Application of Enzymes and Microorganisms for Organic Synthesis

MCB 113

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Enzymes as catalyst for organic synthesis

- Single steps in organic synthesis can be accomplished using enzymes
- Preserves stereochemical centers, which can be important for drugs
- Eliminates the need for protection/deprotection
- Can be done in an aqueous environment – green chemistry

Types of enzymes

- Oxidoreductases
  - Remove H from (oxidize) a donor molecule and add H to (reduce) an acceptor molecule

$$-CH_2OH \leftrightarrow -CH=O, \quad >CHOH \leftrightarrow -C=O$$

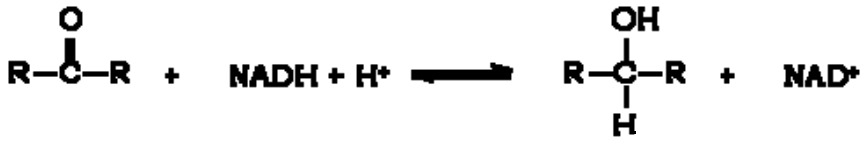
- Transferases
  - Catalyze the transfer of groups (i.e., acyl or phosphoryl) from one molecule to another

Types of enzymes (continued)

- Hydrolases
  - Cleave a bond by adding the atoms from a water molecules across it.
- Lyases
  - Remove a group of atoms (i.e., CO or HOH) from a substrate, leaving a double bond in its place.
  - They can also add a group of atoms to a double bond.
- Isomerases
  - Change the configuration of atoms within a molecule.
- Ligases
  - Catalyze the joining of two molecules at the expense of the cleavage of a pyrophosphate bond in ATP

Some examples of oxidoreductases

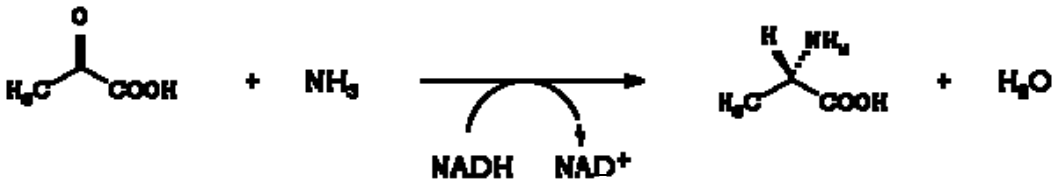
Alcohol Dehydrogenase (EC 1.1.1.1) from *Candida parapsilosis*



Carbonyl reductase. Various carbonyl com-pounds can be reduced stereospecifically to the corresponding (S)-alcohols by using NADH.

Oxidoreductases (continued)

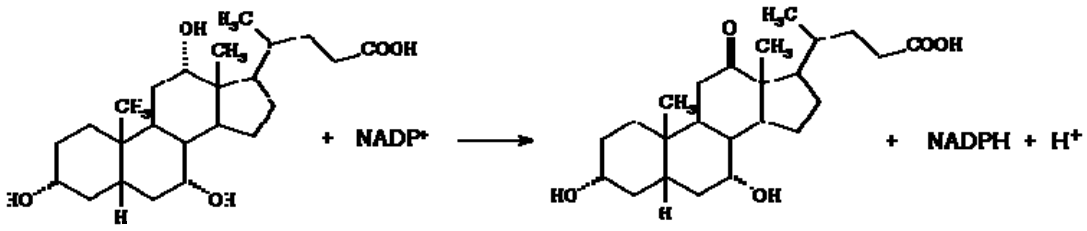
L-Alanine Dehydrogenase EC 1.4.1.1 from *Bacillus cereus*



L-Alanine:NAD^+ oxidoreductase (deaminating)

Oxidoreductases (continued)

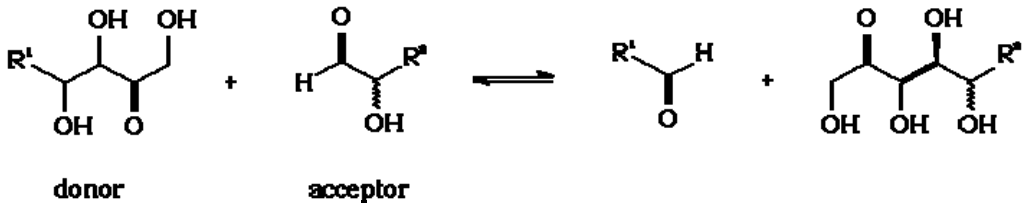
12-Alpha-Hydroxysteroid Dehydrogenase PP
EC 1.1.1.176 from *Clostridium* spec.



Catalyses the oxidation of the 12-alpha-hydroxyl group of bile acids, both in their free and conjugated form, also acts on bile alcohols.

Transferase example

Transaldolase EC 2.2.1.2 from *E. coli* K12 (rec.)



Transfer of a dihydroxyacetone moiety derived from a donor substrate to an acceptor substrate.

Hydrolytic enzymes

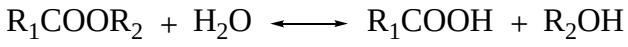
- Important in macroscopic degradation (food spoilage, starch thinning, waste treatment) and chemistry (fruit ripening, autolysis of cells, cheese curing)
- Three basic groups of hydrolytic enzymes:
  - Ester hydrolysis (esterases)
  - Glycosidic bond hydrolysis (carbohydrases)
  - Nitrogen bond hydrolysis

Examples

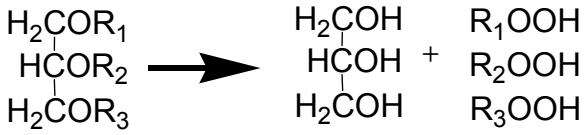
- Esterases
  - Lipases: hydrolyze fats
- Carbohydrases
  - Amylases: hydrolyze starch
  - Cellulases: hydrolyze cellulose
- Nitrogen bond hydrolases
  - Proteases: hydrolyze proteins
  - Ureases: Urea -> CO_2 + NH_3

