acid (—) production along with sucrose (—) and biomass (---) concentration in relation to time.

Production of Citric Acid from Alkanes:

Both yeasts and bacteria can be used for citric acid production from n-alkanes (C_9 - C_{23} hydrocarbons). The citric acid yield is better from hydrocarbons compared to sugars i.e. 145% of citric acid from paraffin. The most commonly used organism is *Candida lipolytica*. The fermentation can be carried out in batch, semi-continuous or continuous modes. The pH should be kept above 5. The major limitations of citric acid production from alkanes are—very low solubility of alkanes and increased production of unwanted isocitric acid.

Recovery of Citric Acid:

The steps for the recovery of citric acid either from surface process or submerged process are comparable (Fig. 24.5). The recovery starts with the filtration of the culture broth and washing of mycelium (which may contain about 10% of citric acid produced). Oxalic acid is an unwanted byproduct and it can be removed by precipitation by adding lime at pH < 3.

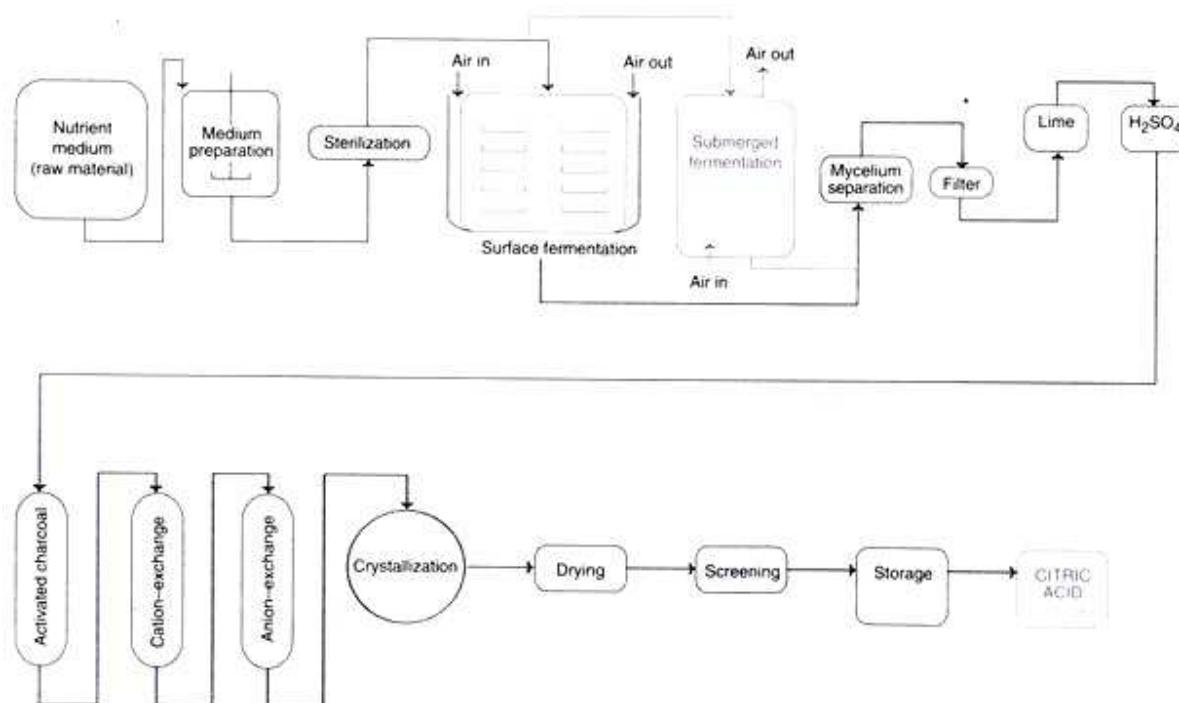


Fig. 24.5 : Flow chart for industrial production of citric acid by surface or submerged processes.

The culture broth is then subjected to pH 7.2 and temperature 70-90°C for precipitating citric acid. For further purification, citric acid is dissolved in sulfuric acid (calcium sulfate precipitate separates). The final steps for citric acid recovery are — treatment with activated charcoal, cation and anion-exchangers and crystallization.

Citric acid monohydrate formed below 36°C is the main commercial product. Above 40°C, citric acid crystallizes in an anhydrous form. The degree of purity of citric acid produced depends on

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the purpose for which it is required. For instance, pure forms of citric acid are needed for use in food preparations, while for industrial use it can be crude form.

Lactic Acid:

Lactic acid occurs in two isomeric forms i.e. L (+) and D (-) isomers, and as a racemic mixture (DL-lactic acid). The isolation of lactic acid from milk was done in 1798. It was the first organic acid produced by microorganisms in 1880. Today, lactic acid is competitively produced both by microbiological and chemical methods.

Applications of Lactic Acid:

There are different grades of lactic acid mainly based on the percentage of lactic acid. The grades and their applications are given in Table 24.2.

TABLE 24.2 Commercial grades of lactic acid along with their applications	
Grade (% lactic acid)	Application(s)
Technical grade (20–50%)	Ester manufacture, textile industry
Food grade (>80%)	Food additive (sour flour and dough)
Pharmaceutical grade (>90%)	Intestinal treatment (metal ion lactates)

Microorganisms for Production of Lactic Acid:

Lactic acid producing bacteria are broadly categorized into two types.

Hetero-fermentative bacteria—produce other byproducts, besides lactic acid, and therefore are not useful for industrial production of lactic acid. These bacteria are employed in food or feed preservation.

Homo-fermentative bacteria—specialized for exclusive production of lactic acid and therefore are suitable for industrial purpose.

Lactobacillus sp are used for lactic acid production. However, there are variations in the substrates utilised as indicated below.

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<i>L. delbrueckii</i>	}	Glucose
<i>L. leichamanni</i>		
<i>L. bulgaricus</i>	}	Whey (lactose)
<i>L. helveticus</i>		
<i>L. lactis</i>	—	Maltose
<i>L. amylophilus</i>	—	Starch
<i>L. pentosus</i>	—	Sulfite waste liquor

Biosynthesis of lactic acid:

The synthesis of lactic acid occurs through glucose oxidation by glycolysis to produce pyruvate which on reduction gives lactic acid. The reducing equivalents ($\text{NADH}^+ + \text{H}^+$) produced during the oxidation of glyceraldehyde 3-phosphate are utilised by the enzyme lactate dehydrogenase to form lactate (Fig. 24.7). Most of the lactic acid producing microorganisms normally produce only one isomer of lactic acid L(+) or D(-). However, some bacteria which usually occur as infection can form racemic mixture.

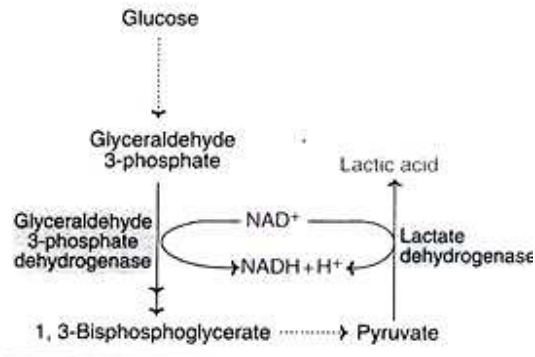


Fig. 24.7 : An outline of the pathway (glycolysis) for the biosynthesis of lactic acid.

Production Process for Lactic Acid:

The fermentation medium contains 12-15% of glucose, nitrogen and phosphate containing salts and micronutrients. The process is carried out at pH 5.5-6.5 and temperature 45-50°C for about 75 hours. Generally, the strains operating at higher temperature (45-60°C) are preferred, since it reduces the need for medium sterilization.

As the lactic acid is produced, it has to be removed since it is toxic to the organisms. This can be achieved either by a continuous culture technique or by removal of lactic acid by electro dialysis. Theoretically, every molecule of glucose forms two molecules of lactic acid. About 90% of

theoretical yield is possible in fermentation industry. L(+) Lactic acid is predominantly produced. The outline of the steps involved in the recovery lactic acid is depicted in Fig. 24.8.

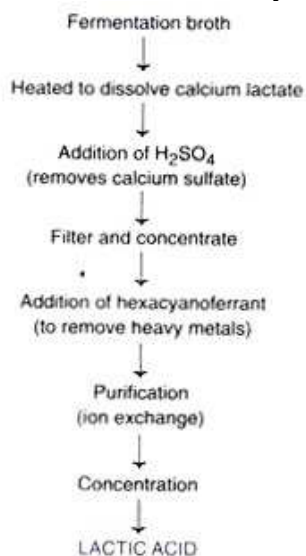


Fig. 24.8 : Flow chart for recovery of lactic acid from fermentation broth.

4. Acetic Acid:

The production of acetic acid, in the form of vinegar (used as a refreshing drink), from alcoholic liquids has been known for centuries.

Microorganisms Used for Production of Acetic Acid:

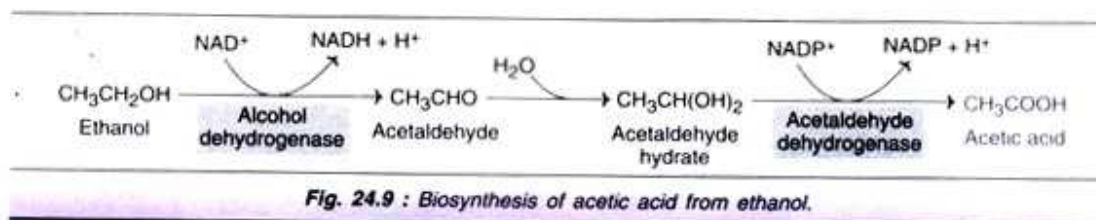
The commercial production of acetic acid is carried out by a special group of acetic acid bacteria, which are divided into two genera.

Gluconobacter that oxidizes ethanol exclusively to acetic acid.

Acetobacter that oxidizes ethanol first to acetic acid, and then to CO₂ and H₂O. These over-oxidizers are Gram-negative and acid tolerant e.g. *A. aceti*, *A. peroxidans*, *A. pasteurianus*.

Biosynthesis of acetic acid:

Acetic acid is a product of incomplete oxidation of ethanol. Ethanol is first oxidized by alcohol dehydrogenase to acetaldehyde which then gets hydrated to form acetaldehyde hydrate. The latter is then acted upon by acetaldehyde dehydrogenase to form acetic acid (Fig. 24.9).



Production Process for Acetic Acid:

For every molecule of ethanol oxidised, one molecule of acetic acid is produced. Thus, high-yielding strains can produce 11-12% acetic acid from 12% alcohol. For optimal production, adequate supply of oxygen is very essential. Insufficient O₂, coupled with high concentration of alcohol and acetic acid result in the death of microorganisms. Surface fermentation or submerged fermentation processes can be carried out to produce acetic acid. Trickling generation process, a type of surface fermentation, is very commonly used.

Recovery:

The acetic acid produced is clarified by filtration and then subjected to decolourization by K₄(FeCN)₆.

Production of Vinegar:

Vinegar is an aqueous solution containing about 4% by volume acetic acid and small quantities of alcohol, salts, sugars and esters. It is widely used as a flavoring agent for processed liquid foods such as sauces and ketchups. The starting materials for vinegar production are wine, whey, malt (with low alcohol content). Vinegar production can be carried out either by surface process (trickling generator) or by submerged process.

Surface process:

The fermentation material is sprayed over the surface which trickles through the shavings that contain the acetic acid producing bacteria. The temperature is around 30°C on the upper part while it is around 35°C on the lower part. Vinegar is produced in about 3 days.

Submerged process:

The fermentation bioreactors are made up of stainless steel. Aeration is done by a suction pump from the top. The production rate in the submerged process is about 10 times higher than the surface process.

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L-Ascorbic Acid:

L-Ascorbic acid is the commonly used chemical name for the water soluble vitamin C. This vitamin forms a redox system and participates in several biological processes. It is intimately involved in the biosynthesis of collagen, the most abundant protein in the human body. Vitamin C also protects the body against carcinogenic nitrosamines and free radicals. The deficiency of ascorbic acid causes scurvy.

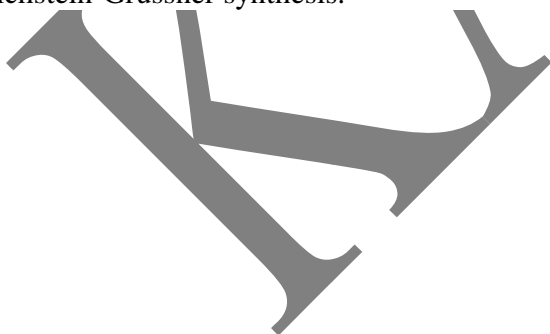
Applications of Ascorbic Acid:

Because of the wide range of physiological and beneficial functions of ascorbic acid, its commercial production assumes significance. Vitamin C is mainly used in food and pharmaceutical industries.

Industrial Production of Ascorbic Acid:

Ascorbic acid is commercially produced by a combination of several chemical steps, and one reaction of biotransformation brought out by microorganisms. This process is referred to as Reichstein-Grussner synthesis (Fig. 24.10B). D-Glucose is first converted to D-sorbitol. Oxidation of D-sorbitol to L-sorbose is carried out by *Acetobacter xylinum* or *A. suboxydans* (The enzyme being sorbitol dehydrogenase).

A submerged bioreactor fermentation process is ideal for this reaction. It takes about 24 hours at temperature 30-35°C. Sorbose by a couple of chemical reactions can be finally converted to L-ascorbic acid. Normally, about 100 g of ascorbic acid is produced from 200 g of glucose in Reichstein-Grussner synthesis.



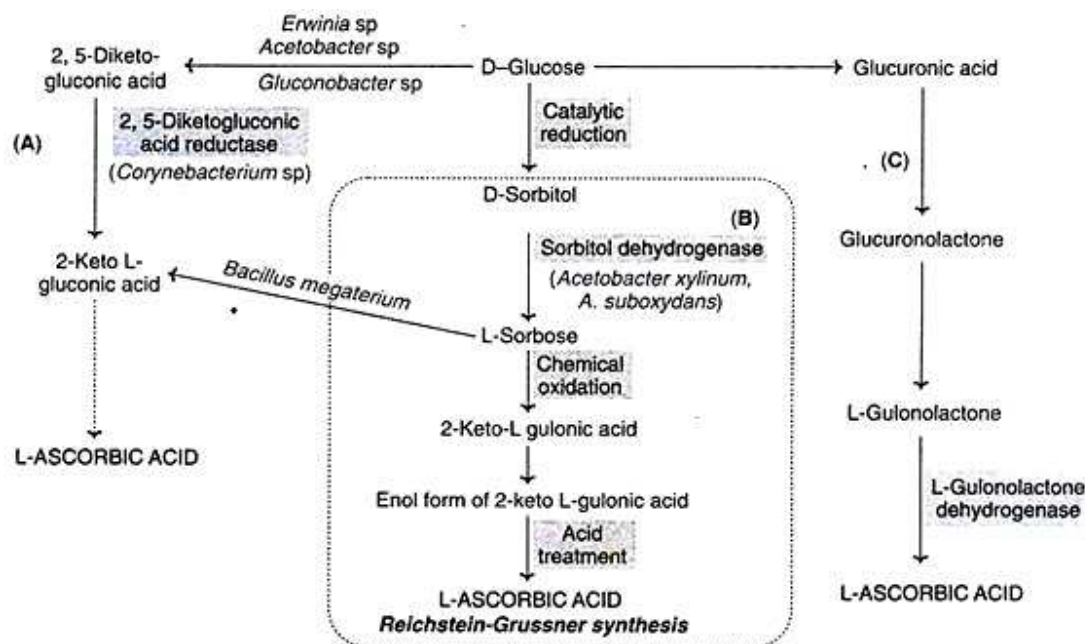


Fig. 24.10 : Pathways for the commercial production of ascorbic acid. (A) Two-step fermentation process (B) Reichstein-Grussner synthesis (C) Production via L-gulonolactone.

