Production of metabolites and Enzymes

I. Industrial Microorganisms and Product Formation

- Industrial Microorganisms and Their Products
- Primary and Secondary Metabolites
- Characteristics of Large-Scale Fermentations
- Scale-Up of Industrial Fermentations

Industrial Microbiology

- *Industrial Microbiology*
  - Uses microorganisms, typically grown on a large scale, to produce products or carry out chemical transformation
  - Originated with alcoholic fermentation processes
    - Later on, processes such as production of pharmaceuticals, food additives, enzymes, and chemicals were developed
  - Major organisms used are fungi and *Streptomyces*
  - Classic methods are used to select for high-yielding microbial variants

Industrial Microorganisms

- Properties of a useful industrial microbe include
  - Can be easily inoculated
  - Grows rapidly on a large scale in inexpensive medium
  - Produces desired product quickly
  - Should not be pathogenic
  - Amenable to genetic manipulation

Microbial Products

- Microbial products of industrial interest include
  - Microbial cells
  - Enzymes
  - Antibiotics, steroids, alkaloids
  - Food additives
  - *Commodity chemicals*
    - Inexpensive chemicals produced in bulk
    - Include ethanol, citric acid, and many others

Products of Industrial Microbiology

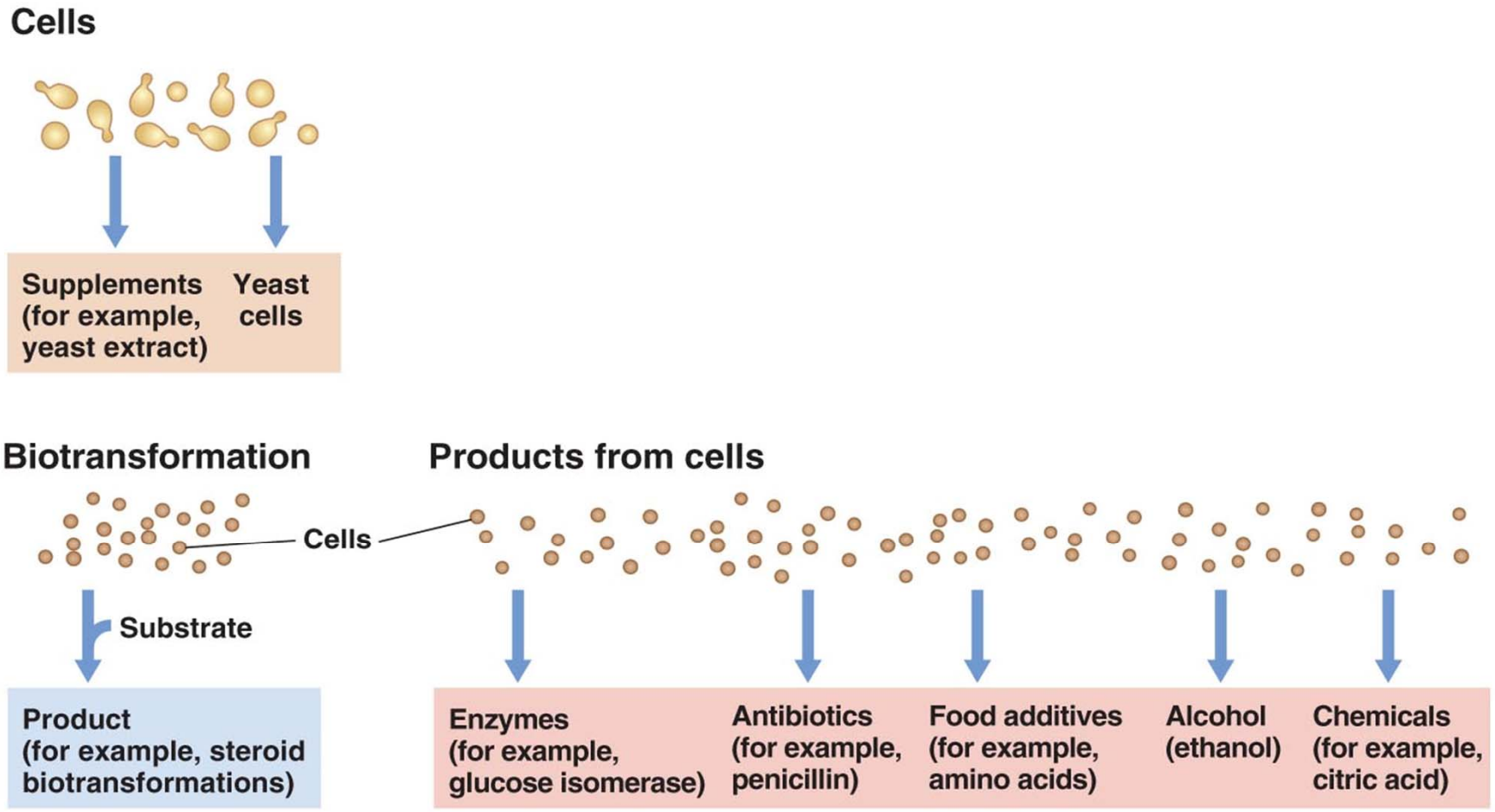
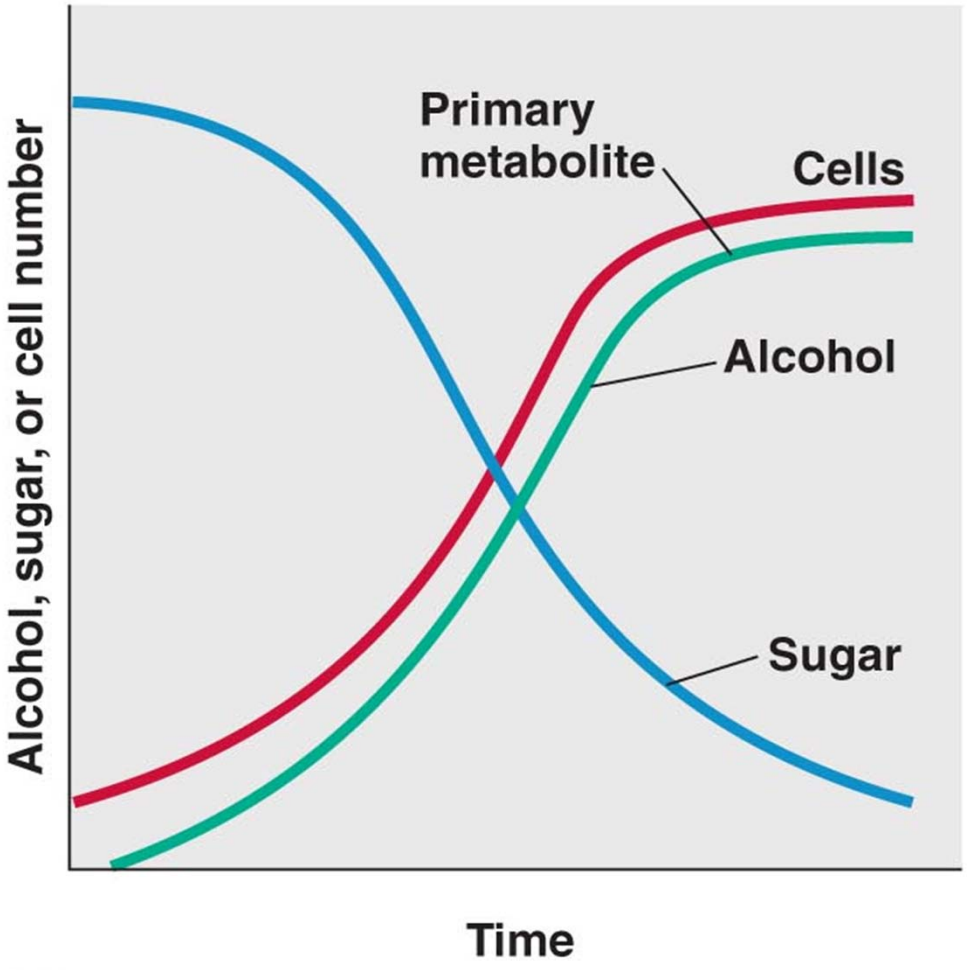


Figure 25.1

Primary and Secondary Metabolites

- *Primary Metabolite*
  - Produced during exponential growth
  - E.g., alcohol
- *Secondary Metabolite*
  - Produced during stationary phase
  - Not essential for growth
  - Formation depends on growth conditions
  - Produced as a group of related compounds