Esterases

- Cleavage of ester bonds to yield an acid and an alcohol:



- Lipases are the most important subgroup. Lipases cleave fats into glycerol and fatty acids:

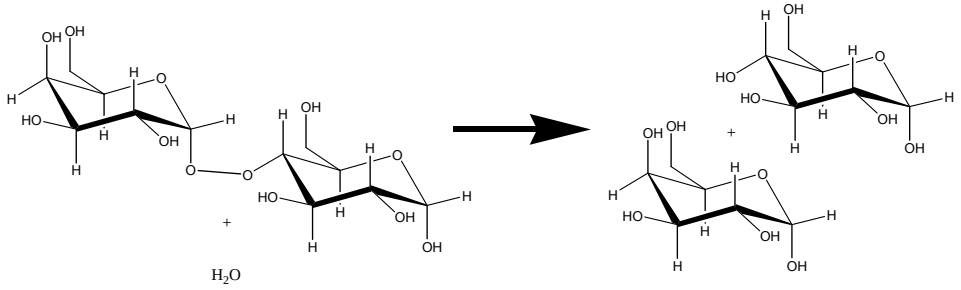


Esterases (continued)

- Lipases are most active against high MW fats and least active against low MW fats
- The activity of lipases is increased by the addition of surfactants
- Can be used in production of fat-free meats and other foods

Hydrolysis of starch and cellulose

- Amylases hydrolyze glycosidic bonds in starch and other glucose-containing compounds



- Amylases are also called starch-liquifying enzymes because they reduce the viscosity of the starch when they hydrolyze the bonds

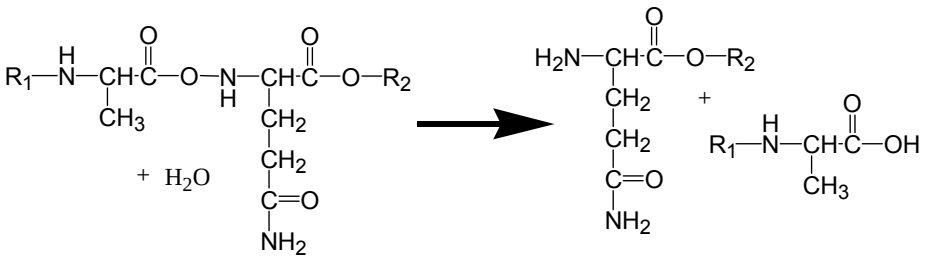
Applications of amylases

- Hydrolysis of corn starch to make syrup
- Conversion of crushed grain starch to maltose for brewing
- Hydrolysis of insoluble starch in juice – reduces turbidity
- Liquify starch coating in paper
- Production of candy with the desired softness

Proteolytic enzymes

- Exopeptidases: cleave terminal groups from polypeptide
- Endopeptidases: cleave internal linkages
- Some proteolytic enzymes are autocatalytic: the enzyme activated after it is cleaved
  - Pepsin is produced in its inactive pepsinogen form
  - Pepsinogen is cleaved by pepsin to produce pepsin

Peptidases



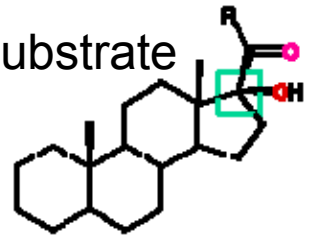
Hydrolase examples

Tannase EC 3.1.1.20 from *Aspergillus ficuum*

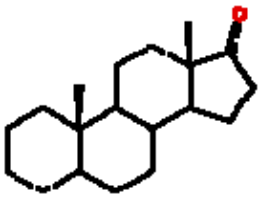
Tannin acylhydrolase, hydrolyzes polymeric gallate into gallate and alcohol or glucose; also hydrolyzes ester links in other tannins.

Lyase example

Substrate



NADPH
steroid 17ά-hydroxylase/17,20-lyase



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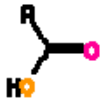
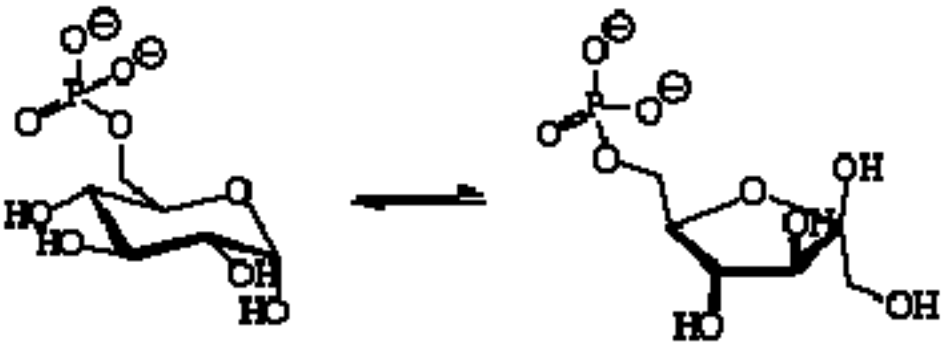


Product

Carboxylic acid

Isomerase example

Glucose 6-phosphate isomerase



glucose 6-phosphate

fructose 6-phosphate

Isomerase example

Triosephosphate isomerase

Dihydroxyacetone phosphate (DHAP)

Glyceraldehyde 3-phosphate (G3P)

Single-step biotransformations

- Transformation of steroids and sterols
- Semi-synthetic penicillin
- Synthesis of optically pure molecules

Transformation of sterols & steroids

- Steroids are used extensively
  - –to treat arthritis
  - –to treat allergies and other inflammatory diseases
  - –for contraception
  - –for hormonal insufficiencies
- Cortisone is used to treat arthritis
  - –Chemical synthesis requires 31 steps with extremely low yield ($200/g)