Two-step fermentation process:

In this, D-glucose is converted to 2, 5-diketogluconic acid by *Erwinia*, *Acetobacter* or *Gluconobacter sp*. In the second step, *Corynebacterium sp* converts 2, 5-diketogluconic acid to 2-keto-L-gluconic acid, (Fig. 24.10A). It is also possible to involve *Bacillus megaterium* for converting L-sorbose to 2-keto-L- gluconic acids. The latter, by chemical reactions, can be converted to ascorbic acid.

Production via L-gulonolactone:

Ascorbic acid can also be synthesized via- gulonolactone which can be directly converted to L- ascorbic acid by the enzyme L-gulonolactone dehydrogenase (Fig. 24.10C).

Direct Production of Ascorbic Acid by Fermentation:

Several workers are trying to produce ascorbic acid directly from glucose. Microalgae of *Chlorella* have shown some promising results, although the yield is very low.

Genetic Engineering for Ascorbic Acid Production:

Biotechnologists have been successful in cloning and expressing the gene for 2, 5-diketogluconic acid reductase of *Corynebacterium sp* into *Erwinia herbicola*. By doing this, the two step

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fermentation process (Fig. 24.10A) has been reduced to one. The genetically engineered *Erwinia* cells were able to convert D-glucose directly to 2-keto-L-gluconic acid.

This is certainly advantageous since the metabolic capabilities of two different microorganisms could be combined into one organism. However, the yield of ascorbic acid by the hybrid strain was very low. Scientists are now trying to alter certain amino acids in 2-5 diketogluconic acid reductase and increase the catalytic activity of this enzyme.

Production of antibiotics: Penicillin and Tetracyclin.

Antibiotics are the chemical substances that can kill microorganisms or inhibit their growth, and are therefore used to fight infections in humans or animals. Most of the antibiotics are produced by microorganisms (i.e. product of one organism that can kill other organism). Certain semi-synthetic antibiotics are the chemically modified natural antibiotics.

Antibiotics have undoubtedly changed the world we live in, and have certainly contributed to the increase in the human life-span. This is mainly due to the fact that several life-threatening infectious diseases could be conveniently cured by administration of antibiotics.

Antibiotics — General:

A brief history of antibiotics along with the microorganisms producing them, and their applications are given hereunder.

History of antibiotic discovery:

It was in 1928, Alexander Fleming made an accidental discovery that the fungus *Penicillium notatum* produced a compound (penicillin) that selectively killed a wide range of bacteria without adversely affecting the host cells. There are records that in some parts of Europe (in 1908) extracts of moldy bread were applied to wounds or abrasions to prevent infections, although the biochemical basis was not known. The penicillin discovery of Fleming has revolutionized antibiotic research.

Wide range of antibiotics:

Antibiotics are the most important class of pharmaceuticals produced by microbial biotechnological processes. They are the products of secondary metabolism. Around 10,000 different antibiotics are known, and 200-300 new ones are being added each year.

Most of these antibiotics are not of commercial importance due to various reasons—toxicity, ineffectiveness or high cost of production. There are around 50 antibiotics which are most widely used. In Table 25.1, a selected list of important antibiotics, their properties and the producing organisms is given.

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TABLE 25.1 A selected list of important antibiotics along with producing organisms	
<i>Antibiotic activity spectrum and antibiotic</i>	<i>Producing microorganism</i>
Antibacterial	
Penicillin G	<i>Penicillium</i> sp
Cephalosporin	<i>Acremonium</i> sp
Streptomycin	<i>Streptomyces</i> sp
Tetracycline	<i>Streptomyces</i> sp
Chloramphenicol	<i>Cephalosporium</i> sp
Bacitracin	<i>Bacillus</i> sp
Antitumor	
Actinomycin D	<i>Streptomyces</i> sp
Mitomycin C	<i>Streptomyces</i> sp
Bleomycin	<i>Streptomyces</i> sp
Adriamycin	<i>Streptomyces</i> sp
Daunomycin	<i>Streptomyces</i> sp
Antifungal	
Griseofulvin	<i>Penicillium</i> sp
Food preservative	
Natamycin	<i>Streptomyces</i> sp
Nisin	<i>Streptomyces</i> sp
Antiprotozoal	
Daunorubicin	<i>Streptomyces</i> sp
Antituberculosis	
Rifamycin	<i>Nocardia</i> sp
Antiamoebic	
Tetracycline	<i>Streptomyces</i> sp
Fumagillin	<i>Aspergillus</i> sp

Broad spectrum antibiotics:

They can control the growth of several unrelated organisms e.g. tetracycline's, chloramphenicol.

Narrow spectrum antibiotics:

They are effective against selected species of bacteria e.g. penicillin, streptomycin.

Microorganisms producing antibiotics:

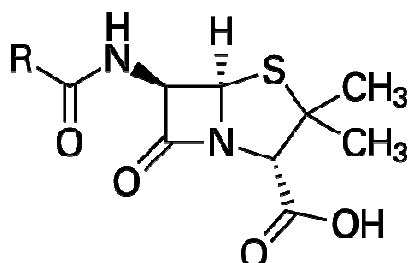
A great majority of antibiotics are produced by actinomycetes particularly of the genus *Streptomyces* e.g. tetracycline's, actinomycin D. The bacteria other than *actinomyces* also produce certain antibiotics e.g. bacitracin. Among the fungi, the two groups *Aspergillaceae* and *Moniliales* are important for antibiotic production e.g. penicillin, cephalosporin, griseofulvin.

Fermentation of Penicillin Antibiotic

Penicillin is an antibiotic produced by microorganisms. This antibiotics inhibit growth and development of other micro-organism. Generally the penicillin antibiotic is produced by some actinomycetes and some filamentous fungi. The antibiotics produced by these micro-organism can be used medicine field, veterinary as well as agricultural field. Penicillin antibiotic was the first antibiotic used in large amount during world war second for treatment of soldiers. Penicillin is a antibiotic used against Gram positive bacteria as well as high dosage can be used against

Gram negative bacteria. Penicillin is not harmful to plants, animals or human beings except in some cases of allergies.

Structure of Penicillin

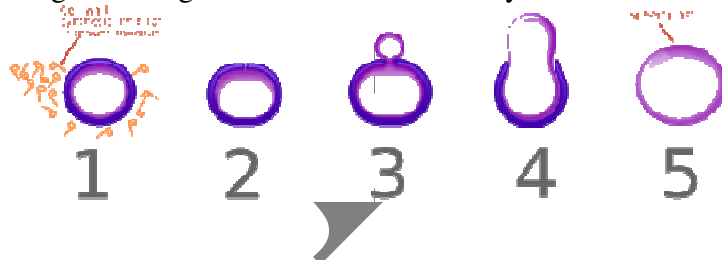


Mode of Action of penicillin on micro-organism

The penicillin antibiotic acts on the cell wall synthesis of growing Gram positive bacteria. Penicillin is a β lactamase antibiotic. The structure of penicillin contain β lactam ring and this β lactam ring gets attach to enzyme DD transpeptidase enzyme present in the cell. The enzyme DD transpeptidase play an important role in formation of cross linkage in cell wall synthesis of Gram positive bacteria.

The β lactamase ring binds the enzyme DD transpeptidase and result in inhibition of formation of cross linkage in cell wall. Due to which the cell wall is not formed and the growth and development of cell is stopped. The gram positive cell completely loses its cell wall and cell without cell wall are called as protoplast. Whereas gram negative cell do not loss its cell wall completely so called as spheroplast. After inhibition of cell wall the protoplast and spheroplast are formed and due to increase in osmotic pressure the cell undergoes its lysis.

Image showing inhibition of Cell Wall synthesis and formation of spheroplast.



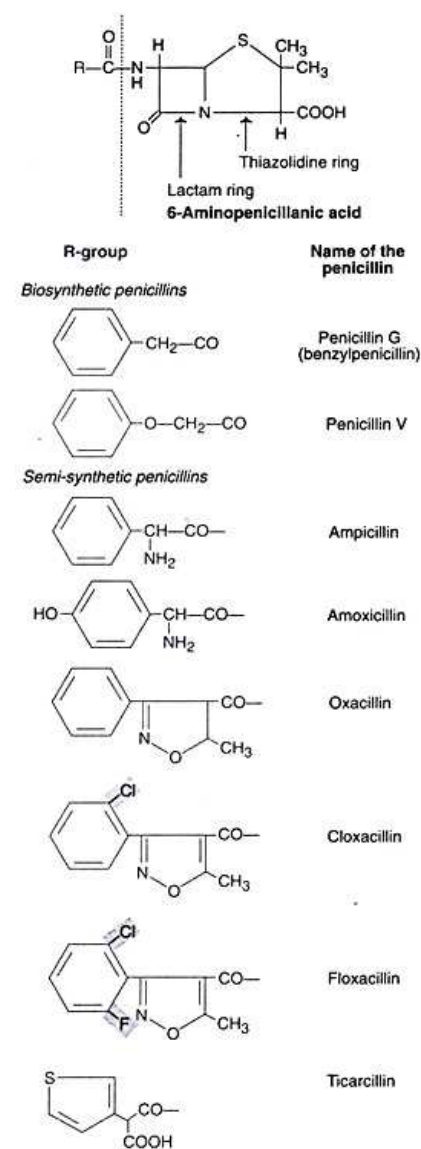


Fig. 25.1 : Structures of important penicillins.

Action of Penicillins:

Natural penicillins (penicillins V and G) are effective against several Gram-positive bacteria. They inhibit the bacterial cell wall (i.e. peptoglycan) synthesis and cause cell death. Some persons (approximately 0.5-2% of population) are allergic to penicillin.

Natural penicillins are ineffective against microorganisms that produce β -lactamase, since this enzyme can hydrolyse penicillins e.g. *Staphylococcus aureus*. Several semi-synthetic penicillins

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that are resistant to β -lactamase have been developed and successfully used against a large number of Gram-negative bacteria.

Cloxacillin, ampicillin, floxacillin and azlocillin are some examples of semi-synthetic penicillins. These are quite comparable in action to cephalosporins. From the huge quantities of penicillins produced by fermentation, about 40% are used for human healthcare, 15% for animal healthcare and 45% for the preparation of semi-synthetic penicillins.

Organisms for Penicillin Production:

In the early days, *Penicillium notatum* was used for the large-scale production of penicillins. Currently, *Penicillium chrysogenum* and its improved mutant strains are preferred. Previously, the penicillin production used to be less than 2 units/ml, and with the new strains, the production runs into several thousands of units/ml. One of the high yielding strains was Q176 is preferred by several penicillin manufacturers.

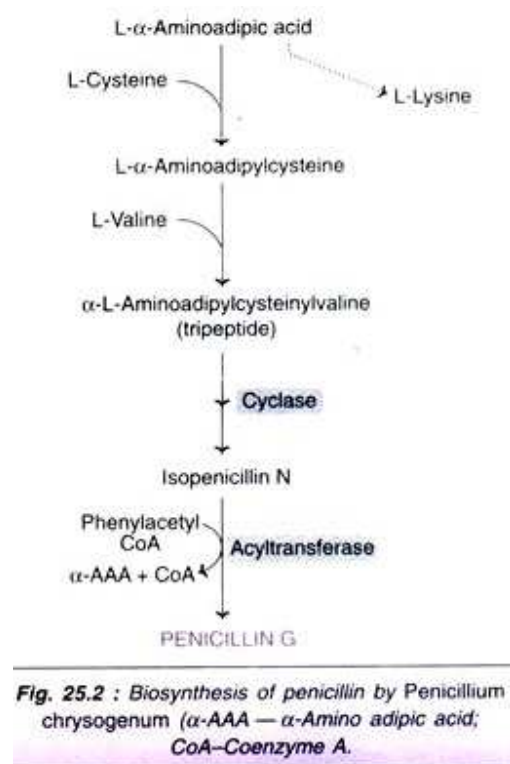
Genetic engineering for improved penicillin production:

Some of the genes involved in penicillin biosynthesis by *P. chrysogenum* have been identified. Genetic manipulations were carried out so as to substantially increase the penicillin production. For instance, extra genes coding for the enzymes cyclase and acyltransferase have been inserted into *C. chrysogenum*.

Biosynthesis of Penicillin:

L- α -Aminoadipic acid combines with L-cysteine, and then with L-valine to form a tripeptide namely α -L-aminoadipylcysteinylvaline. This compound undergoes cyclization to form isopenicillin which reacts with phenyl acetyl CoA (catalysed by the enzyme acyltransferase) to produce penicillin G (benzyl penicillin). In this reaction, aminoadipic acid gets exchanged with phenylacetic acid (Fig. 25.2).





Regulation of biosynthesis:

Some of the biochemical reactions for the synthesis of penicillin and lysine are common. Thus, L- α -aminoadipic acid is a common intermediate for the synthesis of penicillin and lysine. The availability of aminoadipic acid plays a significant role in regulating the synthesis of penicillin. Penicillin biosynthesis is inhibited by glucose through catabolite repression. For this reason, penicillin was produced by a slowly degraded sugar like lactose. The concentrations of phosphate and ammonia also influence penicillin synthesis.

Production Process of Penicillin:

An outline of the flow chart for the industrial production of penicillin is depicted in Fig. 25.3. The lyophilized culture of spores is cultivated for inoculum development which is transferred to pre-fermenter, and then to fermenter.

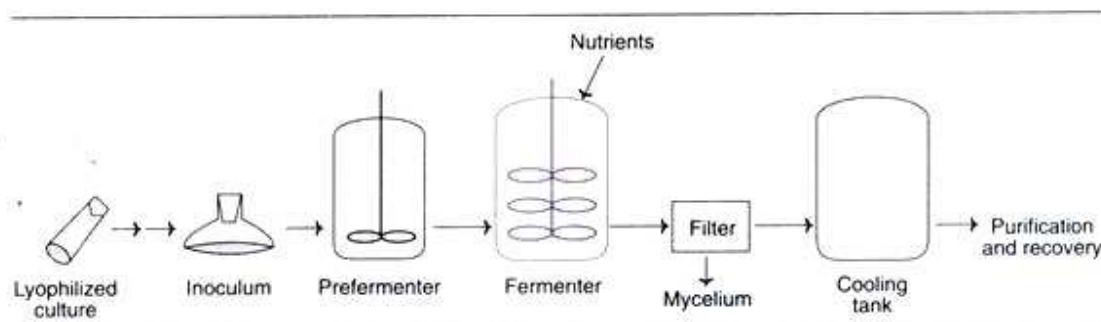


Fig. 25.3 : An outline of the flow chart for penicillin fermentation.

Penicillin production is an aerobic process and therefore, a continuous supply of O_2 to the growing culture is very essential. The required aeration rate is 0.5-1.0 vvm. The pH is maintained around 6.5, and the optimal temperature is in the range of 25-27°C. Penicillin production is usually carried out by submerged processes. The medium used for fermentation consists of corn steep liquor (4-5% dry weight) and carbon source (usually lactose). An addition of yeast extract, soy meal or whey is done for a good supply of nitrogen.