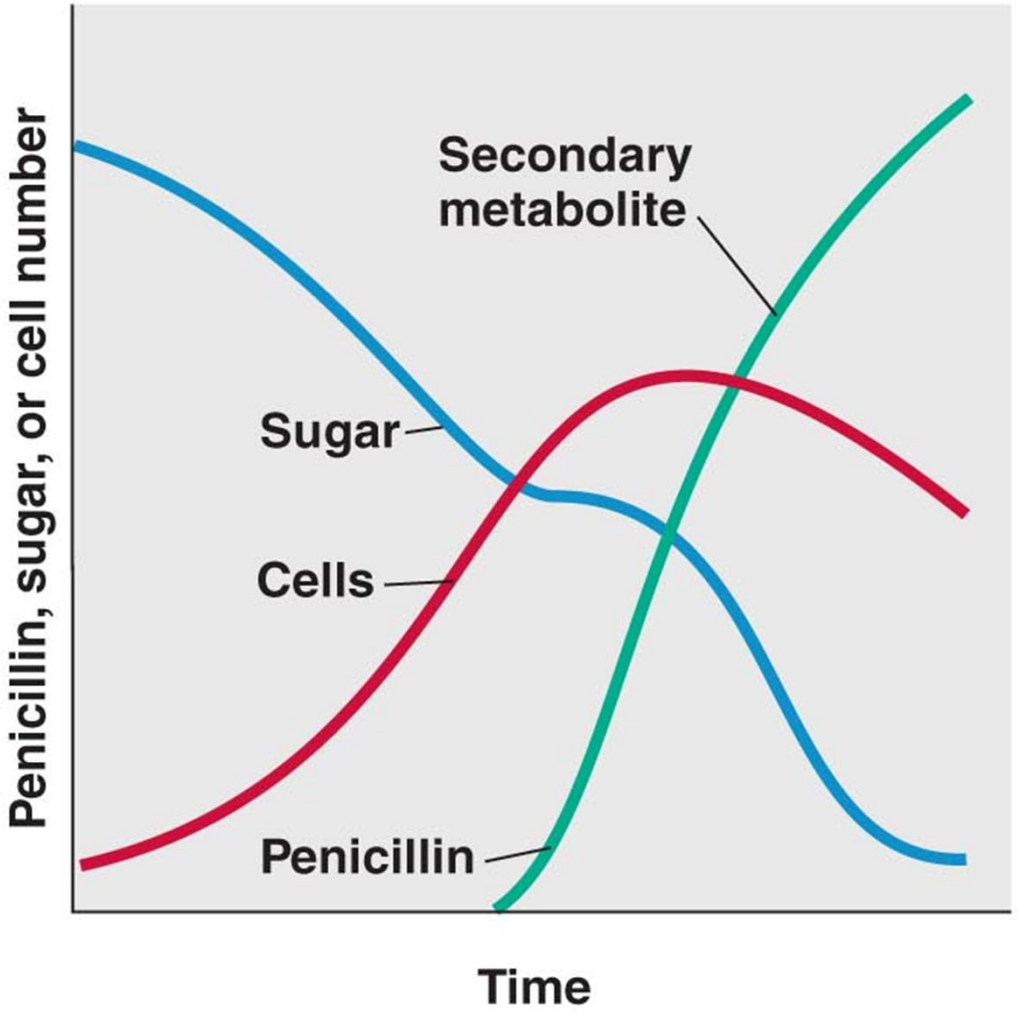
Primary Metabolite Production



(a) Formation of Alcohol by Yeast

Figure 25.2a

Secondary Metabolite Production



(b) Penicilin Production by the Mold *Penicillium Chrysogenum*

Figure 25.2b

Secondary Metabolites

- Secondary metabolites are often large organic molecules that require a large number of specific enzymatic steps for production
  - Synthesis of tetracycline requires at least 72 separate enzymatic steps
  - Starting materials arise from major biosynthetic pathways

Aromatic Antibiotics

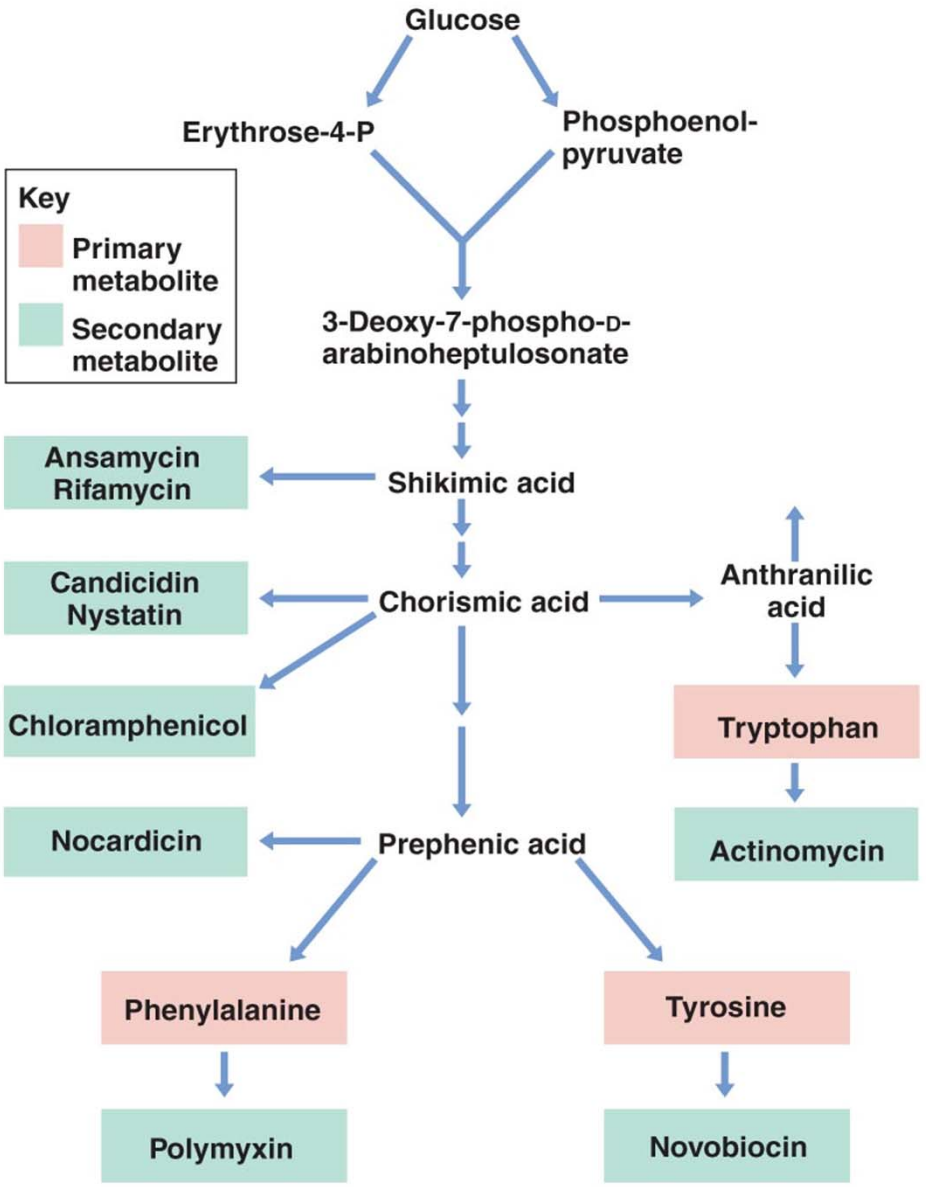


Figure 25.3

Characteristics of Large-Scale Fermentations

- *Fermentor* is the vessel where the microbiology process takes place
- Any large-scale reaction is referred to as a fermentation
  - Most are aerobic processes
- Fermentors vary in size from 5 to 500,000 liters
  - Aerobic and anaerobic fermentors
- Large-scale fermentors are almost always stainless steel
  - Impellers and spargers supply oxygen to the culture

Fermentor Sizes for Various Industrial Processes

Table 25.1 Fermentor sizes for various industrial processes

Size of fermentor (liters)	*Product*
1–20,000	Diagnostic enzymes, substances for molecular biology
40–80,000	Some enzymes, antibiotics
100–150,000	Penicillin, aminoglycoside antibiotics, proteases, amylases, steroid transformations, amino acids, wine, beer
200,000–500,000	Amino acids (glutamic acid), wine, beer

A Small Research Fermentor



Queue Systems, Inc.

(a)