Microbial transformation of steroids

- Primarily hydroxylations and dehydrogenations
- Hydroxylations can occur on any number of C atoms
- Microbes include
  - *Penicillium* sp.
  - *Xylaria* sp.
  - *Diaporthe celastrinia*
  - *Rhizopus* sp.
- Microbial transformation reduced the cost to $0.46/g

Some sterol transformations

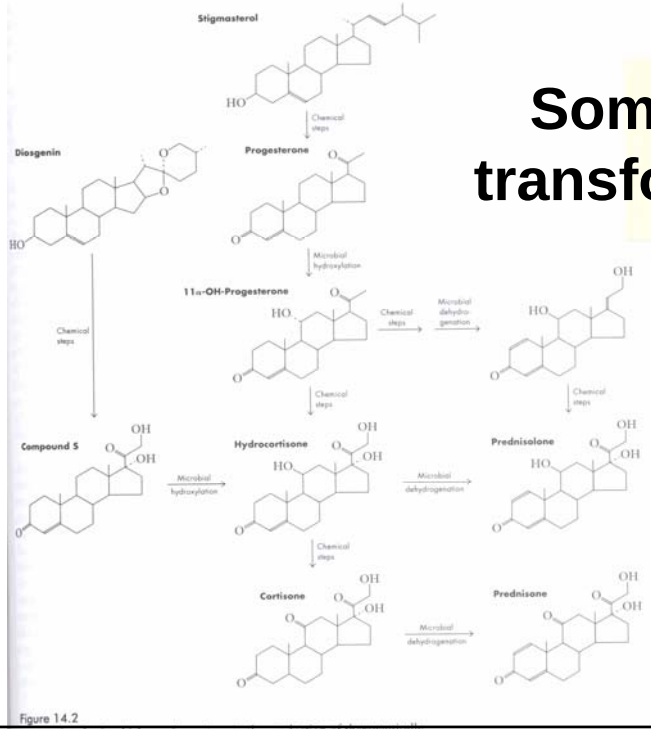


Figure 14.2

Semisynthetic penicillins

- Penicillins and cephalosporins are antibacterial agents
- Worldwide sales of $13B in 1988
- Because of the emergence of antibiotic resistant strains of bacteria, new antibiotics are required
- Derivatization of existing antibiotics to produce new antibiotics
- Ampicillin
  - Uses Penicillin G amidase, L-specific aminopeptidase, and Penicillin G acylase

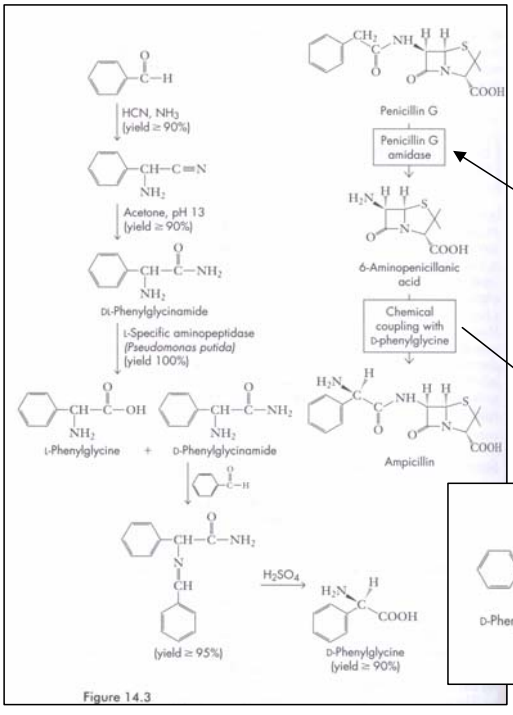


Figure 14.3

Biosynthesis of ampicillin

Immobilized enzyme
(see later)

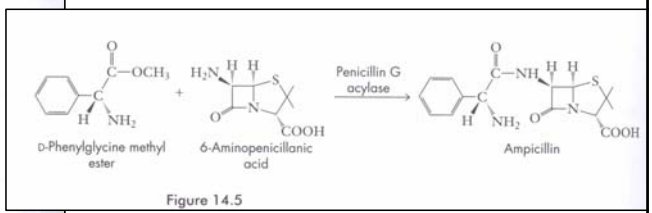


Figure 14.5

Synthesis of optically pure drugs

- Chiral synthons: optically active building blocks
- Difficult to produce using chemical synthesis
- Example of drug:
  - –Atenolol is a drug used to treat hypertension
  - –*Nocardia, Rhodococcus, Corynebacterium,* or *Mycobacterium* is used to resolve the racemic mixture of a key precursor