Sometimes, ammonium sulfate is added for the supply of nitrogen. Phenylacetic acid (or phenoxyacetic acid) which serves as a precursor for penicillin biosynthesis is continuously fed. Further, continuous feeding of sugar is advantageous for a good yield of penicillin. The penicillin production profiles are depicted in Figs. 25.4 and Fig. 25.5.

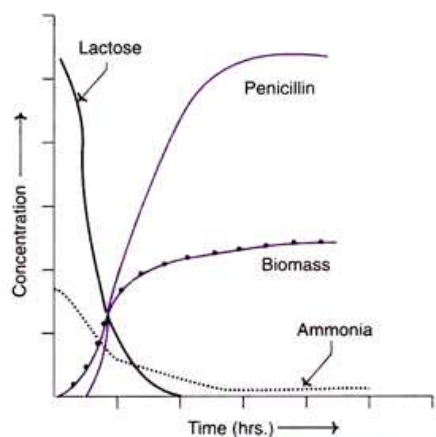


Fig. 25.4 : Penicillin production in relation to substrates utilization and biomass formation.

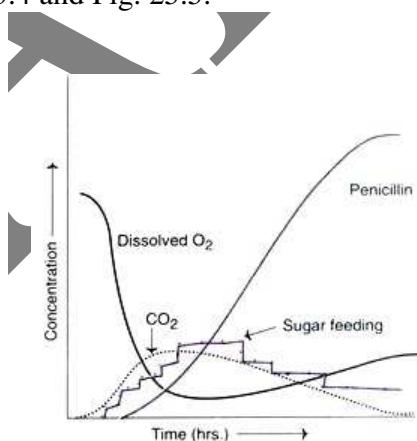


Fig. 25.5: Penicillin production in relation to continuous feeding of sugar, O_2 utilization, and CO_2 formation.

It is estimated that approximately 10% of the metabolised carbon contributes to penicillin production, while 65% is utilised towards energy supply and 25% for growth of the organisms.

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The efficiency of penicillin production can be optimized by adequate supply of carbon source. Thus, by adding glucose and acetic acid, the yield can be increased by about 25%.

For efficient synthesis of penicillin, the growth of the organism from spores must be in a loose form and not as pellets. The growth phase is around 40 hours with a doubling time of 6-8 hours. After the growth phase is stabilized, the penicillin production exponentially increases with appropriate culture conditions. The penicillin production phase can be extended to 150-180 hours.

Recovery of Penicillin:

As the fermentation is complete, the broth containing about 1% penicillin is processed for extraction. The mycelium is removed by filtration. Penicillin is recovered by solvent (n-butyl acetate or methyl ketone) extraction at low temperature (<10°C) and acidic pH (<3.0). By this way, the chemical and enzymatic (bacterial penicillinase) degradations of penicillin can be minimized.

The penicillin containing solvent is treated with activated carbon to remove impurities and pigments. Penicillin can be recovered by adding potassium or sodium acetate. The potassium or sodium salts of penicillin can be further processed (in dry solvents such as n-butanol or isopropanol) to remove impurities. The yield of penicillin is around 90%.

As the water is totally removed, penicillin salts can be crystallized and dried under required pressure. This can be then processed to finally produce the pharmaceutical dosage forms. Penicillins G and H are the fermented products obtained from the fungus *Penicillium chrysogenum*.

Production of 6-Amino Penicillanic Acid:

The penicillins G and H are mostly used as the starting materials for the production of several synthetic penicillins containing the basic nucleus namely 6-amino penicillanic acid (6-APA). About 10 years ago, only chemical methods were available for hydrolysis of penicillins to produce 6-APA. Now a days, enzymatic methods are preferred.

Immobilized penicillin amidases enzymes have been developed for specific hydrolysis of penicillin G and penicillin V. Penicillin salt of either G or V can be used for hydrolysis by immobilized enzyme system. The pH during hydrolysis is kept around 7-8, and the product 6-APA can be recovered by bringing down the pH to 4.

At pH 4, 6-amino penicillanic acid gets precipitated almost completely in the presence of a water immiscible solvent. In general, the enzymatic hydrolysis is more efficient for penicillin V than for penicillin G. However, penicillin G is a more versatile compound, as it is required for ring expansions.

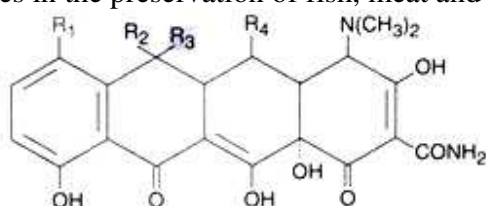
Tetracycline

Tetracycline are broad spectrum antibiotics with widespread medical use. They are effective against Gram-positive and Gram-negative bacteria, besides other organisms (*mycoplasmas*, *chlamydias rickettsias*). Tetracycline are used to combat stomach ulcers (against *Helicobacter pylori*). They are the most commonly used antibiotics, next to cephalosporin's and penicillins.

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Tetracyclines inhibit protein biosynthesis by blocking the binding of aminoacyl tRNA to ribosomes (A site).

The basic structure of tetracyclines is composed of a naphthalene ring (a four ring structure). The substituent groups of the common tetracyclines are given in Fig. 25.10. Among these, chlortetracycline and oxy-tetracycline are most commonly used in the treatment of human and veterinary diseases, besides in the preservation of fish, meat and poultry (in some countries).



Tetracycline	R ₁	R ₂	R ₃	R ₄	Examples of producing organisms
Tetracycline	H	CH ₃	OH	H	<i>Streptomyces aureus</i> , <i>S. flavus</i> , <i>S. antibioticus</i>
Chlortetracycline	Cl	CH ₃	OH	H	<i>S. aureofaciens</i> , <i>S. vindifaciens</i> , <i>S. flavus</i>
Oxytetracycline	H	CH ₃	OH	OH	<i>S. antibioticus</i> , <i>S. cellulosa</i> , <i>S. parvus</i> , <i>S. rimosus</i>
Minocycline	N(CH ₃) ₂	H	H	H	Semisynthetic
Doxycycline	H	CH ₃	H	OH	Semisynthetic

Fig. 25.10 : Structures of some important tetracyclines along with the examples of organisms for their production.

Organisms for Tetracycline Production:

The first tetracycline antibiotic that was isolated was chlortetracycline from the cultures of *Streptomyces aureofaciens* (in 1945). There are at least 20 *streptomycetes* identified now that usually produces a mixture of tetracyclines. In the Fig. 25.10, a selected list of these organisms for producing tetracyclines is also given.

High-yielding strains of *S. aureofaciens* and *S. rimosus* have been developed by using ultraviolet radiation and/or other mutagens (nitrosoguanidine). Such strains are very efficient for the production of chlortetracycline. Further, genetically engineered strains of *S. rimosus* have been developed for increased synthesis of oxytetracycline.

Biosynthesis of Tetracyclines:

The pathway for the biosynthesis of tetracyclines is very complex. An outline of the synthesis of chlortetracycline by *S. aureofaciens* is given in Fig. 25.11. There are at least 72 intermediates formed during the course of chlortetracycline biosynthesis, some of them have not been fully characterized.

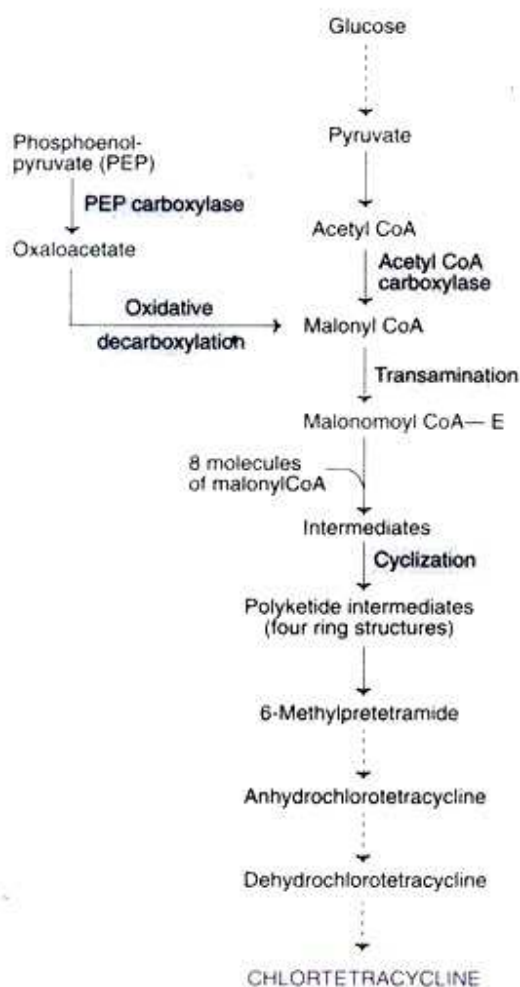


Fig. 25.11 : An outline of the biosynthesis of chlortetracycline by *S. aureofaciens*
(E —Anthracene synthase enzyme complex)

Polyketide antibiotic synthesis:

The term polyketide refers to a group of antibiotics that are synthesized by successive condensation of small carboxylic acids such as acetate, butyrate, propionate and malonate. The synthesis of polyketide antibiotics is comparable to that of long chain fatty acids. That is the carbon chain grows by cyclic condensation process. The synthesis of tetracyclines is a good example of polyketide antibiotic synthesis.

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As glucose gets oxidised, it forms acetyl CoA and then malonyl CoA. On transamination, the later gives malonomoyl CoA. The enzyme anthracene synthase complex binds to malonomoyl CoA and brings out the condensation of 8 molecules of malonyl CoA to form a polyketide intermediates (four ring structures). These intermediates undergo a series of reactions to finally produce chlortetracycline.

Regulation of biosynthesis:

Carbohydrate metabolism (particularly glycolysis) controls chlortetracycline synthesis. For more efficient synthesis of the antibiotic, glycolysis has to be substantially low. The addition of phosphate reduces chlortetracycline production.

Production Process of Chlortetracycline:

The fermentation medium consists of corn steep liquor, soy flour or peanut meal for the supply of nitrogen and carbon sources. Continuous feeding of carbohydrate is desirable for good growth of the organism and production of the antibiotic. This can be done either by addition of crude carbon sources or by supplying glucose or starch. For more efficient production of chlortetracycline, the supply of ammonium and phosphate has to be maintained at a low concentration.

An outline of the production process for chlortetracycline is depicted in Fig. 25.12. The ideal fermentation conditions are — temperature 27-30°C, pH-6.5-7.5, and aeration 0.8-1.0 vvm. The duration of fermentation is around 4 days.



Fig. 25.12 : An outline of production chart for chlortetracycline.

Recovery of chlortetracycline:

At the end of the fermentation, the culture broth is filtered to remove the mycelium. The filtrate is treated with n-butanol or methylisobutylketone in acidic or alkaline condition for extracting the antibiotic. It is then absorbed to activated charcoal to remove other impurities.

Chlortetracycline is eluted and crystallized.

Production of Tetracycline —Different Processes:

The production of tetracycline can be achieved by one or more of the following ways.

- By chemical treatment of chlortetracycline.
- By carrying out fermentation in a chloride-free culture medium.
- By employing mutants in which chlorination reaction does not occur.
- By blocking chlorination reaction by the addition of inhibitors e.g. thiourea, 2-thiouracil.

Bio-insecticides: Production of Bacterial and fungal polysaccharides

Bio-insecticides are those biological agents that are used to control harmful insects. They include the following.

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(a) Predators:

Destructive insects or plant pests can be brought under control through introduction of their natural predators. The predators should be specific and unable to harm the useful insects. Introduction of ladybugs (Lady Bird Beetles) and Praying Mantis has been successful in combating scale insects or aphids which feed on plant sap.

(b) Parasites and Pathogens:

This is alternate biological control of plant pests through the search of their natural parasites and pathogens. They include viruses, bacteria, fungi and insect parasitoids. Parasitoids are organisms that live as parasites for some time (as early or larval stage) and free living at other times, e.g., *Trichogramma*. *Nucleopolyhedrovirus* (NPV) are species specific.

For example, *Baculovirus heliothis* (a virus) can control Cotton bollworm (*Heliothis Zea*). Similarly, *Bacillus thuringensis* (a bacterium) is effective against the cabbage looper (*Trichoplusia ni*) and *Entomophthora ignobilis* (a fungus) the green peach aphid of Potato (*Myzus persicae*). In U.S.S.R. the fungus *Beauveria bassiana* has been successfully employed in controlling Potato beetle and Codling moth.

Polysaccharides:

The microorganisms can produce large amounts of polysaccharides in the presence of surplus carbon source. Some of these polysaccharides (e.g. glycogen) serve as storage compounds. The polysaccharides excreted by the cells, referred to as exopolysaccharides, are of commercial importance. The exopolysaccharides may be found in association with the cells or may remain in the medium.

The microbial polysaccharides may be neutral (e.g. dextran, scleroglucan) or acidic (xanthan, gellan) in nature. Acidic polysaccharides possessing ionized groups such as carboxyl, which can function as polyelectrolytes, are commercially more important.

Applications of Microbial Polysaccharides:

Microbial polysaccharides have immense commercial importance. They are employed in the stabilization of foods, and production of several industrial and pharmaceutical compounds. The commercial value of a polysaccharide is based on its ability to modify the flow characteristics of solutions (technically known as rheology). Polysaccharides can increase the viscosity and, are therefore useful as thickening and gelling agents.

Microbial polysaccharides are of great importance in oil industry. By conventional methods, only 50% of the oil can be extracted. And the rest is either trapped in the rock or too viscous to be pumped out. It is now possible to recover such oils also by a technique called microbial enhanced oil recovery (MEOR). This can be done by injecting surfactants and viscosity decreasing biological agents (i.e. the microbial polysaccharides e.g. xanthan and emulsan).

Production of Microbial Polysaccharides:

The synthesis of polysaccharides favourably occurs in the excess supply of carbon substrate in the growth medium while limiting nitrogen supply. A carbon/nitrogen ratio of around 10: 1 is considered to be favourable for optimal polysaccharide synthesis. The production process is mostly carried out by batch culture fermentation.

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By manipulating the nutrient supply, differential synthesis of polysaccharides can be achieved. By limiting nitrogen supply in the medium, mostly neutral polysaccharides are produced. When metal ions are limited, acidic polysaccharides are mainly synthesized. Molecular oxygen supply of around 90% saturation is ideal for good growth and polysaccharide synthesis.

Biosynthesis of polysaccharides:

Microorganisms are capable of producing a large number of polysaccharides. The pathways for their biosynthesis are comparable to the processes that occur for the formation bacterial cell wall. It is estimated that there are well over 100 enzymatic reactions, directly or indirectly involved in the synthesis of polysaccharides. Starting with glucose, appropriate sugars (by transforming glucose to others) are incorporated in the formation of polysaccharides.

Recovery of polysaccharides:

As the polysaccharide production increases, there occurs a marked increase in viscosity of the culture broth. The polysaccharides can be precipitated by salts, acids or organic solvents, and recovered by employing appropriate techniques.