Figure 25.4a

Diagram of a Fermentor

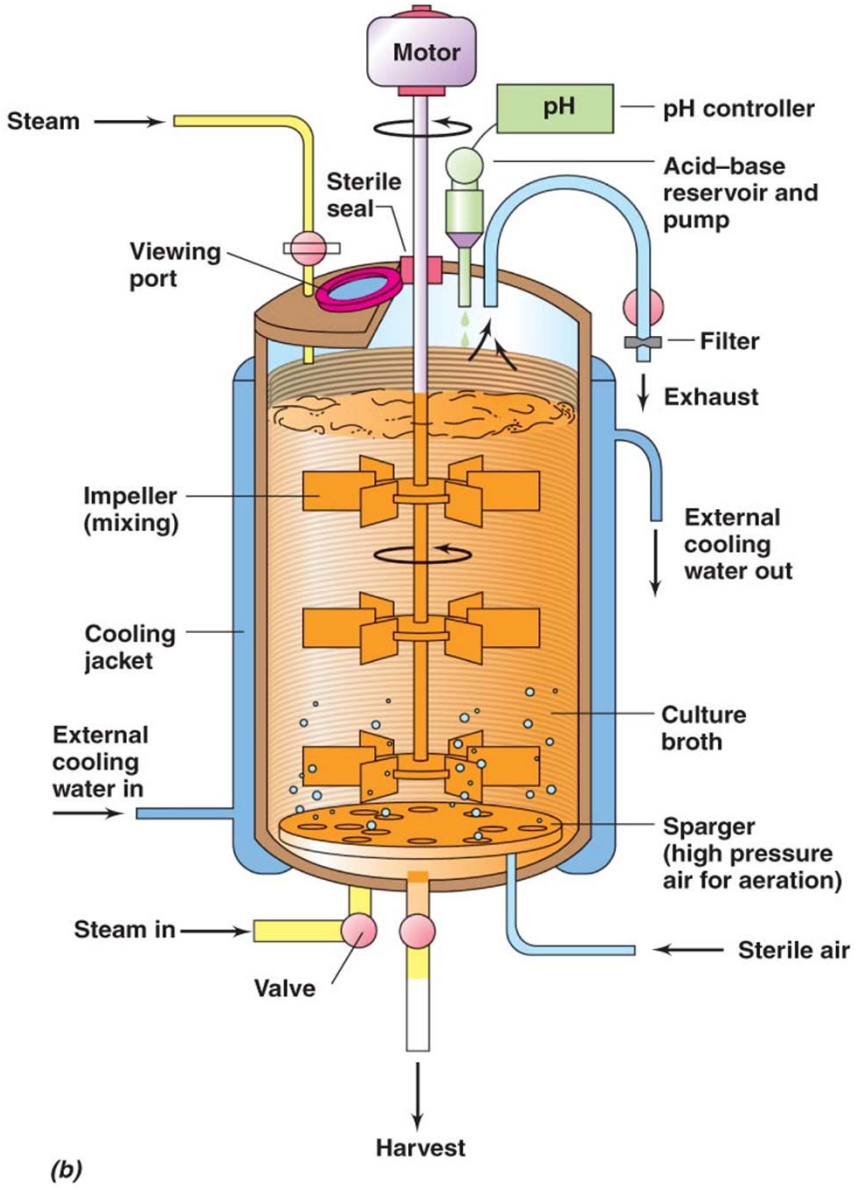
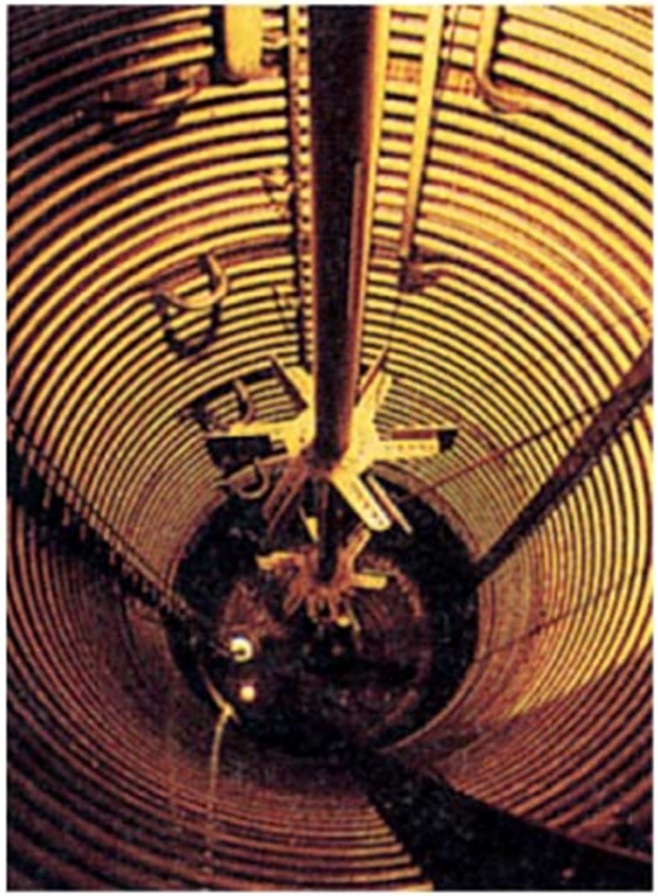


Figure 25.4b

The Inside of an Industrial Fermentor



Novo Nordisk

(c)

Figure 25.4c

Industrial Fermentors

- *Industrial Fermentors*
  - Must be closely monitored during production run
  - Growth and product formation must be measured
  - Environmental factors must be controlled and altered as needed
    - Including temperature, pH, cell mass, nutrients, and product concentration
  - Data on the process must be obtained in real time

Scale-Up of Industrial Fermentations

- *Scale-up*
  - The transfer of a process from a small laboratory scale to large-scale commercial equipment
  - Major task of the biochemical engineer
    - Requires knowledge of the biology of producing organism and the physics of fermentor design and operation
  - Many challenges in scale-up arise from aeration and mixing
- Flask → laboratory fermentor → pilot plant → commercial fermentor

II. Products for the Health Industry

- Antibiotics: Isolation and Characterization
- Industrial Production of Penicillins and Tetracyclines
- Vitamins and Amino Acids
- Steroids and Other Biotransformations
- Enzymes as Industrial Products

Antibiotics: Isolation and Characterization

- *Antibiotics*
  - Compounds that kill or inhibit the growth of other microbes
  - Typically secondary metabolites
  - Most antibiotics in clinical use are produced by filamentous fungi or actinomycetes
  - Still discovered by a laboratory screening process
    - Microbes are obtained from nature in pure culture
    - Assayed for products that inhibit growth of test bacteria

Isolation and Screening of Antibiotic Producers

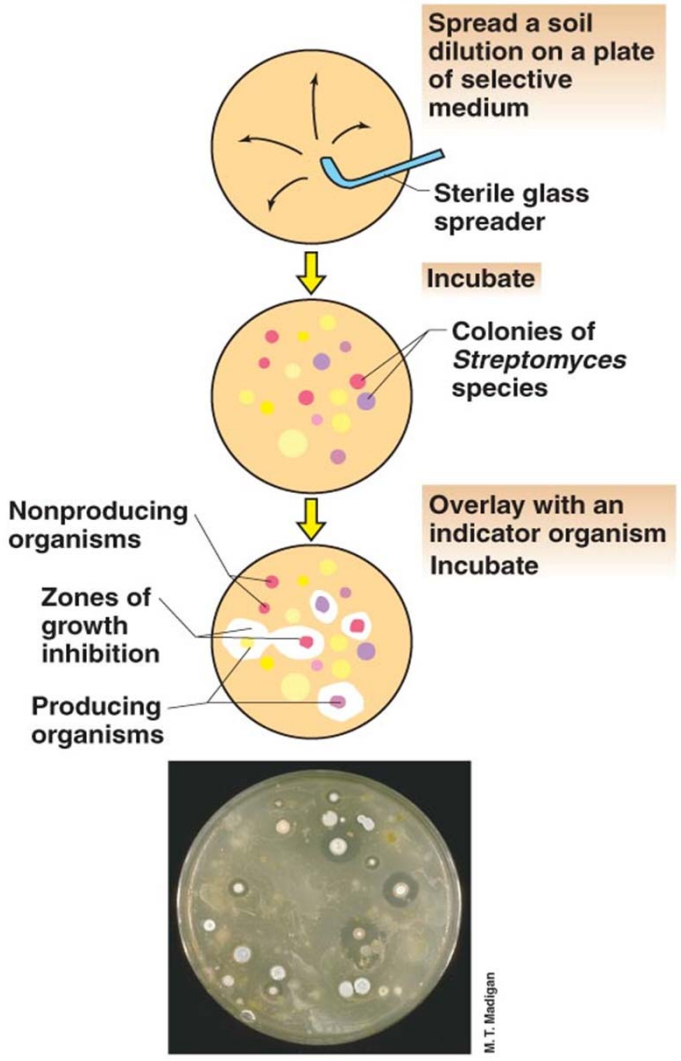


Figure 25.7a

Process of Extraction and Purification of an Antibiotic

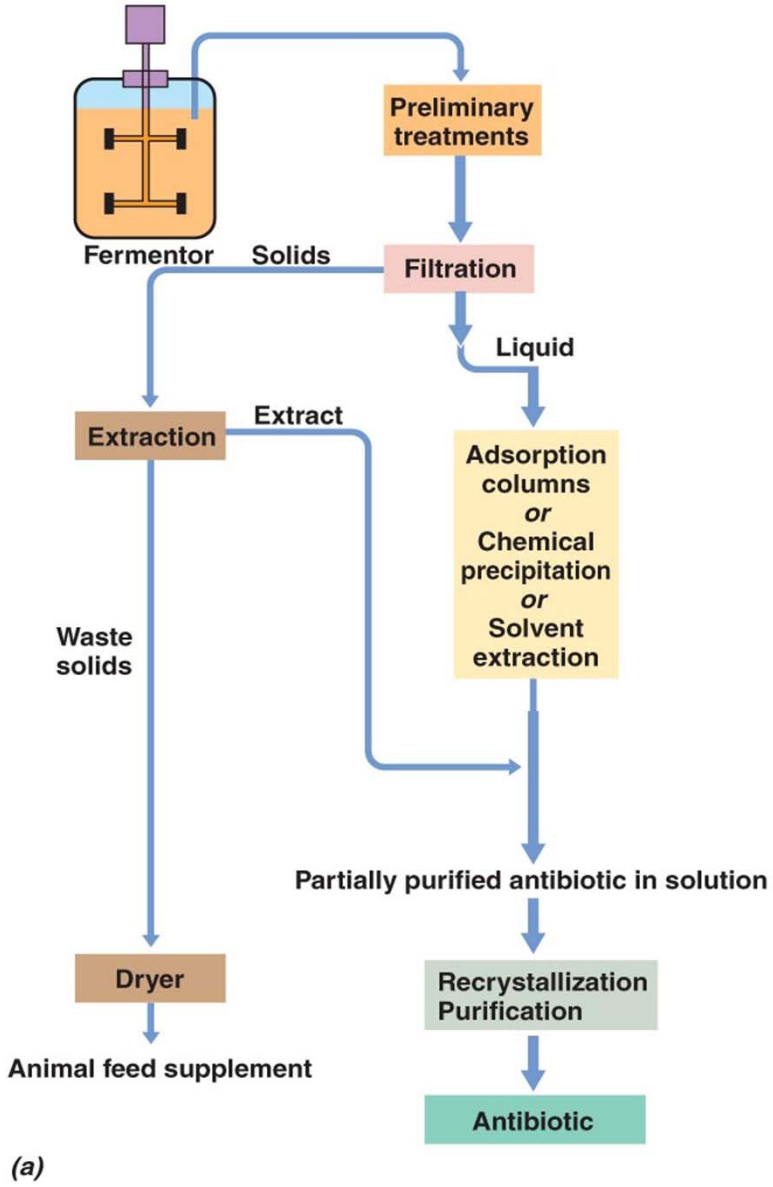


Figure 25.8a

Industrial Production of Penicillins

- *Penicillins are β-lactam antibiotics*
  - *Natural and biosynthetic penicillins*
  - *Semisynthetic penicillins*
    - *Broad spectrum* of activity
- Penicillin production is typical of a secondary metabolite
  - Production only begins after near-exhaustion of carbon sour
  - High levels of glucose repress penicillin production