Biosynthesis of Atenolol

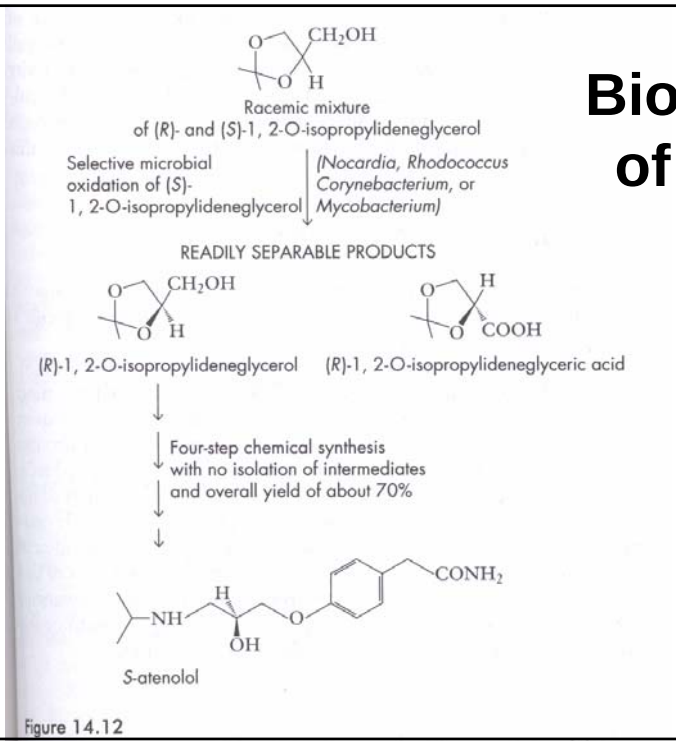


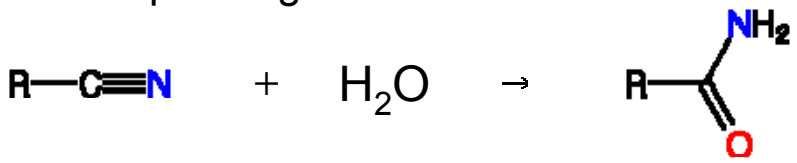
Figure 14.12

Biosynthesis of commodity chemicals

- Acrylamide
  - a building block of polymers used in petroleum recovery or as flocculants or additive in various products.
  - 200,000 tons produced worldwide each year
  - Chemical synthesis requires use of copper salts to catalyze the hydration of acrylonitrile
  - Gives rise to undesirable side products (hydrocyanic acid)
  - Large amounts of salts are formed
  - Reaction must be carefully controlled to avoid polymerization

Acrylamide production

- Nitrile hydratases are mononuclear iron or (non-corrinoid) cobalt enzymes that catalyse the hydration of a large number of diverse nitriles to their corresponding amides



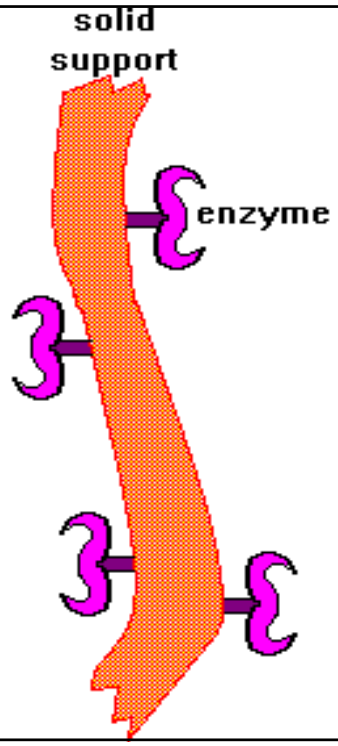
- Bacterial nitrile hydratase converts acrylonitrile to acrylamide
  - Avoids the use of copper salts and the production of by-products such as hydrocyanic acid

Immobilized enzyme technology

- Immobilization of an enzyme confines or localizes it
- Multiple or repetitive use of a single batch of enzymes
- The ability to stop the reaction rapidly by removing the enzyme from the reaction solution (or vice versa)
- Enzymes are usually stabilized by immobilization
- Product is not contaminated with the enzyme (especially useful in the food and pharmaceutical industries)
- Analytical purposes - long half-life, predictable decay rates, elimination of reagent preparation, etc.

Methods of immobilization

- **Carrier-Binding**: the binding of enzymes to water-insoluble carriers.
- **Cross-Linking** : intermolecular cross-linking of enzymes by bi-functional or multi-functional reagents.
- **Entrapping** : incorporating enzymes into the lattices of a semi-permeable gel or enclosing the enzymes in a semi-permeable polymer membrane.



Carrier binding

- most commonly used carriers for enzyme immobilization are polysaccharide derivatives such as cellulose, dextran, agarose, and polyacrylamide gel.
- the carrier-binding method can be further sub-classified into:
  - **Physical Adsorption**
  - **Ionic Binding**
  - **Covalent Binding**

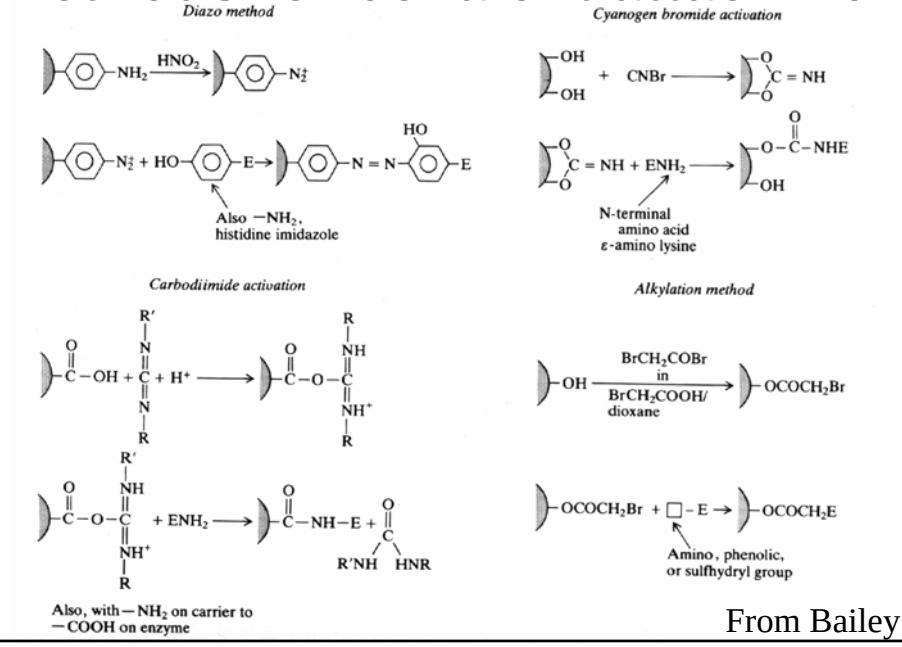
Carriers used for physical adsorption

- Activated carbon
- Silica gel
- Alumina
- Starch
- Clay
- Glass
- Modified cellulose
- Modified sepharose