Microbial polysaccharides versus plant polysaccharides:

There is a lot of competition between microbial and plant polysaccharides for industrial applications. Production of plant polysaccharides is relatively cheap, although it is uncontrolled and occurs for a short period in a year. In contrast, production of microbial polysaccharides is well controlled and can be continued throughout the year. However, fermentation processes for manufacture of cheap (from plant sources) polysaccharides is not advisable.

General Features of Microbial Polysaccharides:

Of the several microbial polysaccharides, around 20 are of industrial importance. As already stated, the commercial value of a polysaccharide is mostly dependent on its rheological properties i.e. its ability to modify the flow characteristics of solutions. A selected list of commercially important polysaccharides, the microorganisms used for their production, and their applications are given in the Table 30.1. Some of the important features of individual microbial polysaccharides are briefly described hereunder.

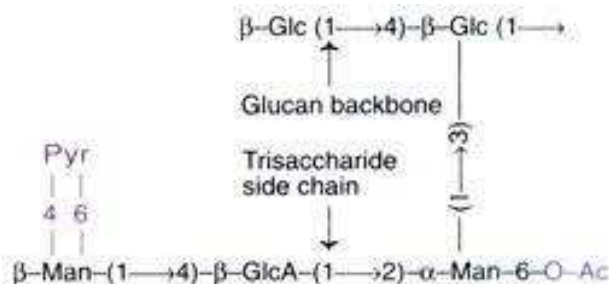
Commercial production of Xanthan gum and pullulan

Xanthan:

Xanthan or more frequently referred to as xanthan gum was the first polysaccharide available commercially. It is a well-studied and most widely used hexopolysaccharide.

Chemistry:

Xanthan has a molecular weight in the range of $2-15 \times 10^4$ Daltons. The basic repeating unit of xanthan is a pentasaccharide containing glucose (Glc), mannose (Man) and glucuronic acid (GlcA) with acetate (Ac) and pyruvate (Pyr) as depicted below.



Basically, xanthan is a branched polymer with β (1 \rightarrow 4) linked glucan (glucose polymer) backbone bound to a trisaccharide (Man, GlcA, Man) side chain on alternate glucose residues. The mannose has either acetate or pyruvate groups.

The number of acetate or pyruvate molecules in xanthan is variable and is dependent on the bacterial strain used. The culture conditions and the recovery processes also influence the quantities of pyruvate and acetate residues. It is believed that the viscosity of xanthan gum is influenced by the contents of pyruvate and acetate.

TABLE 30.1 Commercially important microbial polysaccharides and their applications		
Polysaccharide	Producing organism(s)	Application(s)
Xanthan	<i>Xanthomonas campestris</i>	As a food additive for stabilization, gelling and viscosity control, i.e. for the preparation of soft foods e.g. ice cream, cheese. In oil industry for enhanced oil recovery. In the preparation of toothpastes, and water based paints.
Dextran	<i>Leuconostoc mesenteroides</i> , <i>Acetobacter</i> sp., <i>Streptococcus mutans</i>	Blood plasma expander Used in the prevention of thrombosis, and in wound dressing (as adsorbent). In the laboratory for chromatographic and other techniques involved in purification. As a foodstuff.
Alginate	<i>Pseudomonas aeruginosa</i> <i>Azobacter vinelandii</i>	In food industry as thickening and gelling agent. Alginate beads are employed in immobilization of cells and enzymes. Used as ion-exchange agent.
Scleroglucan	<i>Sclerotium glaucum</i> <i>S. rolfsii</i> , <i>S. delphinii</i>	Used for stabilizing latex paints, printing inks, and drilling muds.
Gellan	<i>Pseudomonas elodea</i>	In food industry as a thickener and solidifying agent.
Pullulan	<i>Aureobasidium pullulans</i>	Being a biodegradable polysaccharide, it is used in food coating and packaging.
Curdian	<i>Alcaligenes faecalis</i>	As a gelling agent in cooked foods (forms a strong gel above 55°C) Useful for immobilization of enzymes.
Emulsan	<i>Acinetobacter calcoaceticus</i> <i>Arthrobacter</i> sp	In oil industry for enhanced recovery. For cleaning of oil spills.

Applications:

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Xanthan gum is used as a food additive for the preparation of soft foods (ice cream, cheese). It is also used in oil industry for enhancing oil recovery. Further, xanthan is useful for the preparation of tooth pastes and water based paints.

Biosynthesis:

For the biosynthesis of xanthan, the monomers are bound to a carrier lipid molecule and then transferred to a growing polymer chain. The activated monosaccharide nucleotides (e.g. uridine diphosphate glucose, UDP-glucose) supply energy for the formation of glycosidic bonds between adjacent units. The biosynthesis of other exopolysaccharides is comparable with that of xanthan. Dextran synthesis however is much simpler as described later.

Production:

Xanthan is commercially produced by the Gram-negative bacterium, *Xanthomonas campestris*. The culture medium usually consists of 4-5% carbohydrate (glucose, sucrose, corn starch hydrolysate), 0.05-0.1% nitrogen (ammonium nitrate, urea, yeast extract) and salts.

The pH is maintained around 7.0, and the fermentation is carried out by batch culture for 2-3 days. Xanthan in the culture broth is precipitated by isopropanol or methanol. These agents also kill the microorganisms. The precipitated xanthan can be dried and used for commercial purposes.

Genetic engineering of *Xanthomonas campestris* for xanthan production:

The wild type *X. campestris* can efficiently utilize glucose, sucrose or starch as a carbon source. They are however, unable to use lactose as a carbon substrate. Whey is a byproduct obtained in the manufacture of cheese. Disposal of large quantities of whey is a major problem in dairy industry. Fortunately, whey is rich in lactose, besides containing small quantities of proteins, vitamins and minerals. Attempts are made to use whey in fermentation industries.

Genetically engineered *X. campestris* have been developed that can utilize lactose (from whey) for the production of xanthan. For this purpose, the *E. coli* *lacZ* genes (encoding the enzyme β -galactosidase and lactose permease respectively) were cloned under the transcriptional control of *X. campestris* bacteriophage promoter. This construct was first introduced into *E. coli*, and then transferred to *X. campestris*.

The genetically engineered strains of *X. campestris* expressed the genes and produced high quantities of the enzymes β -galactosidase and lactose permease. These new strains utilize lactose or whey very efficiently for the industrial production of xanthan. This is a good example of successfully converting a waste product (whey) into a commercially important and valuable product (a biopolymer namely xanthan gum).

Pullulan:

Pullulan is an α -glucose polymer (α -glucan) with α 1 \rightarrow 4, and a few α , 1 \rightarrow 6 glycosidic bonds. Pullulan is produced by using the fungus, *Aureobasidium pullulans*. It is estimated that about 70% of glucose (the substrate) is converted to pullulan during fermentation, although the time taken is rather long (5-7 days). Pullulan is mainly used in food coating and packaging. Pullulan has been produced in good yield from carbohydrate substrates such as sucrose, α -glucose, α -fructose, and maltose. The use of spent sulphite liquor from pulp and paper mills is being

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investigated as a possible substrate. Pullulan is a water-soluble polymer which gives colourless and adhesive solutions with properties depending on the strain of organism and the fermentation conditions employed. It can be used to give texture, viscosity, and moisture retention in foods and also as a dispersant and flocculent.

Production of edible mushroom and SCP

Edible Mushrooms:

Mushrooms are fungi belonging to the classes basidiomycetes (*Agaricus sp*, *Auricularia sp*, *Tremella sp*) and ascomycetes (*Morchella sp*, *Tuber sp*). Majority of edible mushrooms are the species of basidiomycetes. It is estimated that there are around 4,000 species of basidiomycetes. Of these, around 200 are edible, and a dozen of them are cultivated on large scale. Some of the most important edible mushrooms, their common names and the substrates used are given in Table 29.3.

TABLE 29.3 A selected list of the edible mushrooms cultivated on commercial scale		
Mushroom species	Common name	Substrate(s)
<i>Agaricus bisporus</i>	Button mushroom	Straw, horse manure, compost
<i>Leutinus edodes</i>	Oak or shitake mushroom	Saw dust, wooden logs, rice bran
<i>Pleurotus ostreatus</i>	Oyster mushroom	Straw, saw dust, paper
<i>Volvarela volvacea</i>	Chinese mushroom or padi-straw mushroom	Straw, cotton
<i>Auricularia sp</i>	Wood-ear mushroom	Saw dust, rice bran
<i>Coprinus sp</i>	— — —	Straw

The cultivation of edible mushrooms is one of the rare examples of a microbial culture wherein the cultivated macroscopic organism itself is directly used as human food. Mushroom growing is one of the fastest developing biotechnological industries world over. Further growth of mushroom industry is expected for the production of enzymes, and pharmaceutical compounds, including antitumor agents and antibiotics.

Poisonous mushrooms:

There are certain poisonous mushrooms also. They usually possess unpleasant taste and odour. These mushrooms produce some poisonous substances like phallin and muscarine. The examples of poisonous mushrooms are *Amanita phalloides*, *A. muscaria*, *A. viraosa*, *Lepiota morgani* and *Boletus satanas*.

Nutritive value of edible mushrooms:

Some people regard edible mushrooms as vegetable meat. Mushrooms contain 80-90% water, depending on the growth conditions (temperature, humidity). Edible mushrooms are rich sources of protein (35-45% of dry weight). However, all these proteins are not easily digestible by humans. Mushrooms also contain fats and free fatty acids (7-10%), carbohydrates (5-15%) and minerals in good concentration. Certain undesirable substances may also be present in edible mushrooms e.g. cadmium, chromium.

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Advantages of edible mushroom biotechnology:

1. Mushrooms can be produced by utilizing cheap and often waste substrates (industrial and wood wastes).
2. They are of high nutritive value being rich in proteins, vitamins and minerals.
3. Many delicious recipes can be prepared from mushrooms.
4. Due to low carbohydrate content, consumption of mushrooms is advocated to diabetic patients.

Production of Edible Mushrooms:

Mushroom production is basically a fermentation process. This is mostly carried out by solid-substrate fermentation. A wide range of substrates (straw, saw dust, compost, wooden logs) depending the organism can be used (Refer Table 29.3). Mushroom production is a good example of a low technology utilization in an otherwise sophisticated modern biotechnology.

TABLE 29.3 A selected list of the edible mushrooms cultivated on commercial scale		
<i>Mushroom species</i>	<i>Common name</i>	<i>Substrate(s)</i>
<i>Agaricus bisporus</i>	Button mushroom	Straw, horse manure, compost
<i>Lentinula edodes</i>	Oak or shiitake mushroom	Saw dust, wooden logs, rice bran
<i>Pleurotus ostreatus</i>	Oyster mushroom	Straw, saw dust, paper
<i>Volvarella volvacea</i>	Chinese mushroom or padi-straw mushroom	Straw, cotton
<i>Auricularia</i> sp.	Wood-ear mushroom	Saw dust, rice bran
<i>Coprinus</i> sp.	— — —	Straw

The most common edible mushroom cultivated world over (that may constitute about 20% world mushroom produce) is the white button mushroom, *Agaricus bisporus*. *Lentinula edodes* is the second most cultivated mushroom in the world. The substrates straw, compost or horse manure can be used. The substrate selection depends on the local factors.

A schematic representation of mushroom production is depicted in Fig. 29.5. The compost with desired formulation is prepared and sterilized. It is spread into the trays which are then transferred to production room and inoculated with spawn. Spawn is the term used for the mushroom inoculum containing spores and/or small pieces of fruiting body.

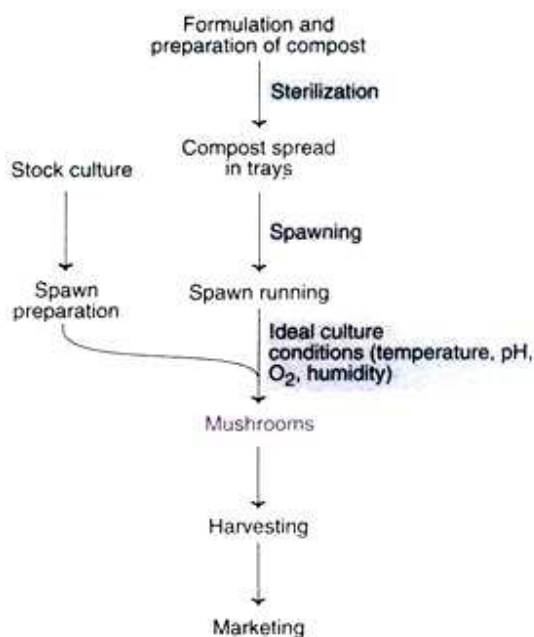


Fig. 29.5 : An overview of edible mushroom production.

After inoculation (spawning), the culture is maintained at optimal growth conditions. The trays are regularly watered to maintain 70-80% humidity. The ideal temperature is about 15°C, and pH about 7.0. It takes about 7-10 days for each crop of mushroom production. It is possible to have 3-4 crops, before terminating the production process. The mushrooms can be harvested and marketed.

Mushrooms have a very short life 8-12 hours, unless stored at low temperature (refrigerator 2-5°C). Therefore, they should be immediately consumed, stored or canned. Variations in culturing mushrooms: The production of mushrooms is highly variable and mostly depends on the organism and the substrate used, besides several other local factors. There are distinct differences in the mushroom cultivation methods between different countries. For instance, garden and field cultivation methods are used in Europe, while in USA, cave and house cultivation techniques are employed.

Some mushrooms (e.g. *Volvariella sp*) are suitable for cultivation in summer and rainy season while others grow well in winter (*Agaricus bisporus*, *Pleurotus sp*). It is however, possible to grow these mushrooms any time in a year with appropriate temperature and humidity control arrangements.

Single-Cell Protein (SCP) :

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Single-cell protein (SCP) refers to the microbial cells or total protein extracted from pure microbial cell culture (monoculture) which can be used as protein supplement for humans or animals. The word SCP is considered to be appropriate, since most of the microorganisms grow as single or filamentous individuals. This is in contrast to complete multicellular plants and animals.

If the SCP is suitable for human consumption, it is considered as food grade. SCP is regarded as feed grade, when it is used as animal feed supplement, but not suitable for human consumption. Single-cell protein broadly refers to the microbial biomass or protein extract used as food or feed additive. Besides high protein content (about 60-80% of dry cell weight), SCP also contains fats, carbohydrates, nucleic acids, vitamins and minerals.

Another advantage with SCP is that it is rich in certain essential amino acids (lysine, methionine) which are usually limiting in most plant and animal foods. Thus, SCP is of high nutritional value for human or animal consumption.

It is estimated that about 25% of the world's population currently suffers from hunger and malnutrition. Most of these people live in developing countries. Therefore, SCP deserves a serious consideration for its use as food or feed supplement. In addition to its utility as a nutritional supplement, SCP can also be used for the isolation of several compounds e.g. carbohydrates, fats, vitamins, minerals.

Advantages of Using Microorganisms for SCP Production:

The protein-producing capabilities of a 250 kg cow and 250 g of microorganisms are often compared. The cow can produce about 200 g protein per day. On the other hand, microorganisms, theoretically, when grown under ideal conditions, could produce about 20-25 tonnes of protein.

There are many advantages of using microorganisms for SCP production:

1. Microorganisms grow at a very rapid rate under optimal culture conditions. Some microbes double their mass in less than 30 minutes.
2. The quality and quantity of protein content in microorganisms is better compared to higher plants and animals.
3. A wide range of raw materials, which are otherwise wasted, can be fruitfully used for SCP production.
4. The culture conditions and the fermentation processes are very simple.
5. Microorganisms can be easily handled, and subjected to genetic manipulations.