Industrial Production of Penicillins

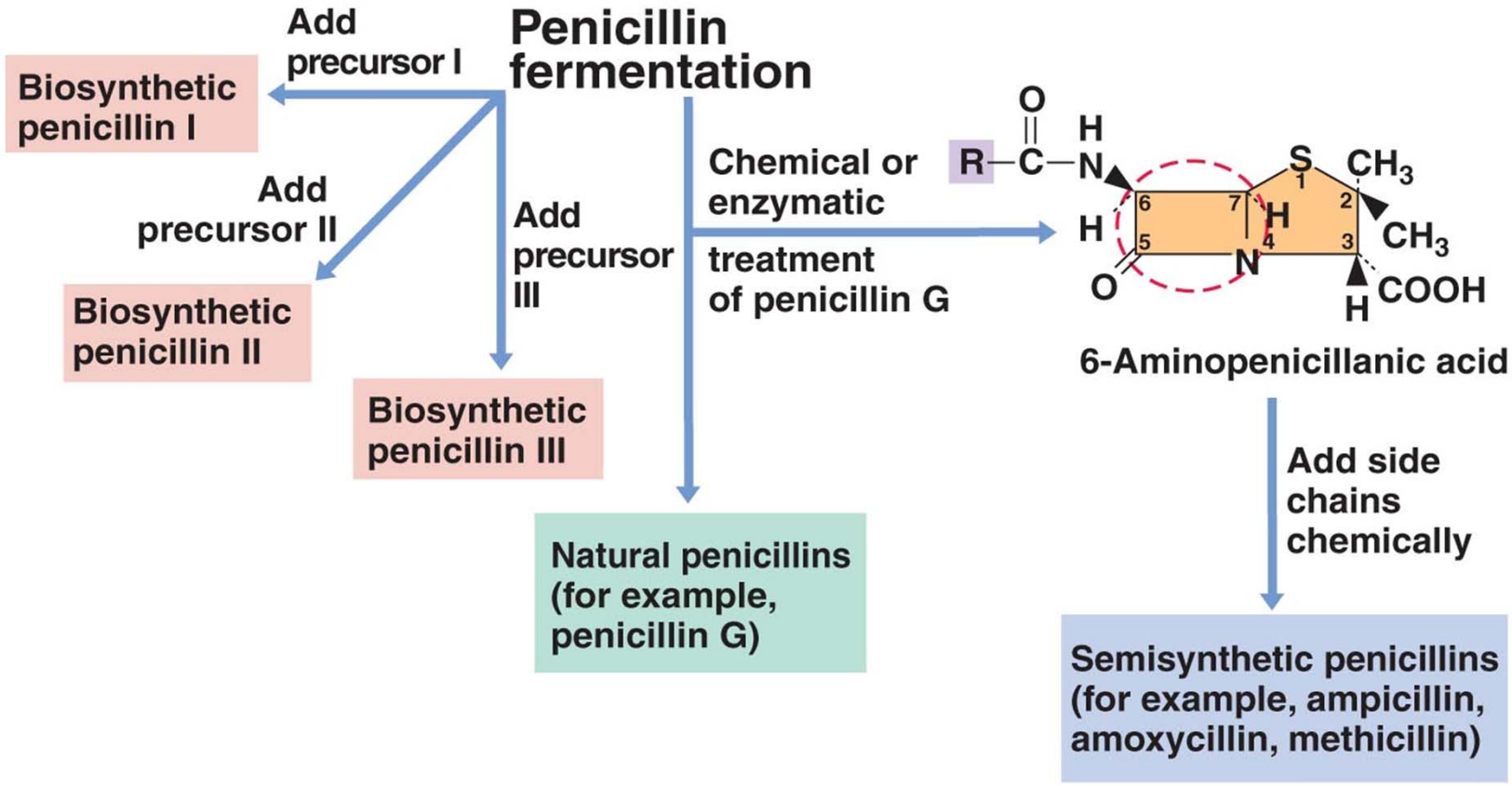


Figure 25.9

Kinetics of Penicillin Fermentation

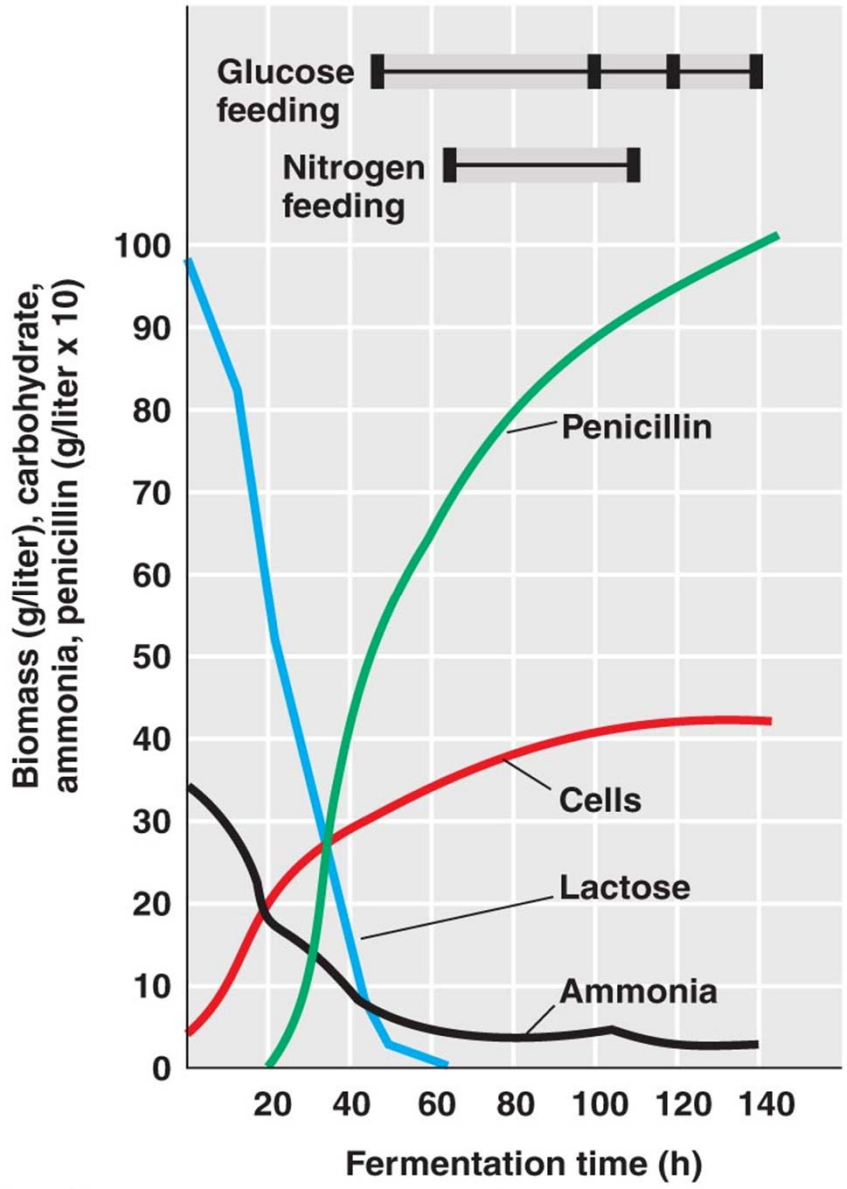


Figure 25.10

Vitamins

- Production of vitamins is second only to antibiotics in terms of total pharmaceutical sales
  - Vitamin B_{12} produced exclusively by microorganisms
    - Deficiency results in *pernicious anemia*
    - Cobalt is present in B_{12}
  - Riboflavin can also be produced by microbes