Carriers for ionic binding

- Cation exchangers
  - CM-cellulose
  - Aberlite
  - Dowex 50
- Anion exchangers
  - DEAE-cellulose
  - DEAE-sephadex
  - polyaminopolystyrene

Methods for covalent attachment



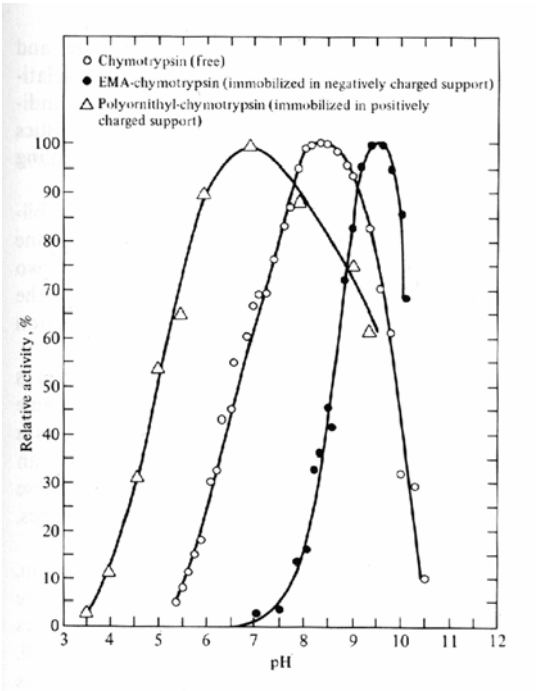
From Bailey. 1986

Carriers for covalent attachment

- Natural supports
  - Cellulose (-OH)
  - CM-cellulose (-COOH)
  - Agarose (-OH)
  - Dextran (-OH)
- Synthetic supports
  - Polyacrylamide derivatives
  - Polyaminopolystyrene (-NH_2)

Protection of enzyme during covalent attachment

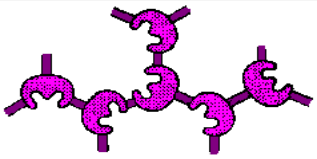
- Covalent attachment to a support matrix must involve only functional groups of the enzyme that are not essential for catalytic action.
- A number of protective methods have been devised:
  - Covalent attachment of the enzyme in the presence of a competitive inhibitor or substrate.
  - A reversible, covalently linked enzyme-inhibitor complex.
  - A chemically modified soluble enzyme whose covalent linkage to the matrix is achieved by newly incorporated residues.



Immobilization of enzymes can change their characteristics

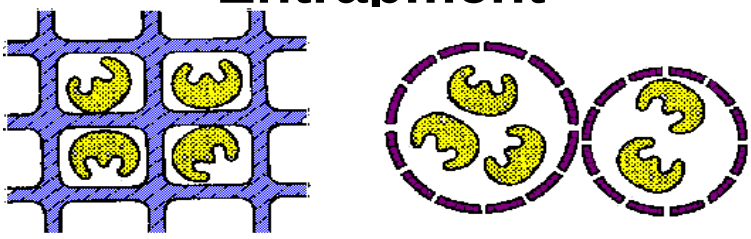
From Bailey. 1986

Cross-linking enzymes



- Immobilization of enzymes has been achieved by intermolecular cross-linking of the protein, either to other protein molecules or to functional groups on an insoluble support matrix.
- Cross-linking an enzyme to itself is both expensive and insufficient, as some of the protein material will inevitably be acting mainly as a support.
- Cross-linking is best used in conjunction with one of the other methods.
- The most common reagent used for cross-linking is glutaraldehyde.

Entrapment



entrapped in a matrix

entrapped in droplets

The entrapment method of immobilization is based on the localization of an enzyme within the lattice of a polymer matrix or membrane.

It is done in such a way as to retain protein while allowing penetration of substrate. It can be classified into **lattice** and **micro capsule** types.

Entrapment methods

- **Lattice-Type** entrapment involves entrapping enzymes within the interstitial spaces of a cross-linked water-insoluble polymer. Some synthetic polymers such as *polyarylamide, polyvinylalcohol, etc...* and natural polymer (starch) have been used to immobilize enzymes using this technique.
- **Microcapsule-Type** entrapping involves enclosing the enzymes within semi permeable polymer membranes. The preparation of enzyme micro capsules requires extremely well-controlled conditions.

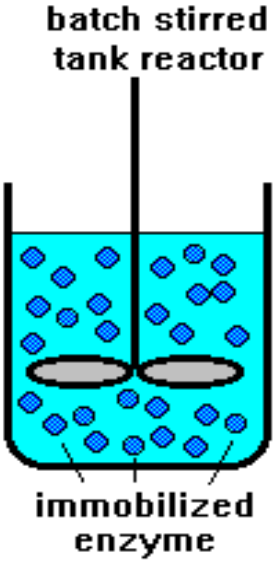
Forms of immobilized enzymes

- Most immobilized enzymes are in particle form for ease of handling and ease of application.
  - **Particles -** The particle form is described in the above section.
  - **Membranes** - Enzyme membranes can be prepared by attaching enzymes to membrane-type carriers, or by molding into membrane form. The molding is done after the enzymes have been enclosed within semi-permeate membranes of polymer by entrapment.
  - **Tubes** - Enzyme tubes are produced using Nylon and polyacrylamide tubes as carriers. The polymer tube is first treated in a series of chemical reactions and the enzyme is bound by diazo coupling to give a tube in a final step.
  - **Fibers** - Enzymes that have been immobilized by entrapment in fibers to form enzyme fibers.