Safety, Acceptability and Toxicology of SCP:

There are many non-technological factors that influence the production of SCP. These include the geographical, social, political and psychological factors. In many countries, there are social and psychological barriers to use microorganisms as food sources. It is desirable to first consider the safety, acceptability and toxicology of SCP, particularly when it is considered for human consumption. There are several limitations for the widespread use of SCP.

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1. The nucleic acid content of microbial biomass is very high (4-6% in algae; 10-15% in bacteria; 5-10% in yeast). This is highly hazardous, since humans have a limited capacity to degrade nucleic acids.
2. The presence of carcinogenic and other toxic substances is often observed in association with SCP. These include the hydrocarbons, heavy metals, mycotoxins and some contaminants. The nature and production of these compounds depends on the raw materials, and the type of organism used.
3. There is a possibility of contamination of pathogenic microorganisms in the SCP.
4. The digestion of microbial cells is rather slow. This is frequently associated with indigestion and allergic reactions in individuals.
5. Food grade production of SCP is more expensive than some other sources of proteins e.g. soy meal. Of course, this mainly depends on the cost of raw materials. In general, SCP for human consumption is 10 times more expensive than SCP for animal feed.

For the above said reasons, many countries give low priority for the use of SCP for human consumption. In fact, mass production of SCP using costly raw materials has been discontinued in some countries e.g. Japan, Britain, Italy. However, these countries continue their efforts to produce SCP from cheap raw materials such as organic wastes.

Microorganisms and Substrates Used for Production of SCP:

Several microorganisms that include bacteria, yeasts, fungi, algae and actinomycetes utilizing a wide range of substrates are used for the production of SCP. A selected list is given in Table 29.1.

KA

TABLE 29.1 A selected list of microorganisms and substrates used for single-cell protein production	
Microorganism	Substrate(s)
Bacteria	
<i>Methylophilus methylotrophus</i>	Methane, methanol
<i>Methylomonas</i> sp	Methanol
<i>Pseudomonas</i> sp	Alkanes
<i>Brevibacterium</i> sp	C ₁ -C ₄ hydrocarbons
Yeasts	
<i>Saccharomycopsis lipolytica</i> (previous name— <i>Candida lipolytica</i>)	Alkanes
<i>Candida utilis</i>	Sulfite liquor
<i>Kluyveromyces fragilis</i>	Whey
<i>Saccharomyces cerevisiae</i> (baker's yeast)	Molasses
<i>Lactobacillus bulgaricus</i>	Whey
<i>Tosulopsis</i> sp	Methanol
Fungi	
<i>Chaetomium cellulolyticum</i>	Cellulosic wastes
<i>Paecilomyces varioti</i>	Sulfite liquor
<i>Aspergillus niger</i>	Molasses
<i>Trichoderma viride</i>	Straw, starch
Algae	
<i>Spirulina maxima</i>	Carbon dioxide
<i>Chlorella pyrenoidosa</i>	Carbon dioxide
<i>Scenedesmus acutus</i>	Carbon dioxide
Actinomycetes	
<i>Nocardia</i> sp	Alkanes
<i>Thermomonospora fusca</i>	Cellulose
Mushrooms (a type of fungi)	
<i>Agaricus bisporus</i>	Compost, rice straw
<i>Morchella crassipes</i>	Whey, sulfite liquor
<i>Auricularia</i> sp	Saw dust, rice bran
<i>Lentinus edodes</i>	Saw dust, rice bran
<i>Volvariella volvaceae</i>	Cotton, straw

The selection of microorganisms for SCP production is based on several criteria. These include their nutritive value, non-pathogenic nature, production cost, raw materials used and growth pattern.

Substrates:

The nature of the raw materials supplying substrates is very crucial for SCP production. The cost of raw material significantly influences the final cost of SCP. The most commonly used raw materials may be grouped in the following categories.

1. High-energy sources e.g. alkanes, methane, methanol, ethanol, gas oil.
2. Waste products e.g. molasses, whey, sewage, animal manures, straw, bagasse.
3. Agricultural and forestry sources e.g. cellulose, lignin.

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4. Carbon dioxide, the simplest carbon source.

Production of SCP from High Energy Sources:

There are a large number of energy-rich carbon compounds or their derivatives which serve as raw materials for SCP production. These include alkanes, methane, methanol, and ethanol and gas oil. Bacteria and yeasts are mostly employed for SCP production from high energy sources. Some scientists question the wisdom of using (rather misusing) high-energy compounds for the production of food, since they regard it as a wasteful exercise.

Production of SCP from alkanes:

Alkanes can be degraded by many yeasts, certain bacteria and fungi. The major limitation of alkanes is that they are not easily soluble, hence they cannot enter the cells rapidly. It is believed that the cells produce emulsifying substances which convert insoluble alkanes into small droplets (0.01-0.5 μ m) that can enter the cells by passive diffusion.

It is observed that when cells are grown on a medium of alkanes enriched with lipids, the diffusion of alkanes into the cells is enhanced. Certain yeasts have been successfully used for producing SCP from alkanes e.g. *Saccharomycopsis lipolytica*, *Candida tropicalis*, *Candida oleophila*.

Petroleum products for SCP production:

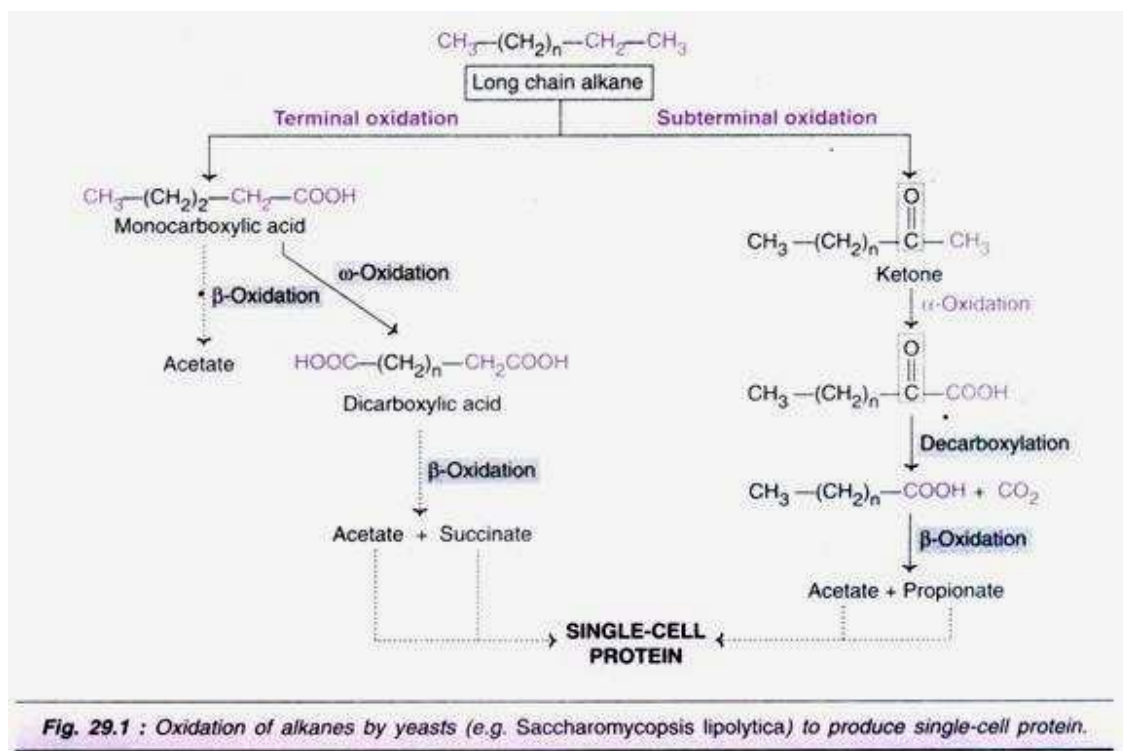
Several oil companies have developed fermentation systems, employing petroleum products for large scale manufacture of SCP by yeasts. Two types of petroleum products are mainly used for this purpose.

1. Gas oil or diesel oil containing 10-25% of alkanes with carbon length C_{15} - C_{30} (i.e. long chain alkanes).
2. Short chain alkanes with carbon length in the range of C_{10} - C_{17} , isolated from gas oil by use of molecular sieves.

Airlift bioreactor system with continuous operation was once used (in France and Britain) to produce SCP from gas oil employing the organism *Saccharomycopsis lipolytica*. But this is now discontinued for political reasons.

Degradation of alkanes:

Alkanes have to be first broken down to appropriate metabolites for their utilization to form SCP. The most important step in this direction is the introduction of oxygen into alkanes which can be brought out by two pathways-terminal oxidation and sub-terminal oxidation (Fig. 29.1).



In terminal oxidation, the terminal carbon gets oxidized to the corresponding monocarboxylic acid. The latter then undergoes β -oxidation to form acetic acid. In some microorganisms, the oxidation may occur at both the terminal carbon atoms (by a process referred to as co-oxidation) to form a dicarboxylic acid. This can be further broken down to acetate and succinate by β -oxidation. Terminal oxidation is the predominant pathway occurring in majority of yeasts and bacteria.

Sub-terminal oxidation involves the oxidation of interterminal carbon atoms (any carbon other than terminal i.e. C_2 , C_3 , C_4 , and so on). The corresponding ketone produced undergoes α -oxidation, decarboxylation, and finally β -oxidation to form acetate and propionate. The individual enzymes responsible for terminal oxidation or sub-terminal oxidation have not been fully identified.

Limitations of SCP production from alkanes:

The production of SCP from alkanes is a very complex biotechnological process and has been extensively studied. The major drawback of alkanes as substrates is the formation of carcinogens, along with SCP which are highly harmful. For this reason, many countries have discontinued alkane-based production of SCP.

Production of SCP from methane:

Methane is the chief constituent of natural gas in many regions. Although methane can be isolated in pure gas form, it cannot be liquefied. The handling and transportation of methane (an explosive gas) are very difficult and expensive. Certain bacteria that can utilize methane for SCP

production have been identified e.g. *Methylococcus capsulatus*, *Methylomonas methanica*, *Methylovibrio soehngenii*. So far, yeasts that can utilize methane have not been identified.

The bacterial enzyme methane oxygenase oxidizes methane to methanol, which can be converted to formaldehyde and then to formic acid. Although methane was extensively researched for its use as a source of SCP, it is not widely used due to technical difficulties.

Production of SCP from methanol:

Methanol is a good substrate for producing SCP. Methanol as a carbon source for SCP has several advantages over alkanes and methane. Methanol is easily soluble in aqueous phase at all concentrations, and no residue of it remains in the harvested biomass. Technically, methanol can be easily handled. The sources for methanol are natural gas, coal, oil and methane.

Many species of bacteria (*Methylobacter*, *Arthrobacter*, *Bacillus*, *Pseudomonas*, *Vibrio*) yeasts (*Candida biodinii*, *Hansenula sp*, *Torulopsis sp*) and fungi (*Trichoderma lignorum*, *Gliocladium delinquescens*) are capable of producing SCP from methanol. Bacteria are mostly preferred because they require simple fermentation conditions, grow rapidly and possess high content of protein.

Oxidation of methanol:

Methanol gets oxidized to formaldehyde, then to formic acid and finally to carbon dioxide, as depicted in Fig. 29.2.

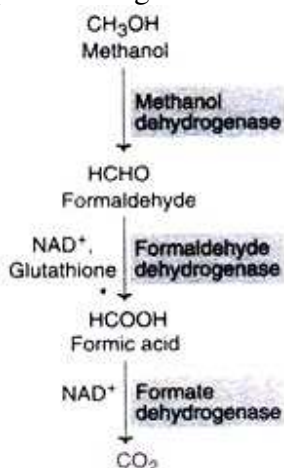


Fig. 29.2 : Oxidation of methanol.

The products obtained from methanol have to form C₃ compounds (such as pyruvate) for final production of SCP. Carbon dioxide formed from methanol can be utilized by photosynthetic organisms for the formation of ribulose diphosphate. Alternately, formaldehyde may condense with ribulose 5-phosphate to form 3-keto 6-phosphohexulose which then gives fructose 6-phosphate and finally pyruvate. This pathway is referred to as ribulose monophosphate (or Quayle) cycle.

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Formaldehyde can condense with glycine to form serine which in a series of reactions forms phosphoenol pyruvate. This is referred to as serine pathway.

Production process:

Imperial Chemical Industries (ICI), U.K. was the first company to develop a process for continuous methanol fermentation for large scale production of SCP. Later, Hoechst (Germany) and Mitsubishi (Japan) also developed similar fermentation systems.

ICI employed *Methylophilus methylotrophus* (formerly called *Pseudomonas methylotrophus*) for producing SCP from methanol. A bioreactor, referred to as ICI pressure cycle fermenter was used for this purpose (Fig. 29.3). This fermenter has three components-airlift column, down-flow tube and gas release space. The operation was carried out at temperature 35-37°C and pH 6.5-7.0. The cells were subjected to disruption by heat or acid treatment. The nutrient solution can be clarified by decanting.

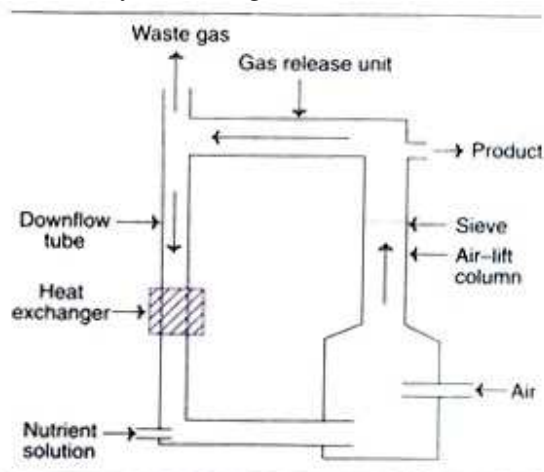


Fig. 29.3 : A diagrammatic representation of ICI pressure cycle fermenter to produce SCP from methanol.

ICI Pruteen:

The single-cell protein produced by ICI from methanol and ammonia using *M. methylotrophus* was referred to as ICI pruteen. This SCP was exclusively used for animal feeding. ICI invested a huge amount (around £40 million) in 1979 and installed a continuous culture system for SCP production. This was the world's largest continuous airlift fermenter. Unfortunately, the plant could not be operated for long due to economic reasons.

For instance, in 1984 the cost of soy meal was around \$125-200 per ton while ICI pruteen was sold at \$600 per ton! This is mainly because of the high cost of methanol which represents approximately half of the production cost expenses. In the Middle East, due to high availability and low cost of methanol, the production of SCP appeared to be attractive. In the erstwhile Russia, there were several plants producing SCP from methanol which were later closed.

Genetic engineering for improved SCP production from methanol:

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The efficiency of SCP production has been improved by using genetic engineering. The assimilation of ammonia by *M. methylotrophus* is an essential step for cellular growth. This organism lacks glutamate dehydrogenase. It possesses glutamine synthase and glutamine ketoglutarate transaminase to utilize ammonia for the formation of glutamate (Fig. 29.4A).