Vitamin B_{12} Produced by Microorganisms

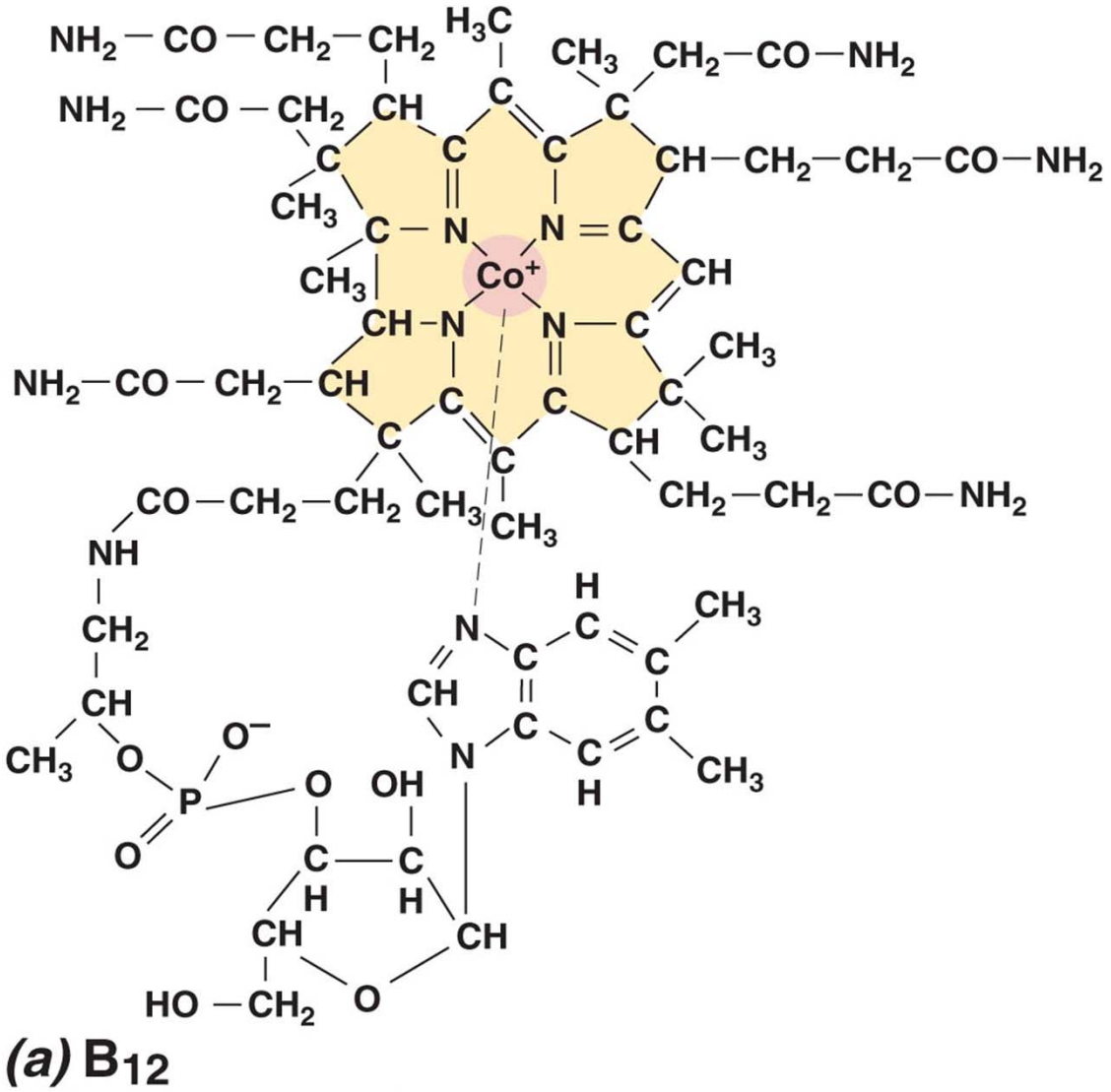
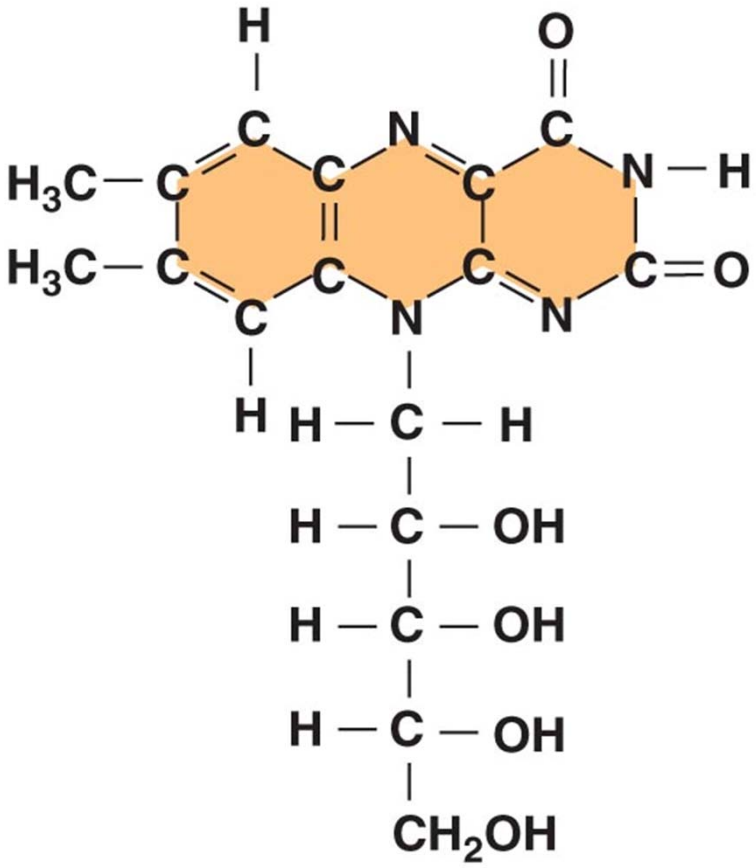


Figure 25.12a

Vitamin B_2 Produced by Microorganisms



***(b)* Riboflavin**

Figure 25.12b

Amino Acids

- *Amino Acids*
  - Used as feed additives in the food industry
  - Used as nutritional supplements in nutraceutical industry
  - Used as starting materials in the chemical industry
  - Examples include
    - Glutamic acid (MSG)
    - Aspartic acid and phenylalanine (aspartame (Nutrasweet))
    - Lysine (food additives)