Characteristics of enzyme immobilization methods

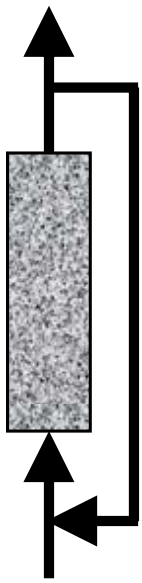
Characteristic	Physical adsorption	Ionic binding	Covalent binding	Cross-linking method	Entrapping method
Preparation	Easy	Easy	Difficult	Difficult	Difficult
Enzyme activity	Low	High	High	Moderate	High
Substrate specificity	Un-change-able	Unchange-able	Changeable	Changeable	Unchange-able
Binding force	Weak	Moderate	Strong	Strong	Strong
Regeneration	Possible	Possible	Impossible	Impossible	Impossible
General applicability	Low	Moderate	Moderate	Low	High
Cost of immobilization	Low	Low	High	Moderate	Low

Immobilized enzyme bioreactors



- Batch stirred tank reactor
  - the simplest type
  - useful for substrate solutions of high viscosity and for immobilized enzymes with relatively low activity.
  - immobilized enzyme tends to decompose on physical stirring

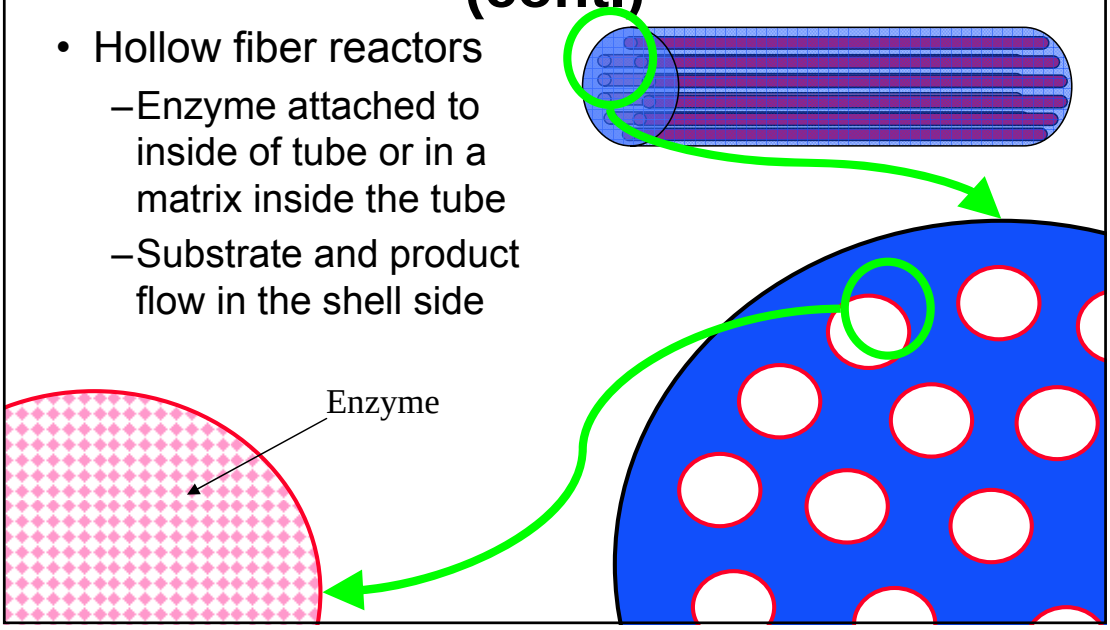
Immobilized enzyme reactors (cont.)



- Packed bed reactors
  - the most widely used reactors for immobilized enzymes
  - it is necessary to consider the pressure drop across the packed bed or column, and the effect of the column dimensions on the reaction rate
  - three substrate flow possibilities in a packed bed:
    1. Downward flow method
    2. Upward flow method
    3. Recycling method

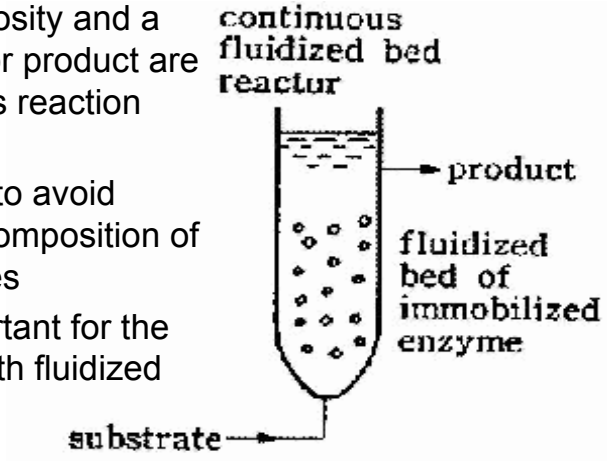
Immobilized enzyme reactors (cont.)

- Hollow fiber reactors
  - Enzyme attached to inside of tube or in a matrix inside the tube
  - Substrate and product flow in the shell side



Immobilized enzyme reactors (cont.)

- Fluidized bed reactor
  - most suitable when a substrate solution of high viscosity and a gaseous substrate or product are used in a continuous reaction system
  - care must be taken to avoid destruction and decomposition of immobilized enzymes
  - particle size is important for the formation of a smooth fluidized bed



Immobilized enzymes currently in use or being considered

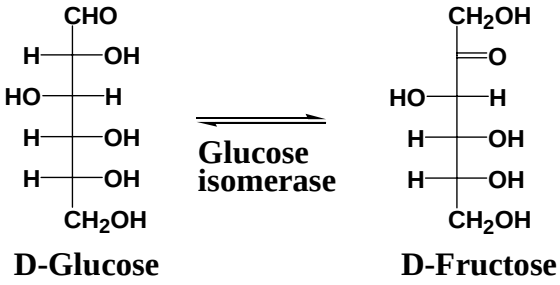
Enzyme	Application
Glucose isomerase	Isomerization of glucose to fructose (high fructose corn syrup; 8 million tons)
Aminoacylase	Optical resolution of DL-amino acids
B-galactosidase	Hydrolysis of lactose to galactose and glucose (treatment of milk and whey)
Lipase	Interesterification of fats
Nitrile hydratase	Production of acrylamide from acrylonitrile; 15,000 tons