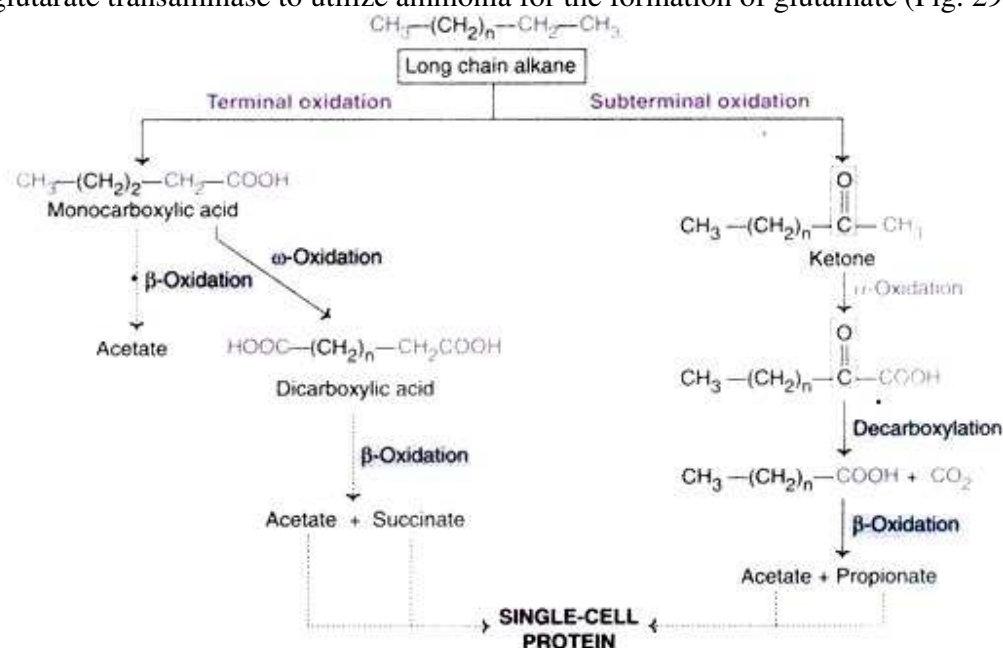


Fig. 29.1 : Oxidation of alkanes by yeasts (e.g. *Saccharomycopsis lipolytica*) to produce single-cell protein.

This is an energy (ATP) dependent reaction'. By employing recombinant DNA technology, the gene for the enzyme glutamate dehydrogenase from *E. coli* was cloned and expressed in *M. methylotrophus*. These genetically transformed organisms were more efficient in assimilating ammonia. They could grow rapidly and convert more methanol to SCP. However, the overall increase in the production of SCP did not exceed 10%.

Production of SCP from ethanol:

Ethanol is a good substrate for the production of SCP for human consumption (feed grade SCP). However, this process, as such, is not economically feasible. However, several factors-local raw materials, innovative fermentation technology, political decisions and foreign trade balances influence production of SCP. It may not be surprising if large scale production of SCP commences, on one day, from ethanol for a variety of reasons.

Production of SCP from Wastes:

There are several materials that serve no useful purpose and they are collectively referred to as wastes e.g. molasses, whey, animal manures, sewage, straw, date wastes. These waste products,

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formed in various industries and other biological processes, largely contribute to environmental pollution. There are several advantages of utilizing wastes for the production of SCP.

These include the conversion of low-cost organic wastes to useful products, and reduction in environmental pollution. However, there has been very limited success for the large scale production of SCP from wastes. This is mainly because of transportation cost and technical difficulties. The technology adopted and the organism employed for SCP production depends on the waste being used as the substrate. Thus, *Saccharomyces cerevisiae* is used for molasses, *Kluyveromyces fragilis* for cheese whey.

Symba process:

Symba process is a novel technology developed in Sweden to produce SCP by utilizing starchy wastes by employing two yeasts, *Endomycopsis fibuligira* and *Candida utilis*. The Symba process is carried out in three phases.

Phase I:

The waste material containing starch is sterilized by passing through a heat exchanger.

Phase II:

The sterilized material is passed through two bioreactors. The first reactor contains *E. fibuligira* which hydrolyses starch. When this hydrolysate is passed to the second bioreactor, the organism, *C. utilis* grows to form biomass.

Phase III:

The microbial biomass can be separated by centrifugation. The samples of SCP can be dried, packaged and stored.

Applications of Symba product:

The yeast biomass produced in Symba process is of good nutritive value. It is widely used as an animal feed for pigs, calves and chicken. The animals grow quite well and no adverse effects have been reported.

Pekilo — a fungal protein rich product:

A filamentous fungus, *Paecilomyces variotii*, with good fibrous structure was used for the production of Pekilo. This protein, rich in fungal biomass, was produced by fermentation of wastes such as molasses, whey, sulfite liquor and agricultural wastes. It can be produced by a continuous fermentation process. Pekilo is rich in proteins (containing essential amino acids), vitamins and minerals.

It was used as an animal feed in supplementing the diets of calves, pigs, chickens and hens without any adverse effects. It is unfortunate that the production of Pekilo has been discontinued at most places due to economic and commercial considerations.

Quorn-the mycoprotein for humans:

The protein Quorn is the mycoprotein produced by the fungus *Fusarium graminearum*. Many companies in the developed countries are engaged in the production of fungal proteins for human consumption. Quorn is the trade name for *Fusarium* mycoprotein produced in Britain by Marlow Foods

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The fungus *Fusarium* can be grown continuously on simple carbohydrate sources (like glucose). Ammonium ions supply nitrogen. Mineral salts and vitamins are also added. The fermentation is carried out at pH 6.0 and temperature 30°C. At the end of fermentation, the culture is heated to 65°C to activate RNases. This is necessary to degrade RNA and reduce the content from 10% to around 1%.

The breakdown products of RNA namely the nucleotides diffuse out from the cells and can be easily removed. (Reduction in RNA content is desirable to make the product acceptable for human consumption. This is because humans have a very limited capacity to digest nucleic acids). It is possible to produce 1 kg of fungal biomass with a protein content of about 135 g from 1 kg glucose utilized in the culture medium.

The dried *Fusarium* product is artificially flavoured and marketed in pieces that resemble beef, pork and chicken. The nutritional composition of mycoprotein when compared to beef is given in Table 29.2. Besides being rich in essential nutrients, mycoprotein has a good content of dietary fiber. There are several advantages of fiber consumption- prevents constipation, decreases intestinal cancers, improves glucose tolerance and reduces serum cholesterol.

TABLE 29.2 Nutritional composition (in percentage) of mycoprotein in comparison to beef

Nutrient	Mycoprotein	Beef (raw lean beefsteak)
Protein	4	68
Lipid	15	30
Carbohydrate	10	0
Fiber	25	Traces
Ash (minerals etc.)	3	2
RNA	1	Traces

Production of SCP from Wood:

The natural waste wood sources containing cellulose, hemicellulose and lignin are attractive natural sources for the production of SCP. It is however, essential to breakdown these cellulosic compounds into fermentable sugars. For this purpose, extracellular cellulases can be used. Certain bacteria (*Cellulomonas sp*) and fungi (*Trichoderma sp*, *Penicillium sp*) are good sources for cellulases.

Techniques for the production of cellulases have been well standardized from several organisms. The cost of production of cellulases is a critical factor in determining the ultimate production cost of SCP. In some instances, the cellulosic materials can be directly used for biomass production. The resultant SCP is used as animal feed.

Production of SCP from CO₂:

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Certain algae grown in open ponds require only CO₂ as the carbon source. In the presence of sunlight, they can effectively carry out photosynthesis, and produce SCP. The examples of these algae are *Chlorella sp*, *Senedesmus sp* and *Spirulina sp*. *Chlorella* is used as a protein and vitamin supplement for enriching ice-creams, breads and yoghurts in some countries. In some parts of the world, the algae in ponds are used for the removal of organic pollutants. The resultant algae biomass can be harvested, dried and powdered. Algae SCP are very useful as animal supplements.

Nutritive value of Spirulina SCP:

Traditionally *Spirulina sp* have been eaten by people in some parts of Africa and Mexico. SCP of *Spirulina* is of high nutritive value (protein-65%, carbohydrate- 20%, fat-4%, fibre-3%, chlorophyll-5%, ash-3%). *Spirulina* is a good source of protein for human consumption, particularly in developing countries.

Production of SCP from Sewage:

Domestic sewage is normally used for large scale production of methane, which in turn may be utilized for the production of SCP. The sewage obtained from industrial wastes in cellulose processing, starch production and food processing can be utilized for the production of SCP.

The organism *Candida utilis* is used to produce SCP by using effluent formed during the course of paper manufacture. Other microorganisms namely *Candida tropicalis*, *Paecilomyces varioti* are employed to use sulfite waste liquor for the production of SCP.

Genetically Engineered Artificial Protein As Animal Feed:

Rumen bacteria can synthesize amino acids. Some workers have developed genetically engineered strains of rumen bacteria that can produce a protein rich in methionine, threonine, lysine and leucine. This artificial protein has a total of 100 amino acids, of which 57 are essential.

The gene for artificial protein was synthesized by 14 overlapping oligonucleotides held to maltose binding protein gene. This gene was expressed in *E. coli* under the transcriptional control of *tac* promoter. The production of this artificial protein accounts to around 12% of the intracellular proteins. However, the large scale production of artificial protein by rumen bacteria is yet to be clearly established and commercialized.

Biofertilizer

'Biofertilizer' is a substance which contains living microorganism which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant. Biofertilizers are not fertilizers.

Fertilizers directly increase soil fertility by adding nutrients. Biofertilizers add nutrients through the natural processes of fixing atmospheric nitrogen, solubilizing Phosphorus, and stimulating plant growth through the synthesis of growth promoting substances.

Biofertilizers can be grouped in different ways based on their nature and function.

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S. No.	Groups	Examples
Nitrogen (N₂) fixing Biofertilizers		
1	Free-living	<i>Azotobacter, Clostridium, Anabaena, Nostoc,</i>
2	Symbiotic	<i>Rhizobium, Frankia, Anabaena azollae</i>
3	Associative Symbiotic	<i>Azospirillum</i>
P Solubilizing Biofertilizers		
1	Bacteria	<i>Bacillus megaterium var. phosphaticum</i> <i>Bacillus circulans, Pseudomonas striata</i>
2	Fungi	<i>Penicillium sp, Aspergillus awamori</i>
P Mobilizing Biofertilizers		
1	Arbuscular mycorrhiza	<i>Glomus sp., Gigaspora sp., Acaulospora sp.,</i> <i>Scutellospora sp. & Sclerocystis sp.</i>
2	Ectomycorrhiza	<i>Laccaria sp., Pisolithus sp., Boletus sp., Amanita sp.</i>
3	Orchid mycorrhiza	<i>Rhizoctonia solani</i>
Biofertilizers for Micro nutrients		
1	Silicate and Zinc solubilizers	<i>Bacillus sp.</i>
Plant Growth Promoting Rhizobacteria		
1	Pseudomonas	<i>Pseudomonas fluorescens</i>

Different types of biofertilizers:

1. Rhizobium -

This belongs to bacterial group and the classical example is symbiotic nitrogen fixation. The bacteria infect the legume root and form root nodules within which they reduce molecular nitrogen to ammonia which is readily utilized by the plant to produce valuable proteins, vitamins and other nitrogen containing compounds. The site of symbiosis is within the root nodules. It has been estimated that 40-250 kg N / ha / year is fixed by different legume crops by the microbial activities of Rhizobium. Table shows the N fixation rates.

Table: Quantity of biological N fixed by Liquid Rhizobium in different crops

Host Group	Rhizobium Species	Crops	N fix kg/ha
Pea group	<i>Rhizobium leguminosarum</i>	Green pea, Lentil	62- 132
Soybean group	<i>R. japonicum</i>	Soybean	57- 105
Lupini Group	<i>R. lupine orinthopus</i>	Lupinus	70- 90
Alfafa grp.Group	<i>R. melliloti</i> <i>Medicago Trigonella</i>	Melilotus	100- 150
Beans group	<i>R. phaseoli</i>	Phaseoli	80- 110
Clover group	<i>R. trifoli</i>	Trifolium	130
Cowpea group	<i>R. species</i>	Moong, Redgram,	57- 105

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		Cowpea, Groundnut	
Cicer group	R. species	Bengal gram	75- 117

2. Azotobacter -

It is the important and well known free living **nitrogen fixing aerobic bacterium**. It is used as a Bio-Fertilizer for all non leguminous plants especially rice, cotton, vegetables etc. Azotobacter cells are not present on the rhizosphere but are abundant in the rhizosphere region. The lack of organic matter in the soil is a limiting factor for the proliferation of Azotobacter in the soil.

3. Azospirillum-

It belongs to bacteria and is known to **fix the considerable quantity of nitrogen** in the range of 20- 40 kg N/ha in the rhizosphere in non- leguminous plants such as cereals, millets, Oilseeds, cotton etc.

4. Cyanobacteria-

A group of one celled to many celled aquatic organisms. Also known as **blue-green algae**

5. Azolla -

Azolla is a free floating water fern that floats in water and **fixes atmospheric nitrogen** in association with nitrogen fixing blue green alga Anabaena azollae. Azolla fronds consist of sporophyte with a floating rhizome and small overlapping bi-lobed leaves and roots. Azolla is considered to be a potential biofertilizer in terms of **nitrogen contribution to rice**.

Long before its cultivation as a green manure, Azolla has been used as a fodder for domesticated animals such as pigs and ducks. In recent days, Azolla is very much used as a sustainable feed substitute for livestock especially dairy cattle, poultry, piggery and fish

6. Phosphate solubilizing microorganisms (PSM)

7. AM fungi-

An arbuscular mycorrhiza (AM Fungi) is a type of mycorrhiza in which the fungus penetrates the cortical cells of the roots of a vascular plant.

8. Silicate solubilizing bacteria (SSB)-

Microorganisms are capable of **degrading silicates and aluminum silicates**. During the metabolism of microbes several organic acids are produced and these have a dual role in silicate weathering.

9. Plant Growth Promoting Rhizobacteria (PGPR)-

The group of bacteria that colonize roots or rhizosphere soil and beneficial to crops are referred to as **plant growth promoting rhizobacteria (PGPR)**.

Liquid Biofertilizers

Benefits:-

The advantages of Liquid Bio-fertilizer over conventional carrier based Bio-fertilizers are listed below:-

- Longer shelf life -12-24 months.
- No contamination.

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- No loss of properties due to storage upto 45° c.
- Greater potentials to fight with native population.
- Easy identification by typical fermented smell.
- Better survival on seeds and soil.
- Very much easy to use by the farmer.
- High commercial revenues.
- High export potential.

Characteristics of different liquid Bio-fertilizers

Rhizobium-

Physical features of liquid Rhizobium:

- Dull white in colour
- No bad smell
- No foam formation, pH 6.8-7.5

Azospirillum-

Physical features of liquid Azospirillum:

- The colour of the liquid may be blue or dull white.
- Bad odours confirms improper liquid formulation and may be concluded as mere broth.
- Production of yellow gummy colour materials confirms the quality product.
- Acidic pH always confirms that there is no Azospirillum bacteria in the liquid.

Role of Liquid Azospirillum under field conditions:

- Stimulates growth and imparts green colour which is a characteristic of a healthy plant.
- Aids utilization of potash, phosphorous and other nutrients.
- Encourage plumpness and succulence of fruits and increase protein percentage.