Industrial Production of Lysine

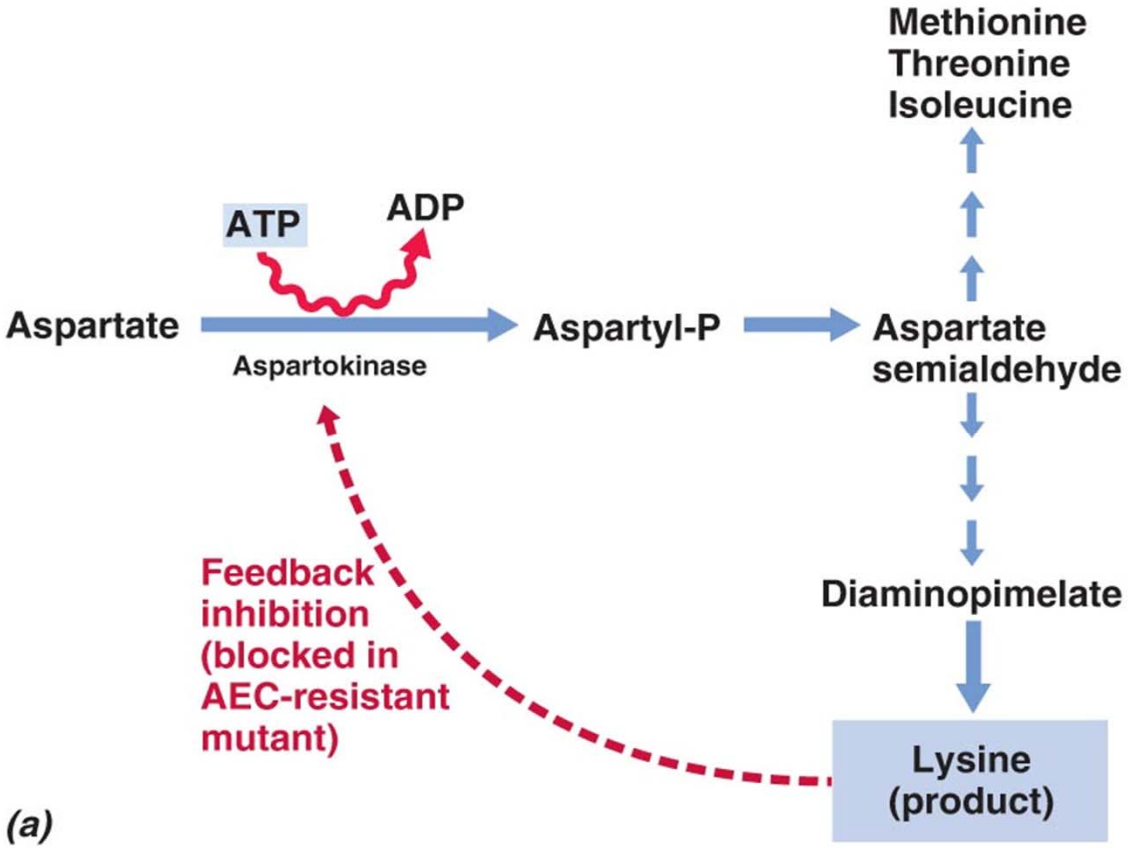


Figure 25.13a

Structure of Lysine and AEC

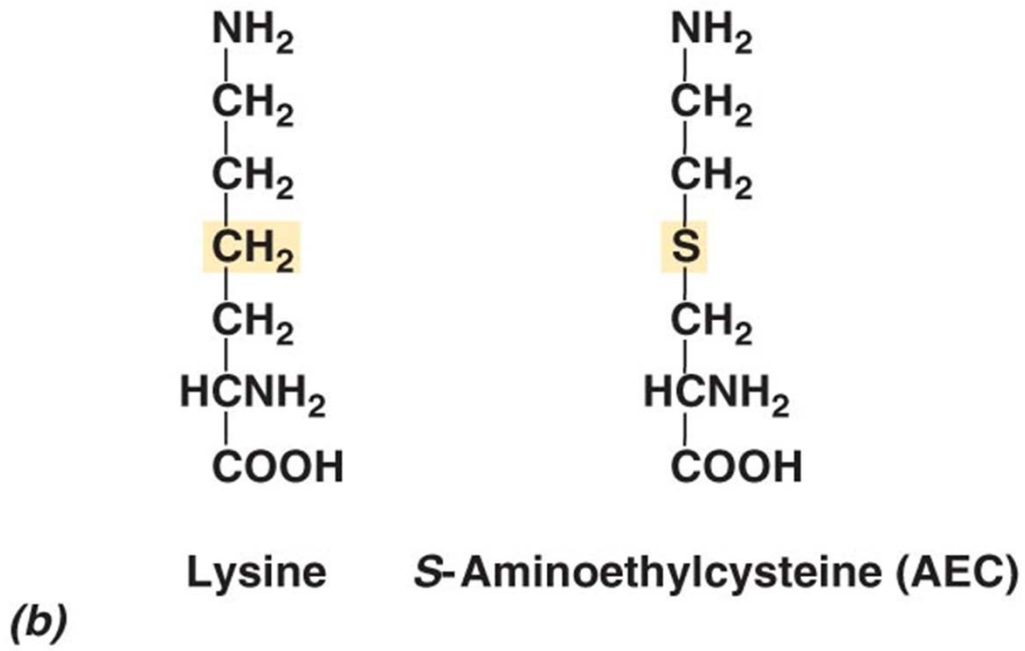


Figure 25.13b

Amino Acids Used in the Food Industry

Table 25.3 Amino acids used in the food industry[a]

Amino acid[b]	*Annual production worldwide (metric tons)*	*Uses*	*Purpose*
L-Glutamate (monosodium glutamate, MSG)	370,000	Various foods	Flavor enhancer; meat tenderizer
L-Aspartate and L-alanine	5,000	Fruit juices	"Round off" taste
Glycine	6,000	Sweetened foods	Improves flavor; starting point for organic syntheses
L-Cysteine	700	Bread Fruit juices	Improves quality Antioxidant
L-Tryptophan + L-Histidine	400	Various foods, dried milk	Antioxidant, prevent rancidity; nutritive additives
Aspartame (made from L-phenylalanine + L-aspartic acid)	7,000	Soft drinks, chewing gum, many other "sugar-free" products	Low-calorie sweetener
L-Lysine	70,000	Bread (Japan), feed additives	Nutritive additive
DL-Methionine	70,000	Soy products, feed additives	Nutritive additive

[a]Data from Glazer, A. N., and H. Mikaido. 1995. *Microbial Biotechnology*, W. H. Freeman, New York.
[b]The structures of these amino acids are shown in Figure 3.12.

Steroids and Other Biotransformations

- *Steroids*
  - Are derivatives of sterols
  - Are important animal hormones with medicinal uses
    - *Corticosteroids* reduce inflammation, and help control allergies, inflammation and arthritis
    - Estrogens and androgenic steroids play a role in human fertility and can stimulate production of muscle mass
  - Production of steroids can be costly
    - Use microbes to reduce cost (*biotransformation*)

Cortisone Production Using a Microbe: Biotransformation

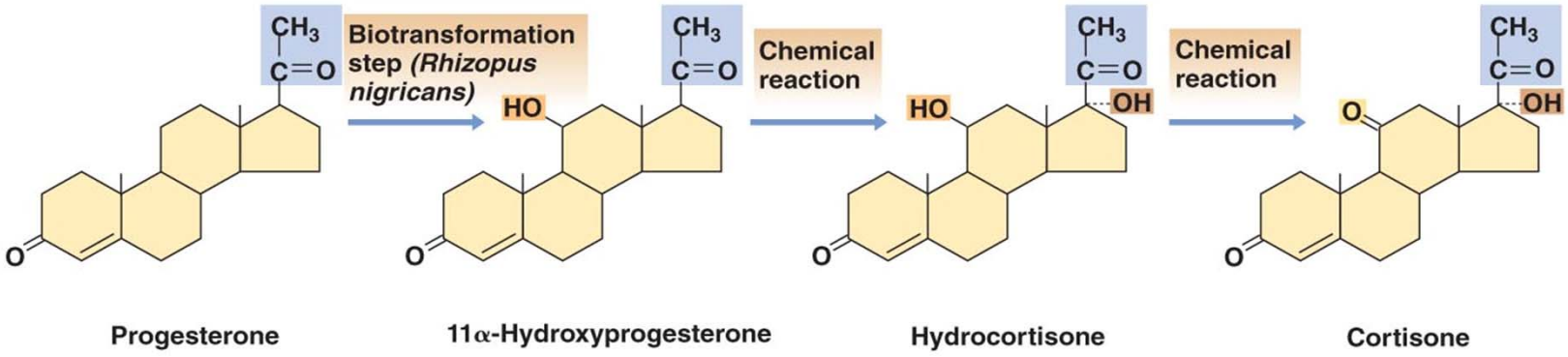


Figure 25.14

Enzymes as Industrial Products

- Exoenzymes
  - Enzymes that are produced in such large amounts that they are excreted into the medium instead of being held within the cell; they are extracellular
  - Can digest insoluble polymers such as cellulose, protein, and starch
- Enzymes are useful as industrial catalysts
  - Produce only one stereoisomer
  - High substrate specificity

Enzymes as Industrial Products

- Enzymes are produced from fungi and bacteria
  - Bacterial proteases are used in laundry detergents (can also contain amylases, lipases, and reductases)
    - Isolated from *alkaliphilic bacteria*
- Amylases and glucoamylases are also commercially important
  - Produce *high-fructose syrup*

Microbial Enzymes and Their Applications

Table 25.4 Microbial enzymes and their applications

Enzyme	*Source*	*Application*	*Industry*
Amylase (starch-digesting)	Fungi	Bread	Baking
	Bacteria	Starch coatings	Paper
	Fungi	Syrup and glucose manufacture	Food
	Bacteria	Cold-swelling laundry starch	Starch
	Fungi	Digestive aid	Pharmaceutical
	Bacteria	Removal of coatings (desizing)	Textile
	Bacteria	Removal of stains; detergents	Laundry
Protease (protein-digesting)	Fungi	Bread	Baking
	Bacteria	Spot removal	Dry cleaning
	Bacteria	Meat tenderizing	Meat
	Bacteria	Wound cleansing	Medicine
	Bacteria	Desizing	Textile
	Bacteria	Household detergent	Laundry
Invertase (sucrose-digesting)	Yeast	Soft-center candies	Candy
Glucose oxidase	Fungi	Glucose removal, oxygen removal	Food
		Test paper for diabetes	Pharmaceutical
Glucose isomerase	*Bacteria*	High-fructose corn syrup	Soft drink
Pectinase	Fungi	Pressing, clarification	Wine, fruit juice
Rennin	Fungi	Coagulation of milk	Cheese
Cellulase	*Bacteria*	Fabric softening, brightening; detergent	Laundry
Lipase	Fungi	Break down fat	Dairy, laundry
Lactase	Fungi	Breaks down lactose to glucose and galactose	Dairy, health foods
DNA polymerase	*Bacteria* *Archaea*	DNA replication in polymerase chain reaction (PCR) technique (∞ Section 12.8)	Biological research; forensics

Immobilized Enzymes

- *Immobilized enzymes* are attached to a solid surface
  - Used in the starch processing industry
- Three ways to immobilize an enzyme
  - Bonding of enzyme to a carrier
  - Cross-linking of enzyme molecules
  - Enzyme inclusion