Immobilized enzyme electrodes

Analyte	Enzyme	Sensor	Immobilization procedure
Galactose	Galactose oxidase	H_2O_2	Covalently bound on collagen membrane
Ethanol	Alcohol dehydrogenase	Pt (+350 mV)/$FeCN_6^{4-}$	Glutaraldehyde/ cellulose triacetate
Urea	Urease	O_2	Entrapped; dialysis membrane
Lactose	β-galactosidease + glucose oxidase	O_2	Glutaraldehyde and BSA; magnetic film

Case 1: Industrial processes: Glucose isomerase

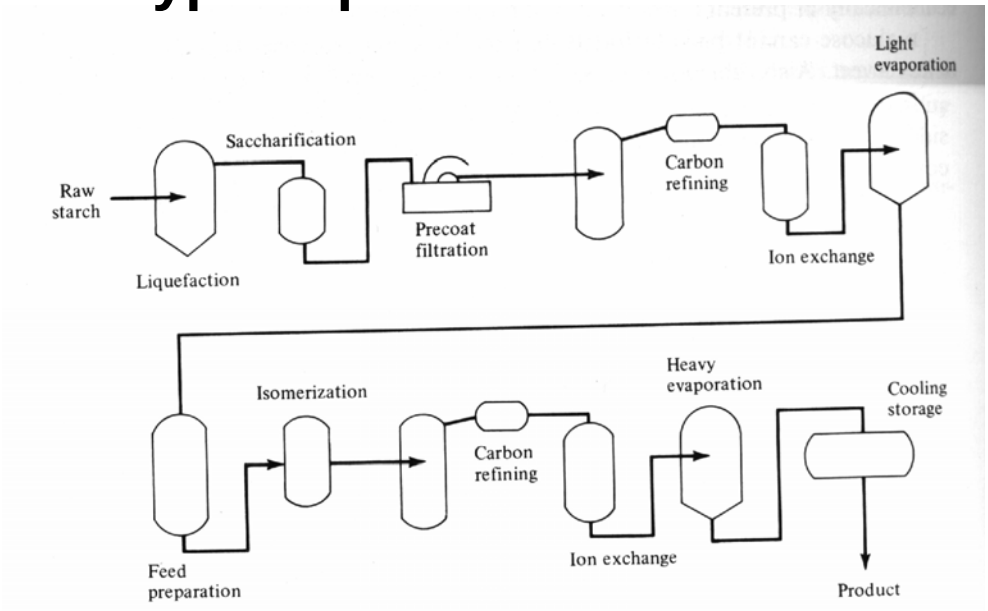
- D-glucose cannot be substituted directly for sugar because glucose is less sweet
- Glucose crystallization is difficult
- One can alleviate the problems by isomerizing glucose to fructose



Glucose isomerase

- Equilibrium constant = 1 at 50°C
  - 1:1 D-glucose:D-fructose is a good substitute for sugar
- Produced by a number of organisms: *Arthrobacter* and *Streptomyces*
- Must disrupt cells to get the enzyme out
- Sensitive to many inhibitors
- Immobilization would allow re-use of the enzyme

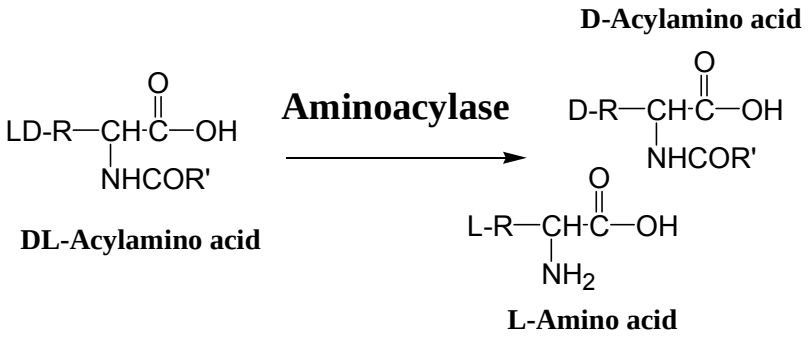
Typical process flow sheet



Case 2: Acylation of D-amino acids

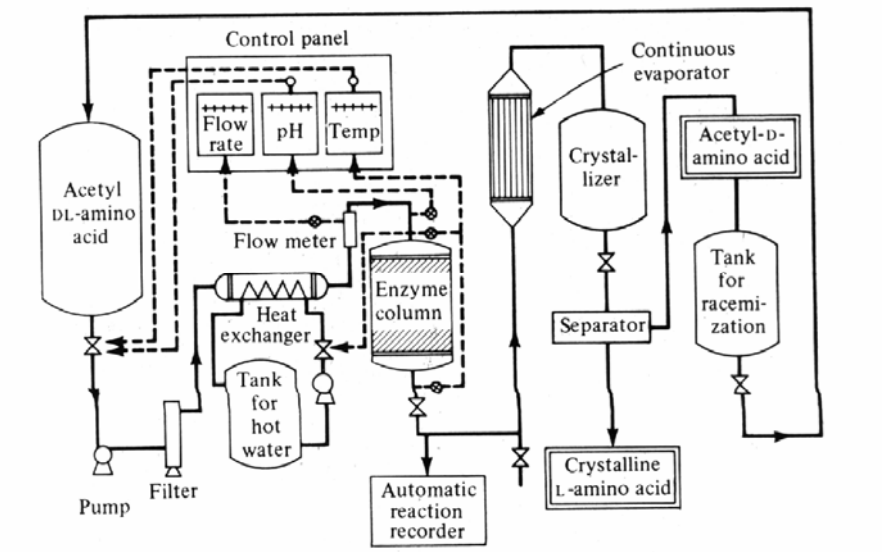
- Demand for L-amino acids for food and medical applications is growing
- Chemical synthesis of amino acids results in racemic mixtures (D- and L-forms)
- The D isomer is generally of no nutritive value