Azotobacter-

Physical features of liquid Azotobacter:

The pigmentation that is produced by Azotobacter in aged culture is melanin which is due to oxidation of tyrosine by tyrosinase an enzyme which has copper. The colour can be noted in liquid forms. Some of the pigmentation are described below-

- *A. chroococcum*: Produces brown-black pigmentation in liquid inoculum.
- *A. beijerinckii*: Produces yellow- light brown pigmentation in liquid inoculum
- *A. vinelandii*: Produces green fluorescent pigmentation in liquid inoculum.
- *A. paspali*: Produces green fluorescent pigmentation in liquid inoculum.
- *A. macrocytogenes*: Produces, pink pigmentation in liquid inoculum.
- *A. insignis*: Produces less, gum less, grayish-blue pigmentation in liquid inoculum.
- *A. agilis*: Produces green-fluorescent pigmentation in liquid inoculum.

Acetobacter-

This is a saccharophilic bacteria and associate with sugarcane, sweet potato and sweet sorghum plants and fixes 30 kgs/ N/ ha year. Mainly this bacterium is commercialized for sugarcane crop. It is known to increase yield by 10-20 t/ acre and sugar content by about 10-15 percent.

Liquid Bio-fertilizer application methodology-

There are three ways of using Liquid Bio-fertilizers

1. Seed treatment
2. Root dipping
3. Soil application

Dosage of liquid Bio-fertilizers in different crops

Recommended Liquid Bio-fertilizers and its application method, quantity to be used in different crops are as follows:

Crop	Recommended Biofertilizer	Application method	Quantity to be used
Field crops	Rhizobium	Seed treatment	200 ml/acre
Pulses			
Chickpea, pea, Groundnut, soybean, beans, Lentil, lucern, Berseem, Green gram, Black gram, Cowpea and pigeon pea			
Cereals	Azotobacter/ Azospirillum	Seed treatment	200 ml/acre
Wheat, oat, barley			
Rice	Azospirillum	Seed treatment	200 ml/acre
Oil seeds	Azotobacter	Seed treatment	200 ml/acre
Mustard, sesamum, Linseeds, Sunflower, castor			
Millets	Azotobacter	Seed treatment	200 ml/acre
Pearl millets, Finger millets, kodo millet			
Maize and Sorghum	Azospirillum	Seed treatment	200 ml/acre
Forage crops and Grasses	Azotobacter	Seed treatment	200 ml/acre
Bermuda grass, Sudan grass, Napier Grass, ParaGrass, StarGrass etc.			

Other Misc. Plantation Crops	Azotobacter	Seedling treatment	500 ml/acre
Tobacco			
Tea, Coffee	Azotobacter	Soil treatment	400 ml/acre
Rubber, Coconuts	Azotobacter	Soil treatment	2-3 ml/plant
Agro-ForestRY/Fruit Plants	Azotobacter	Soil treatment	2-3 ml / plant at nursery
All fruit/agro-forestry (herb, shrubs, annuals and perennial) plants for fuel wood fodder, fruits, gum, spice, leaves, flowers, nuts and seeds puppose			
Leguminous plants/ trees	Rhizobium	Soil treatment	1-2 ml/plant

How to apply or use Biofertilizers

There are three major ways of applying biofertilizers

1. Seed treatment

One packet of the inoculant is mixed with 200 ml of rice kanji to make a slurry. The seeds required for an acre are mixed in the slurry so as to have a uniform coating of the inoculant over the seeds and then shade dried for 30 minutes. The shade dried seeds should be sown within 24 hours. One packet of the inoculant (200 g) is sufficient to treat 10 kg of seeds.

2. Seedling root dip

This method is used for transplanted crops. Two packets of the inoculant is mixed in 40 litres of water. The root portion of the seedlings required for an acre is dipped in the mixture for 5 to 10 minutes and then transplanted.

3. Main field application

Four packets of the inoculant is mixed with 20 kgs of dried and powdered farm yard manure and then broadcasted in one acre of main field just before transplanting.

Rhizobium :- For all legumes, Rhizobium is applied as seed inoculant.

Quantity of Rhizobium as seed application

S. No.	Crop	Total requirement of packets per ha
1	Soybean	5
2	Groundnut	5
3	Bengalgram	5
4	Blackgram	3
5	Greengram	3
6	Redgram	3

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7 Cowpea

3

Azospirillum/Azotobacter

In the transplanted crops, **Azospirillum is inoculated through seed, seedling root dip and soil application methods.** For direct sown crops, Azospirillum is applied through seed treatment and soil application.

Precautions

- Bacterial inoculants should not be mixed with insecticide, fungicide, herbicide and fertilizers.
- Seed treatment with bacterial inoculant is to be done at last when seeds are treated with fungicides.

Constraints in Biofertilizer Technology

Though the biofertilizer technology is a low cost, ecofriendly technology, several constraints limit the application or implementation of the technology the constraints may be environmental, technological, infrastructural, financial, human resources, unawareness, quality, marketing, etc. The different constraints in one way or other affecting the technique at production, or marketing or usage.

1. Technological constraints

- Use of improper, less efficient strains for production.
- Lack of qualified technical personnel in production units.
- Production of poor quality inoculants without understanding the basic microbiological techniques
- Short shelf life of inoculants.

2. Infrastructural constraints

- Non-availability of suitable facilities for production
- Lack of essential equipments, power supply, etc.
- Space availability for laboratory, production, storage, etc.
- Lack of facility for cold storage of inoculant packets

3. Financial constraints

- Non-availability of sufficient funds and problems in getting bank loans
- Less return by sale of products in smaller production units.

4. Environmental constraints

- Seasonal demand for biofertilizers
- Simultaneous cropping operations and short span of sowing/planting in a particular locality
- Soil characteristics like salinity, acidity, drought, water logging, etc.

5. Human resources and quality constraints

- Lack of technically qualified staff in the production units.
- Lack of suitable training on the production techniques.
- Ignorance on the quality of the product by the manufacturer
- Non-availability of quality specifications and quick quality control methods

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- No regulation or act on the quality of the products
- Awareness on the technology
- Unawareness on the benefits of the technology
- Problem in the adoption of the technology by the farmers due to different methods of inoculation.
- No visual difference in the crop growth immediately as that of inorganic fertilizers.

Biopesticides

Generally, biopesticides are made of living things, come from living things, or they are found in nature. They tend to pose fewer risks than conventional chemicals. Very small quantities can be effective and they tend to break down more quickly, which means less pollution.

Some biopesticides are targeted in their activity, often working on a small number of species. However, users need more knowledge to use biopesticides effectively. This is because they are often most effectively used as part of an Integrated Pest Management approach.

Types

Microbes - These are tiny organisms like bacteria and fungi. They tend to be more targeted in their activity than conventional chemicals. For example, a certain fungus might control certain weeds, and another fungus might control certain insects. The most common microbial biopesticide is *Bacillus thuringiensis*.

- **Substances Found in Nature** – These include plant materials like corn gluten, garlic oil, and black pepper. These also some include insect **hormones** that regulate mating, molting, and food-finding behaviors. They tend to control pests without killing them. For example, they might repel pests, disrupt their mating, or stunt their growth. Some synthetic substances are allowed. However, they must be similar in shape and makeup to their natural counterparts. They must also work in the exact same way against pests.
- **Plant-Incorporated Protectants (PIPs)** – These are the genes and proteins, which are introduced into plants by genetic engineering. They allow the genetically modified plant to protect itself from pests, like certain insects or viruses. For example, some plants produce insect-killing proteins within their tissues. They can do this because genes from *Bacillus thuringiensis* were inserted into the plant's DNA. Different types of proteins target different types of insects.

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Leaching of ores by microbes

Microbial ore leaching (bioleaching) is the process of extracting metals from ores with the use of microorganisms. This method is used to recover many different precious metals like copper, lead, zinc, gold, silver, and nickel. Microorganisms are used because they can:

- lower the production costs.
- cause less environmental pollution in comparison to the traditional leaching methods.
- very efficiently extract metals when their concentration in the ore is low.

The Leaching Process

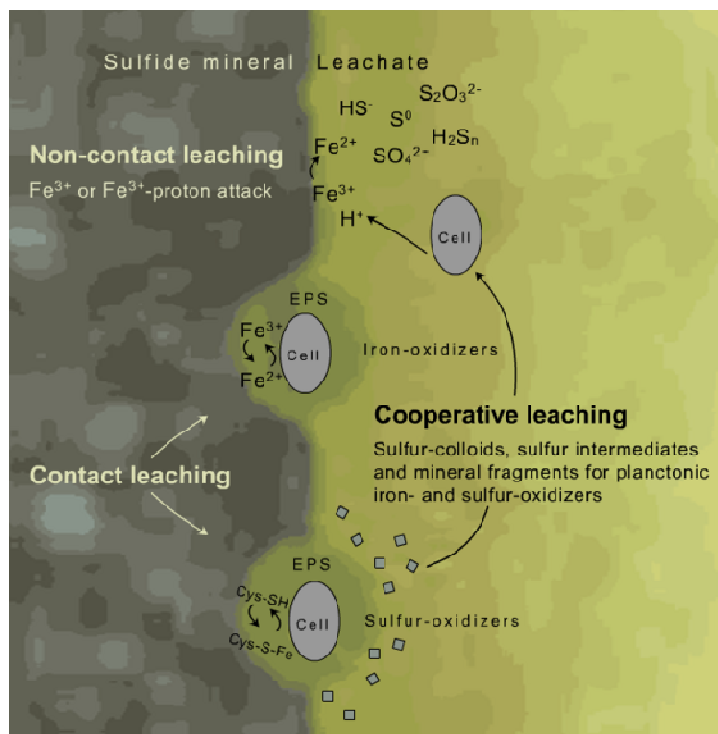
Bacteria perform the key reaction of regenerating the major ore oxidizer which in most cases is ferric iron as well as further ore oxidation. The reaction is performed at the bacterial cell membrane. In the process, free electrons are generated and used for the reduction of oxygen to water which produces energy in the bacterial cell.

Ores, like pyrite (FeS_2), are first oxidized by ferric iron (Fe^{3+}) to thiosulfate ($\text{S}_2\text{O}_3^{2-}$) in the absence of bacteria.

In the first step, disulfide is spontaneously oxidized to thiosulfate by ferric iron (Fe^{3+}), which in turn is reduced to give ferrous iron (Fe^{2+}):



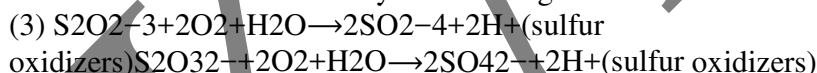
Bacteria are added in the second step and recover Fe^{3+} from ferrous iron (Fe^{2+}) which is then reused in the first step of leaching:



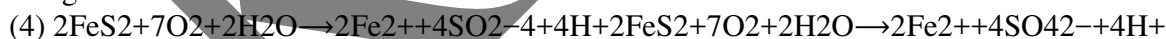
Sulfide mineral bacterial leaching: Bacterial cells oxidizing the ferrous iron back to ferric iron while using slightly different contact mechanisms with the metal.



Thiosulfate is also oxidized by bacteria to give sulfate:



The ferric iron produced in reaction (2) oxidized more sulfide as in reaction (1), closing the cycle and given the net reaction:



The net products of the reaction are soluble ferrous sulfate and sulfuric acid.

The microbial oxidation process occurs at the cell membrane of the bacteria. The electrons pass into the cells and are used in biochemical processes to produce energy for the bacteria while reducing oxygen to water. The critical reaction is the oxidation of sulfide by ferric iron. The main role of the bacterial step is the regeneration of this reactant.

Copper leaching has a very similar mechanism.

Microorganisms Capable of Ore Leaching

Bioremediation reactions industrially are performed by many bacterial species that can oxidize ferrous iron and sulfur. An example of such species is *Acidithiobacillus ferrooxidans*. Some fungi species (*Aspergillus niger* and *Penicillium simplicissimum*) have also been shown to have the

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ability to dissolve heavy metals. When fungi are used, the leaching mechanism is different. The fungi use the acids that they produce in their metabolic reactions to dissolve the metal. In general, bioleaching is cleaner and safer for the environment than chemical processing. However environmental pollution with toxic products, like sulfuric acid from the pyrite leaching, and heavy metals is still possible. Another drawback of microbial leaching is the slow rate at which microbes work.

Microorganisms in Wastewater Treatment:

Bioaugmentation (Use of Blends of Microorganism):

Acceleration of biodegradation of specific compounds by inoculating bacterial cells is called bioaugmentation. Bacterial cells contain specific plasmid which encodes enzymes for degradation of those, compounds.

A variety of plasmids have been reported from *Alcaligenes*, *Acinetobacter*, *Arthrobacter*, *Beijerinckia*, *Klebsiella*, *Flavobacterium* and *Pseudomonas*. Several genetically engineered strains have been developed exploiting *Pseudomonas*.

Microorganisms capable of degrading herbicides/other chemicals in industrial water are isolated from wastewater, compost, sludge, etc. Some of the strains may be irradiated to enhance their ability and mutants are selected.

Before their use in the environment they are tested in laboratory for their biodegradation ability. Bioassays are also used to assess the toxicity of the waste water for commercial preparation of microbial seeds. Selected strains are used in large fermentor to get mass culture. Then they are preserved through lyophilization, drying and freezing.

Commercial bioaugmentation products are single culture of consortia of microorganisms with certain degradative properties or their desirable characters. At present most important users are the industrial wastewater treatment plants.

The selected microorganism is added to a bioreactor so that potential for biodegradation of wastes must be maintained or enhanced. Due to trade secrets information on bioformulation of mixture of microbial cultures are not scanty.

Application of bio augmentation includes:

- (a) The increased BOD removal in wastewater treatment plants,
- (b) Reduction of sludge volume by about 30% after addition of selected microorganisms,
- (c) Use of mixed cultures in sludge digestion,
- (d) Biotreatment of hydrocarbon waste, and
- (e) Biotreatment of hazardous wastes.

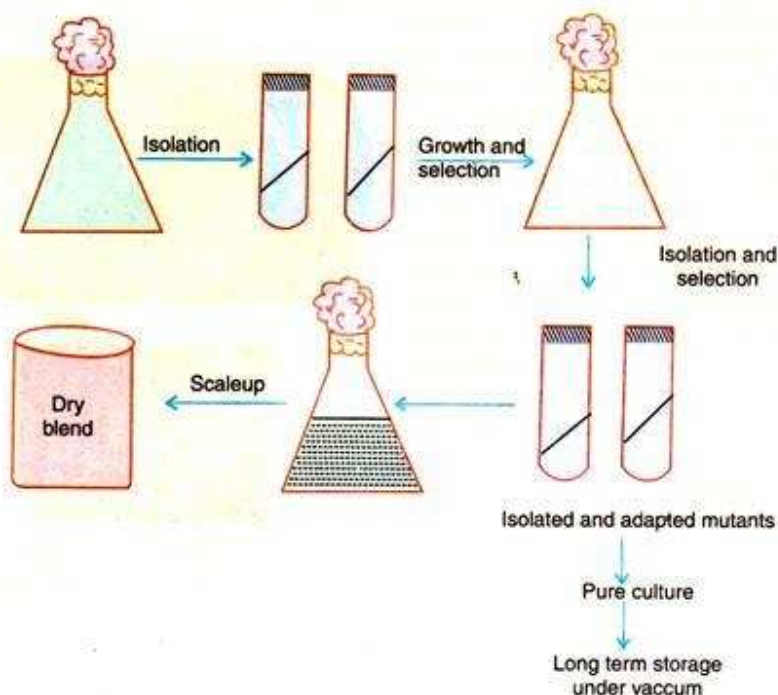


Fig. 33.12 : Isolation and purification of microbial blends used for pollution control.