Procedures for the Immobilization of Enzymes

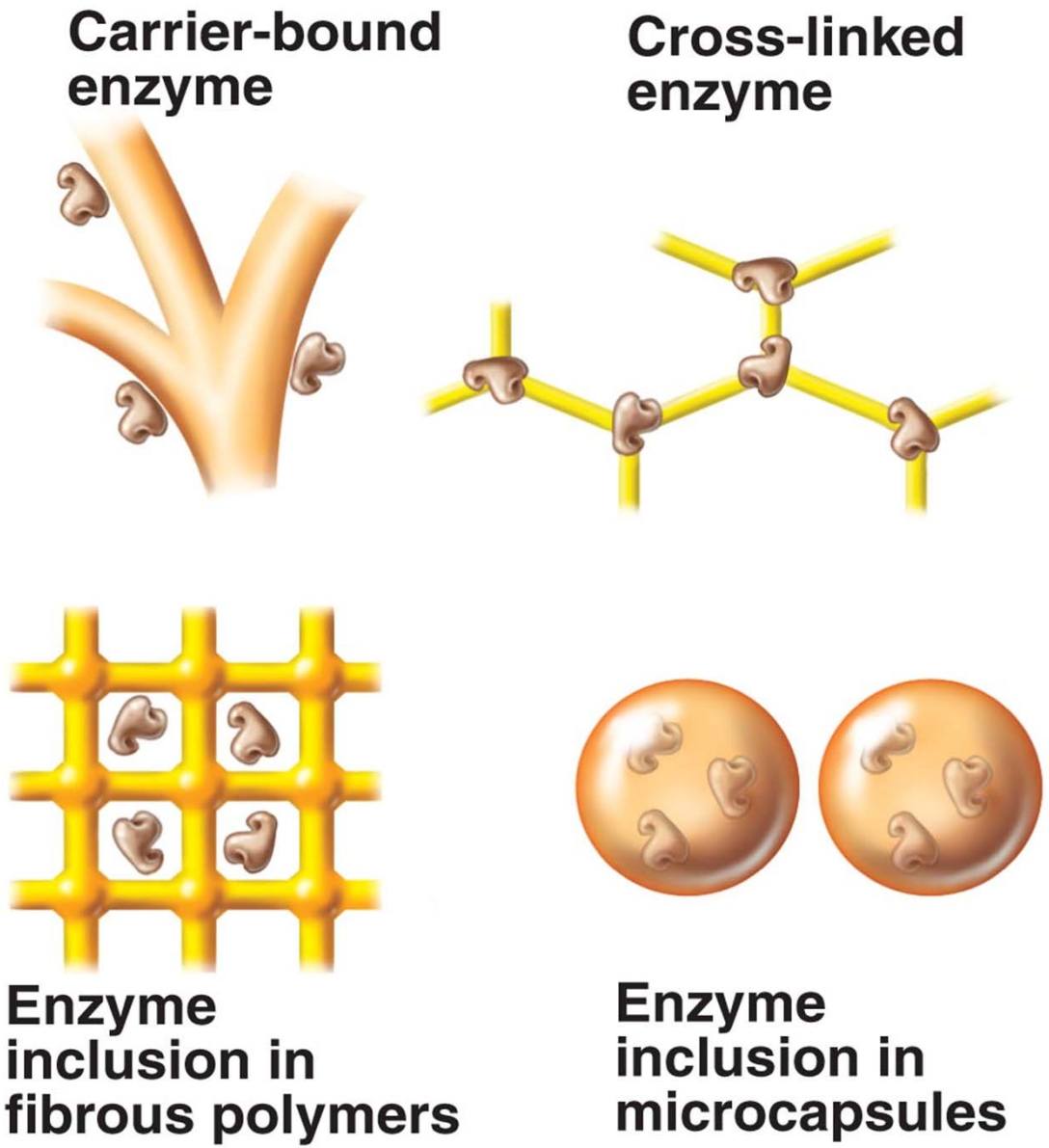


Figure 25.16

III. Products for the Food Industry

- Commodity Alcohol
- Vinegar
- Citric Acid and Other Organic Compounds
- Yeast as a Food and Food Supplement

Ethanol Production Plant, Nebraska (USA)



Chris Standlee and DOE/NREL

Figure 25.21

Vinegar

- *Vinegar*
  - Results from conversion of ethyl acohol to acetic acid; performed by *Acetobacter* and *Gluconobacter*
- Three different processes for the production of vinegar
  - *Open-vat method*
  - *Trickle method; Vinegar generator*
  - *Bubble method*

The Chemistry of Vinegar Production

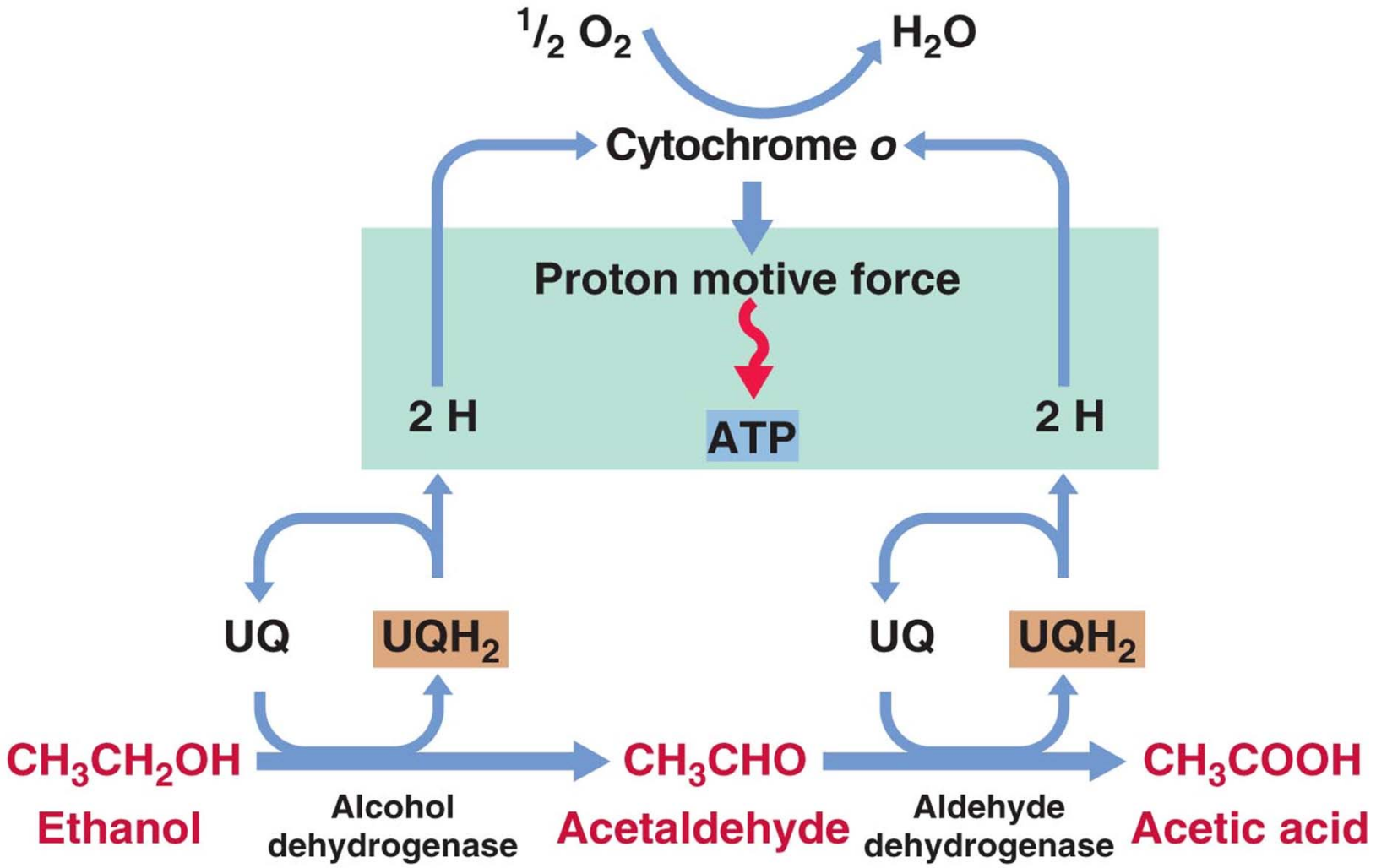
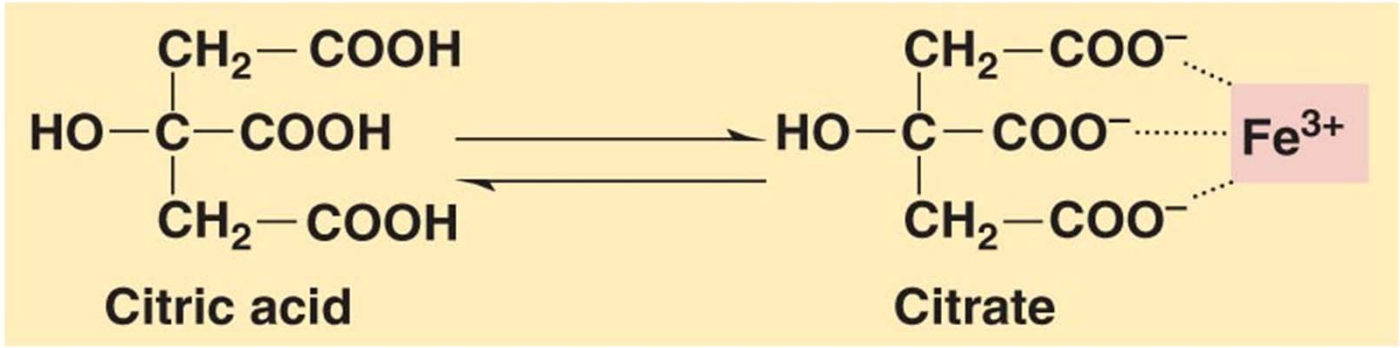


Figure 25.22

Citric Acid and Other Organic Compounds

- *Citric acid*
  - A widely used food industry additive
    - Supplement in beverages, confections, and other foods
    - Used in leavening of bread
  - Produced by the mold *Aspergillus niger*
    - Citrate is a scavenger of iron
  - Purified following removal of fungal cells by addition of lime (CaCO)

Structure of Citric Acid



(a)

Figure 25.24a

Yeast as a Food and Food Supplement

- Yeast for baking or nutritional purposes are grown in aerated fermentors
  - Molasses is the major ingredient of growth medium
- Cells are recovered from broth by centrifugation
  - Compressed yeast cakes
  - Active dry yeast
  - Nutritional yeast

Industrial Uses of Yeast and Yeast Products

Table 25.5 Industrial uses of yeast and yeast products

Production of yeast cells
Baker's yeast for bread making
Dried food yeast, for food supplements
Dried feed yeast, for animal feeds
Yeast products
Yeast extract, for microbial culture media
B vitamins, vitamin D
Enzymes for food industry; invertase (sucrase), galactosidase
Biochemicals for research: ATP, NAD^+, RNA
Fermentaion products from yeast
Ethanol, for industrial alcohol and as a gasoline extender
Glycerol
Beverage alcohol
Beer, wine
Distilled beverages
Whiskey, brandy, vodka, rum