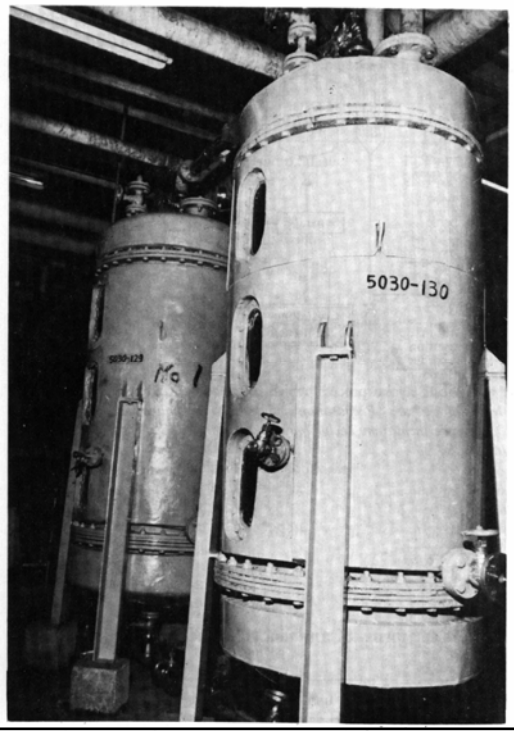
Resolution of racemic amino acid mixtures with aminoacylase



- Following the hydrolysis of the L-acylamino acid, the L-amino acid is separated from the D-acylamino acid because of solubility issues

Flowsheet for aminoacylation

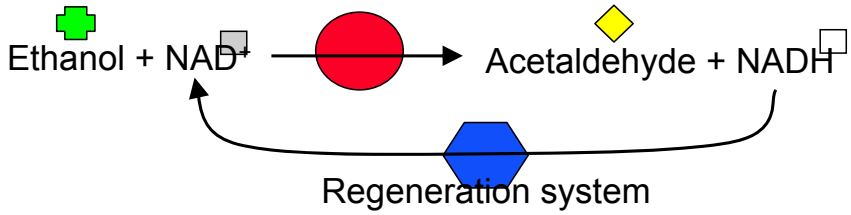




Immobilized enzyme columns for L-amino acid production

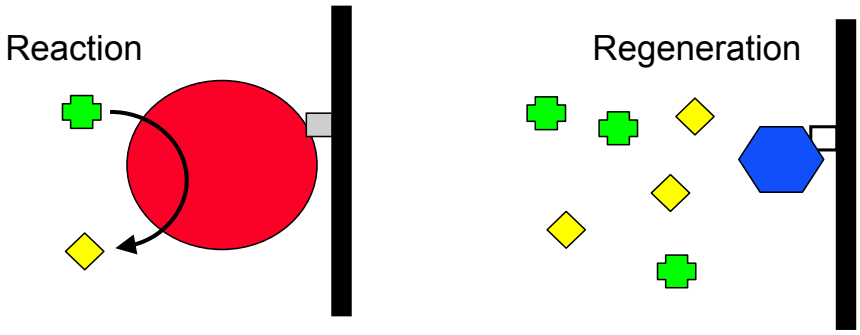
Utilization and regeneration of cofactors

- Many enzymes need cofactors for activity
  - NAD/NADH
  - FAD/FADH
- Need a method to retain/regenerate cofactors
  - Cofactors can be extremely expensive



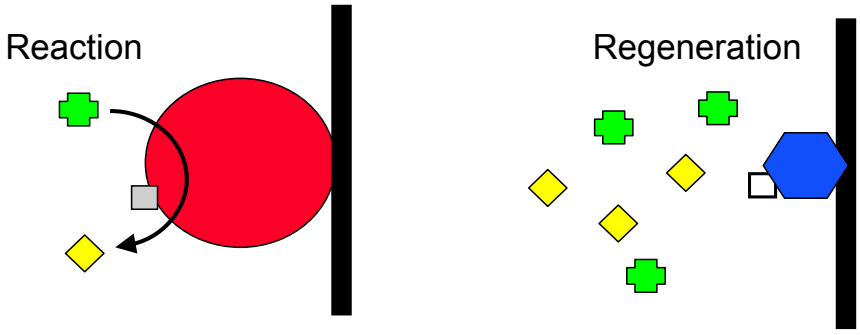
Three approaches

- Immobilize the coenzyme
  - Put enzyme and all necessary substances for regeneration in solution
  - Everything except coenzyme will be contained in the reactor effluent



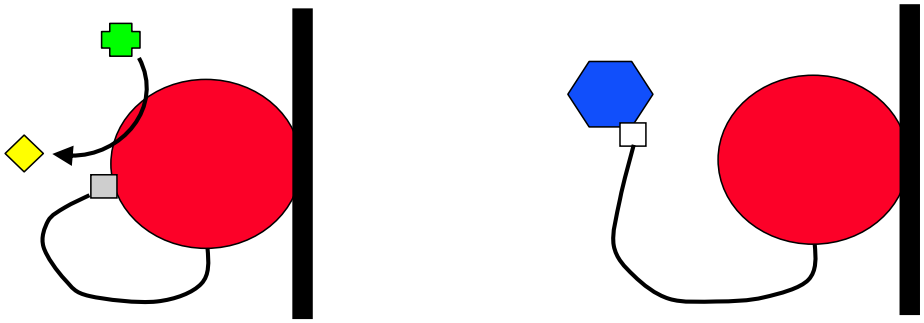
Three approaches (cont.)

- Immobilize enzyme
  - Put coenzyme and all necessary substances for regeneration in solution
  - Regeneration occurs in a separate reactor



Three approaches (cont.)

- Link the enzyme and coenzyme to each other using a long, flexible molecule (coenzyme-on-a-string)
  - Enzyme/coenzyme complex are immobilized
  - Not yet possible



Immobilized enzyme kinetics