The use of added microorganisms for treating hazardous wastes such as phenol, ethylene glycol, formaldehyde has been attempted. Bioaugmentation with parachlorophenol-degrading bacteria decomposed 96% para-chlorophenol in 9 hours. Cells of *Candida tropicalis* have been used for removal of high concentration of phenol present in freshwater.

Ability of a bioreactor to dechlorinate 3-chlorobenzoate was increased after addition of *Desulfomonile tiedjei* to a methanogenic upflow anaerobic granular sludge blanket. Anoxygenic phototrophic bacteria have also been considered for the degradation of toxic compounds in wastes.

Some demerits of bioaugmentation are:

- (a) Need of an acclimation period prior to onset of biodegradation,
- (b) A short survival or lack of growth of microbial inocula in the seeded bioreactors, and
- (c) Sometimes negative or non-conclusion of some of commercial products.

Aerobic Treatment

Aerobic wastewater treatment is a process where bacteria utilize oxygen to degrade organic matter (generally quantified as biochemical oxygen demand or BOD) and other pollutants involved in various production systems. The two most common types of aerated wastewater systems are activated sludge systems and aerated stabilization basins (ASBs). ASBs are

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commonly found as treatment systems in the pulp and paper industry and are used in some municipalities, as well as other industries.

There are eight growth pressures that affect a treatment system but we will review two major ones: oxygen and organic loading (BOD). In a typical wastewater treatment system, the influent coming into the system has the most BOD because it hasn't yet been treated. As the influent reaches the ASB, it enters an aerated environment where the degradation will begin. Different types of aeration are used in ASBs but the most widely used are either surface aerators or diffused aeration systems. When using surface aeration, multiple units are needed to be properly spaced to treat the water. Diffused aeration is normally air that is supplied by compressors or blowers and piped under the surface where the air is released evenly throughout the ASB. Occasionally, pure oxygen is utilized in wastewater treatment, but this is relatively uncommon in ASBs.

The degradation of BOD is achieved through aerobic bacteria in a system. The bacteria utilize oxygen as an electron receptor in order to convert the organic material (BOD or oxygen demand) to carbon dioxide. Via this process they multiply, which in turn creates more bugs to break down more BOD. As the water flows through the system, many changes will occur. As the amount of BOD in the system reduces, the total number of bacteria will also decrease. The oxygen demand, as measured by oxygen uptake rate (OUR) will decrease and the environment will become acceptable for more advanced life forms, such as protozoa or metazoan. A few of the common higher life forms are: flagellates, free swimming ciliates, stalked ciliates, and rotifers. The higher life forms will feed on the dispersed bacteria and flocculated bacteria that have been formed after degradation has occurred. Higher life forms are an indication that most BOD has been removed from the system.

ASBs tend to be very resilient systems and generally produce adequate quality effluent for typical discharge requirements. However, proper aerator placement and routine maintenance are critical to ensuring that system performance does not deteriorate over time.

Whether it is aerobic or anaerobic treatment, each treatment system has its place in the world today. They are very different in the process but both are used to achieve maximum degradation, while meeting the strict regulations set by the environmental agencies that regulate what is released into the air, ground, or water.

Anaerobic Treatment

Anaerobic treatment is a process where wastewater or material is broken down by microorganisms without the aid of dissolved oxygen. However, anaerobic bacteria can and will use oxygen that is found in the oxides introduced into the system or they can obtain it from organic material within the wastewater. Anaerobic systems are used in many industrial systems including food production and municipal sewage treatment systems.

Anaerobic digestion is commonly used to treat sludges in the first areas of a wastewater treatment plant. This process is popular because it is able to stabilize the water with little biomass production. Anaerobic treatment occurs in many different stages. The key

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microorganisms are methane formers and acid formers. The acid formers are microorganisms that create various acids from the sludge. Methane formers convert the acids into methane.

The two main anaerobic systems are batch systems and continuous systems. In a batch system, the biomass is added into a reactor that is sealed for the rest of the digestion process. This is the simplest form of anaerobic treatment but can have odor issues associated with it. As the most simple, it is also one of the least expensive ways to achieve treatment.

A continuous system has organic matter constantly added to the treatment system. Since it is continuously being fed, there is a need for the byproduct to continuously be removed. The byproduct can result in a constant source of biogas, which can be used as an alternative source for energy. This system is usually more expensive to operate because of the need for constant monitoring and manpower.

Biogas is produced as the bacteria feed off the biodegradable material in the anaerobic process. The majority of the biogas produced is methane and carbon dioxide. These gases can be stored and used for energy production. The methane in the biogas can be burned to produce heat and electricity. The heat and electricity can be used to aid the process of the anaerobic system by providing power and heat for the digestion to occur.

Biogas can also be used as alternative source for fuel. This has received a lot of attention due to the ever-rising cost of burning fossil fuels. To produce fuel, the biogas must be treated to reduce or eliminate hydrogen sulfide. The treatment may become expensive but is necessary because the EPA has strict limits on the amount of hydrogen sulfide released into the atmosphere.

Whether it is aerobic or anaerobic treatment, each treatment system has its place in the world today. They are very different in the process but both are used to achieve maximum degradation, while meeting the strict regulations set by the environmental agencies that regulate what is released into the air, ground, or water.

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POSSIBLE QUESTIONS

Two marks

1. What is bioinsecticide? Name any two.
2. Define antibiotic.
3. Name any four antibiotics.
4. What is biofertilizers? Name any two.
5. Name any three applications of single cell protein.
6. Name any three applications of edible mushroom.

Essay type questions

1. Discuss the production of antibiotics.
2. Explain in brief about production of edible mushroom and SCP.
3. Write a brief note on production of xanthan gum and pullulan.
4. Give a short note on biofertilizers.
5. Write a brief note on bioinsecticides.
6. Explain in detail about production of antibiotics.
7. Write short notes on the applications of edible mushroom and SCP.
8. What are the methods involved in the production of alcohol and alcohol beverages?
9. What are the applications of citric acid, lactic acid and acetate?
10. Write the applications of bioinsecticides and biofertilizers.

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MULTIPLE CHOICE QUESTIONS
UNIT V

S.No	Questions	Option A	Option B	Option C	Option D	Answer
1	Penicillin is now produced by strain improvement of	Penicillium notatum	Penicilium chrysogem	Both the above	None	Penicilium chrysogem
2	Streptomyces aureofacines involved in the synthesis of	Streptomycin	Penicillin	Tetracycline	Cephalosporin	Tetracycline
3	The amino acids present in penicillin are	Valine and Cystine	Methionine and Arginine	Lysine and proline	All options	Lysine and proline
4	The lactic acid production through the break down of sugar was shown by	John Needham	Louis Pasteur	Franscesco Redi	John Tyndall	John Tyndall
5	Streptomycin is particularly active against_____	Vibrio cholerae	Salmonella typhi	Mycobacterium tuberculosis	e-coli	Mycobacterium tuberculosis
6	Penicillin f is _____	2-Pentenyl pencillin	Dihydro pencillin	N-pentyl pencillin	Benzyl pencillin	2-Pentenyl pencillin
7	Phenoxy acetic acid is used for the production of_____	penicillin G	penicillin V	penicillin K	N-Heptyl penicillin	penicillin V
8	Penicillin inhibits_____synthesis	Peptido glycan	Nuclear material	N – acetyl glucosamine	N – acetyl galactoseamine	Peptido glycan
9	The optimum pH and temp for Xanthane gum production is	7 And 60°	7 And 25 °– 30°	1 and 10°	14 and 100°	7 And 25 °– 30°
10	Optimum pH for lactic acid bacteria is_____	20° _25°	30°- 35°	27°- 32°	40°-50°	27°- 32°
11	Culture instability resulting from the accumulation of methanol in the SCP broth can be relieved by which organism	hypho microbium	aspergillus	saccharomyces species	zymomonas species	hypho microbium

12	Which strain increase the lysine content of fermented soy milk	L.acidophilus	L.fermetei	L.lactus	both L.acidophilus and L.fermetei	L.acidophilus
13	The Fermented product produced by aspergillus niger is	Lactic acid	Pyruvic acid	Fumaric acid	Citric acid	Citric acid
14	Moist heat at	120°C/ 15 min	121°C/ 15 min	130°C/ 15 min	125°C/ 15 min	125°C/ 15 min
15	Tetracycline is produced by_____	Bacillus	Penicillium	Streptomyces	Aspergillus	Streptomyces
16	The main antibiotic affecting nucleic acid and protein synthesis is	Vanconycin	Bacteriocin	Tetracyclin	Cyclosporin	Tetracyclin
17	----- was first called as “Miracle Drug” by Fleming.	Ampicillin	Penicillin	Cephalosporin	Amoxylin	Penicillin
18	Commercially citric acid is produced by the following except one	Koji fermentation process	Liquid surface culture fermentation process	Solid culture	Submerged culture fermentation processs	Solid culture
19	Organisms used for commercial production of citric acid is	Asperigillus Oryzae	Asperigillus tamarii	Asperigillus terreus	Asperigillus niger	Asperigillus niger
20	Penicillin was first isolated from	Penicillin notat	Penicillin chrysogenum	Penicillin roqueforti	Penicillin citrinum	Penicillin chrysogenum
21	Clostridium acetobutylicum produce	Lactic acid	Acetone-butanol	Acetone	Formic acid	Acetone-butanol
22	Among the following bacteria which is best for production of alcohol	Zymomonas mobilis	streptococcus	Salmonella	Clostridium	Zymomonas mobilis
23	Organism involved in butyric acid fermentation	Pseudomonas	Bacillus popilliac	Candida albicans	Clostridium aceto butilicum	Clostridium aceto butilicum
24	The ratio of Petrol :Ethanol in gaseol is	80:20:00	0.888888889	60:40:00	40 :60	80:20:00
25	Ethanol production is favoured by	Aerobic condition	Anaerobic condition	Moist condition	Dry condition	Moist condition

26	Which substance required pretreatment for the production of alcohol	sugary substance	Starchy substance	cellulosic substance	dibasic substance	cellulosic substance
27	Which yeast strain is commonly employed for the fermentation of alcohol's	Sacchromycopsis lipolytia	Candida milleri	Saccharomyces rouxii	Saccharomyces cerevicea	Saccharomyces cerevicea
28	Food produced by micro organism	Single cell protein	Glucose	Sucrose	Fructose	Single cell protein
29	Blackstrap molasses is prepared from	Ethanol	Synthetic media	Sugar cane	Fruit juice	Sugar cane
30	Fungal amylases are produced by	aspergillus oxyzac	Bacillus substillin	Psuedomonas	Serratia	aspergillus oxyzac
31	Penicillinase hydrolyse penicillin to produce	penicilloic acid	Penicillo aldehyde	Penicillamine	6 amino penicillamic acid	6 amino penicillamic acid
32	In antibiotic industry,conversion of PencillinG to 6-amino pencillanic acid is done by	pencillinase	pencillin acylase	pectinase	pencillin amidase	pencillin acylase
33	In antibiotic industry,conversion of PencillinG to 6-amino pencillanic acid isdone by	pectinase	Penicillo aldehyde	pencillinase	pencillin acylase	pectinase
34	The enzymes present in the membrane of mitochondria are	Flavoproteins and cytochromes	Fumarase and lipase	Enolase and catalase	Hexokinase and zymase	Flavoproteins and cytochromes
35	A mitochondrial marker enzyme is	Aldolase	Amylase	Succinic dehydrogenase	Pyruvate dehydrogenase	Succinic dehydrogenase
36	The active site of an enzyme is formed by	R group of amino acids	NH ₂ group of amino acids	CO group of amino acids	Sulphur bonds which are exposed	CO group of amino acids
37	The tear secretion contains an antibacterial enzyme known as	Zymase	Diastase	Lysozyme	Lipase	Lysozyme
38	The transfer of bacterial extrecellular polysaccharide is	isoprenoid lipid	ABC transponter	GT- Transponter	None	isoprenoid lipid

	accompanied using					
39	The semisynthetic pencillin was produced by action of	pencillin amidase	Penicillin acylase	Pencillinase	All options	Penicillin acylase
40	The antibiotics which are used as a preservative in food and poultry is	Chlortetracycline and oxytetracycline	Oxytetracycline and penicillin	Streptomycin and tetracycline	Penicillin and streptomycin	Chlortetracycline and oxytetracycline
41	Microbial polysaccharide originally used as a blood plasma extender Xanthan	Dextran	Lipopolysaccharide	Dextrin	All options	Dextran
42	In antibiotic production, which system is used to maintain for pure product	Aseptic conditions	Non-Aseptic condition	Batch	None	Aseptic conditions
43	CTC is the abbreviation of	7- chlorotetracyclan	7- carboxy tetracycline	Oxytetracycline	Tetracycline	7- chlorotetracyclan
44	The assay of enzymes require the presence of substrate at	saturating level	optimum level	minimum level	None	optimum level
45	The following are extra cellular enzyme except	amylase	cellulase	asparaginase	protease	asparaginase
46	The major source of citric acid is	Plum	Lemon	Apple	Mango	Lemon
47	One Of the most widely used natural insecticides are the toxins produced by_____	B-thrugiensis	B-cereus	Salmonella	E. coli	B-thrugiensis
48	Which of the following is a common bacterial biocotrol agent?	Bacillus moritai	Entomophthora virulenta	Bacillus thuringenesis	Metarrhizium anisopilac	Bacillus thuringenesis
49	Bacillus thuringenesis is used as insecticide against_____	Lepidoptera	Diptera	Both Lepidoptera and Diptera	None	Both Lepidoptera and Diptera
50	The amino acids present in penicillin are	Valine and Cystine	Methionine and Arginine	Lysine and proline	All the Above	Lysine and proline

51	Bacillus thuringesis is a	Bacterium	Fungu	Algae	Protozoan	Bacterium
52	Streptomycin is active against	M.tuberculosis	S.griseus	P.nonatum	B.subtili	S.griseus
53	In the following , which one is not used as microbial insecticide	Bacillus thurin	Sacromyces ceregenesis	Entomopox virus	Beauveria bassiana	Sacromyces ceregenesis
54	Citric acid are used in	Backing powder	Bread mixes	Blood transfusion	None of the above	Blood transfusion
55	The organism responsible for the production of lactic acid by fermentation is	Bacillus subtilis	Lactobacillus delbrueckii	Bacillus pumilus	Bacillus thuringiensis	Lactobacillus delbrueckii
56	In alcoholic fermentation the pyruvate is converted into----- -----	Ethanol and CO2	Oxygen and methanol	Methanol and oxygen	CO2 and glucose	Ethanol and CO2
57	The following are one carbon compounds except	Formic acid	Methanol	Carbon monoxide	Ethanol	Ethanol
58	Micro organism capable of growing one carbon substrate are called	Methylotroph	Organotroph	lithotroph	phototroph	Methylotroph
59	Following are methanol producing enzymes except	Methane monoxide	Methane dioxygenase	Methane oxido reductase	Methane dehydrogenase	Methane dehydrogenase
60	Following are methanol producing organisms except	Methymonos	Methylobacter	Methylococcus	Methylo bacilli	Methylo bacilli