Stages in Industrial Production of Yeast Cells

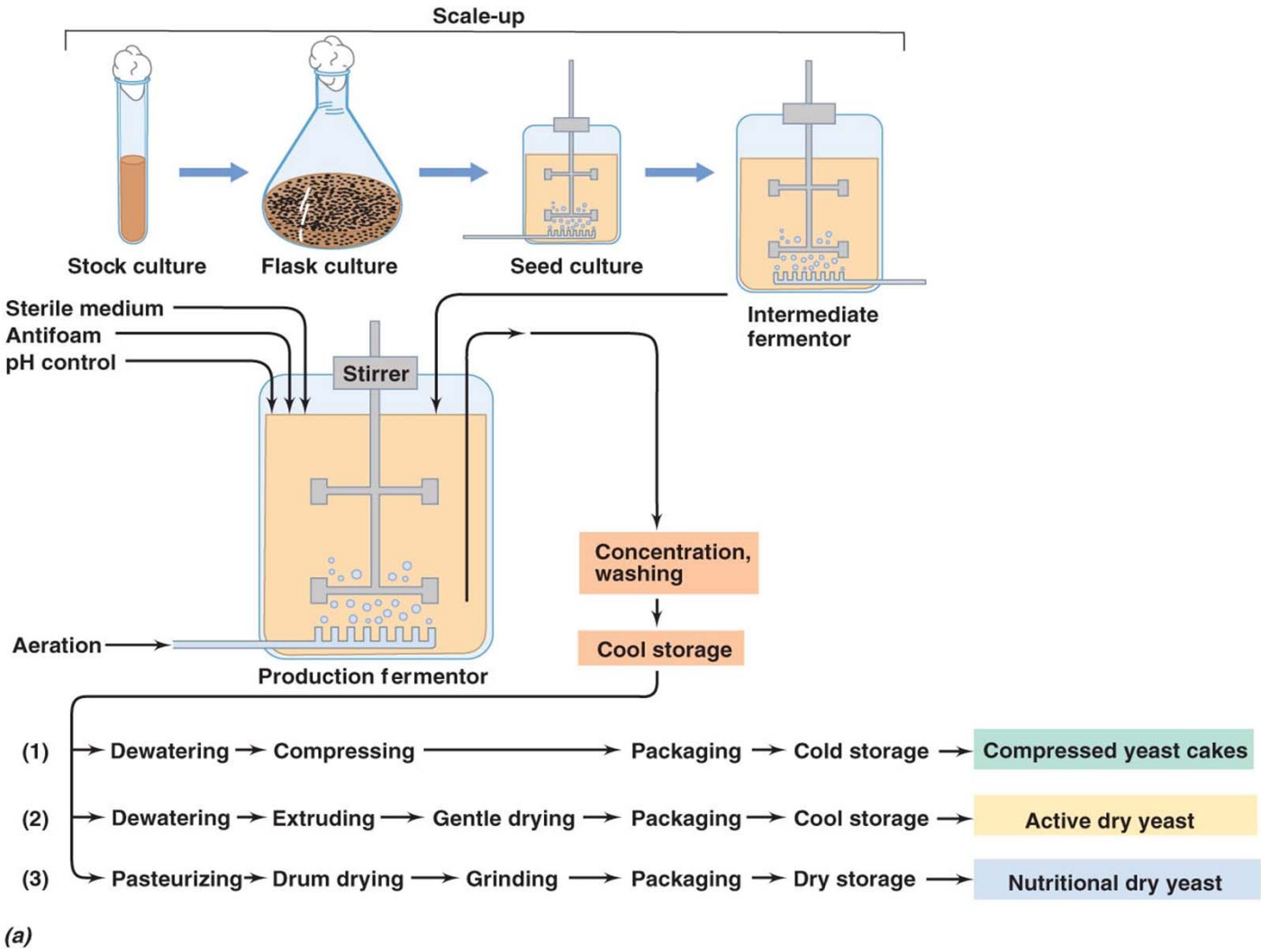


Figure 25.25a

Regulation of metabolism

- Ensures that
  - Only necessary enzymes are made
  - Correct amounts are made
  - Activity controlled by inhibition and activation
- Industrial synthesis requires deregulation
  - By environmental modification
  - By genetic modification
    - Mutation
    - Recombination

Primary metabolites

- Building blocks for macromolecules
  - Amino acids
  - Nucleic acids
  - Sugars
  - Fatty acids
- Precursors of coenzymes
  - Vitamins
- Catabolite products
  - Organic acids
  - Ethanol
  - Acetone
  - butanol

Overproduction of primary metabolites

Product	Growth Required(mg/L)	Produced (mg/L)	Ratio Produced/required
Lysine	250	100,000	$4.0*10^2$
Glutamic acid	300	125,000	$4.2*10^2$
Inosinic acid	25	30,000	$1.2*10^3$
Riboflavin	0.5	25,000	$5.0*10^4$
Vitamin	0.001	150	$1.5*10^4$

Regulatory mechanism

- Inductions
- Nutritional repression
  - Carbon source
  - Nitrogen source
  - Phosphorus source
  - Sulfur source
- Feedback regulation
  - Inhibition
  - Repression
- Metal regulation

Induction

- Inducers
  - Substrates
  - Products
- Selection for constitutive mutants from inducible parents
  - Mutation and selection
  - Genetic changes in promoter, repressor

Carbon source repression

- Ensures enzymes made for catabolism of best C-sources
  - Glucose vs. lactose
- Usually caused by glucose but not always
- C-AMP mediates in enteric bacteria

Combating carbon source repression

- Environmental
  - Avoid repressive carbon source
    - Mannose etc. (case by case)
  - Controlled feeding
    - Maintaining at low level of glucose
- Genetic
  - Mutation and selection
  - Selection methods
    - 2-deoxyglucose selection

Nitrogen source repression

- Observed in the production of enzymes
  - Proteases, amidases, urase
  - Amino acid degrading and converting enzymes
- Obtaining the derepressed mutants
  - Methyl ammonium resistance
  - Zone on casein in the presence of high NH_4^+

Phosphorus source repression

- Usually nucleases, phosphatase
- *Eremothecium ashbyi*: riboflavin

- Mutant selection
  - Growth with a phosphate ester as a sole source of carbon in presence of high phosphate

Feedback regulation

- Feedback inhibition
  - Final metabolite inhibits first enzyme
- Feedback repression
  - Final metabolite (co repressor) combines with regulatory protein (aporepressor)
  - The complex binds at operator and blocks transcription

Combating feedback regulation

- Limit intracellular accumulation of inhibitory or repressive end products
  - Auxotrophic mutants
    - Lysine production by Thr^- and Met^-
- Select for mutant resistant to feedback regulation
  - Use of antimetabolites for selecting mutant
  - To increase antimetabolite effects
    - Change C source
    - Change N source
    - Add detergent

Amino acid production by mutant

Amino acid	Microorganism	Auxotrophic requirement	Analogue resistance	Potency (g/L)
L-Glutamate	Corynebacterium glutamicum	Biotin		100
L-Leucine	Brevibacterium flavum	Methionine, isoleucine	2-thiazolealanine	28
L-Lysine	Brevibacterium flavum	Threonine, methionine		75
L-Phenyl alanine	Arthrobacter paraffineus	tyrosine		15
L-Tryptophan	Corynebacterium glutamicum	Phenylalanine tyrosine	Tryptophan analogues	12

Glutamate overproduction by *Corynebacterium glutamicum*

- Glutamate causes feedback
  - Repression of phosphoenolpyruvate (PEP) carboxylase
  - Repression of citrate synthase
  - Repression and inhibition of glutamate dehydrogenase
- Manipulations which alter permeability (decrease phospholipids content) of plasma membrane
  - Biotin limitation of biotin auxotroph
  - Addition of surfactant (fatty acid derivative)
  - Addition of penicillin
    - Penicillin does not kill corynebacteria because of complex cell wall

Metal regulation

- Riboflavin fermentation
  - Requires iron deficiency
  - GTP cyclohydrolase II repressed by iron
  - Riboflavin synthetase repressed by iron

- Citric acid fermentation
  - Requires Mn^{++} deficiency
  - Mn^{++} deficiency increases intracellular NH4 5-fold which reverses citrate inhibition of phosphofructokinase

Secondary metabolites

- Special metabolites
- Idiolites

- Examples of secondary metabolites
  - Antibiotics
  - Mycotoxins
  - Alkaloids
  - Pigments

Production of secondary metabolites

- Often increased by precursor addition
  - Phenyl acetic acid (PAA) for penicillin production
- Controlled by regulation
  - Nutrient repression
  - Induction
  - Growth rate
  - Feedback control

Carbon source repression of antibiotic biosynthesis

Antibiotics	Interfering carbon source	Noninterfering carbon source
Actinomycin	glucose	galatose
Cephalosporin C	glucose	sucrose
Chloramphenicol	glucose	glycerol
Kanamycin	glucose	galactose
phenicillin	glucose	lactose

Other Factors

- Nitrogen source repression
- Inorganic phosphate inhibition
- Metals
  - Zn ， Mn
- Oxygen
- Light
- Temperature