Chapter 26 Aquatic Microbiology

OUTLINE

Aquatic microbiology is the study of microorganisms and their activities in fresh, estuarine, and marine waters, including springs, lakes, rivers, bays, and seas. It is the study of the microorganisms—viruses, bacteria, algae, protozoa, and microscopic fungi—which inhabit these natural waters. Some of these microorganisms are indigenous to natural bodies of water; others are transient, entering the water from air or soil or from industrial or domestic wastes. For example, wastewater can be pumped into rivers and coastal waters, or it can be disposed of in deep ocean dump sites. Wastewater usually contains microorganisms which will influence the activities of microorganisms already present in the receiving waters. This important aspect of aquatic microbiology, wastewater, will be treated in greater detail in the following chapter. The present chapter will deal primarily with aquatic microorganisms and their habitat.

Aquatic microorganisms and their activities are of great importance in many ways. They may affect the health of humans and other animal life; they occupy a key position in the food chain by providing rich nourishment for the next

higher level of aquatic life; they are instrumental in the chain of biochemical reactions which accomplish recycling of elements, e.g., in mineralization. Aquatic microbiology, which in previous decades was studied by a relatively few microbiologists, has emerged as one of the more important areas of applied microbiology. Urbanization and consequently the growing demand for water by communities, the importance of natural water as a major food source, the offshore exploration for oil and minerals, and other developments have resulted in the establishment of federal agencies which exercise jurisdiction over many aspects of natural bodies of water. The Environmental Protection Agency (EPA) and the National Oceanic and Atmospheric Administration (NOAA) are two of these agencies.

NATURAL WATERS

The earth's moisture is in continuous circulation, a process known as the **water cycle** or **hydrologic cycle** (see Fig. 26-1). It has been estimated that about 80,000 cubic miles of water from oceans and 15,000 cubic miles from lakes and land surfaces evaporate annually. The total evaporation is equaled by the total precipitation, of which about 24,000 cubic miles fall on land surfaces. Microorganisms of various kinds are present at different stages of this cyclic process—in atmospheric water, surface water, and groundwater. Because the kinds of aquatic environments are so different, it is not surprising that different species of microbes are considered to be indigenous to specific habitats.

Atmospheric Water

The moisture contained in clouds and precipitated as snow, sleet, hail, and rain constitutes atmospheric water. The microbial flora of this water is contributed by the air. In effect, the air is "washed" by atmospheric water, which carries with it the particles of dust to which microorganisms are attached. Most of the microorganisms are thus removed from air during the early stages of precipitation.

Surface Water

Bodies of water such as lakes, streams, rivers, and oceans represent surface water. To a greater or lesser degree, these waters are susceptible to contamination

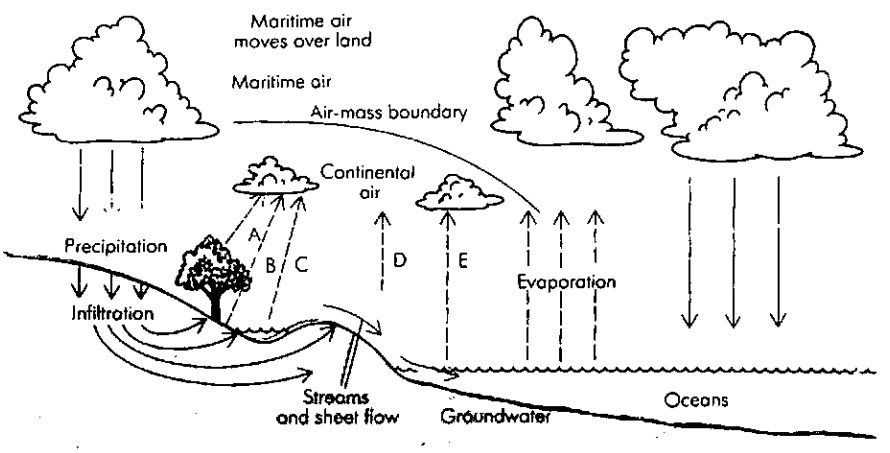


Figure 26-1. Diagram of hydrologic cycle. Water returns chiefly to dry continental air through transpiration (A) and evaporation from soil (B), lakes and ponds (C), and streams (D). Continental air moves over ocean to become more moist (E) with conversion to maritime air with precipitation over the oceans. (*Adapted from B. Holzman.*) (*Courtesy of McGraw-Hill Encyclopedia of Science and Technology, vol. 6, McGraw-Hill, New York, 1982, p. 251.*)

with microorganisms from atmospheric water (precipitation), the surface runoff from soil, and any wastes deliberately dumped into them. Microbial populations vary in both number and kind with the source of water, with composition of the water in terms of microbial nutrients, and with geographical, biological, and climatic conditions.

Groundwater

Groundwater is subterranean water that occurs where all pores in the soil or rock-containing materials are saturated. Bacteria as well as suspended particles are removed by filtration, in varying degrees, depending on the permeability characteristics of the soil and the depth to which the water penetrates. Springs consist of groundwater that reaches the surface through a rock fissure or exposed porous soil. Wells are made by sinking a shaft into the ground to penetrate the groundwater level. Wells less than 100 ft deep are considered to be shallow. Bacteriologically speaking, wells and springs that are properly located produce water of very good quality. If precautions are taken to avoid contamination, the microbial content is negligible.

THE AQUATIC ENVIRONMENT

The microbial population in a body of natural water is, to a large extent, determined by the physical and chemical conditions which prevail in that habitat. This generalization applies to all habitats. It is readily apparent that these conditions vary over wide extremes when one compares streams, estuaries, and the open sea. Some of these conditions are described below.

Temperature

The temperature of surface waters varies from near 0°C in polar regions to 30 to 40°C in equatorial regions. More than 90 percent of the marine environment (by far the major aquatic habitat) is below 5°C, a condition favorable for the growth of psychrophilic microoganisms. Microorganisms do occur in natural hot springs where temperatures as high as 75 to 80°C prevail (*Thermus aquaticus*, a common bacterial inhabitant of hot springs, has an optimum growth temperature of 70 to 72°C). Recently, microbiologists have reported extreme thermophilic microorganisms associated with geothermal vents in the Pacific Ocean floor. These unusual microbes are said to be capable of growing at 250°C and 265 atm of pressure. If these reports can be confirmed, the existence of such organisms will cause all biologists to reevaluate their concept of the maximum temperature that can be tolerated by life on earth! Aside from this extreme, the temperature in lakes, streams, and estuaries is influenced by the seasons, and there are corresponding shifts in the microbial flora.

Hydrostatic Pressure

There are striking differences in the hydrostatic pressure of surface waters and of water in oceanic depths. Hydrostatic pressure affects chemical equilibrium, which, in turn, results in lowering the pH of seawater, resulting in a change in the solubility of nutrients such as bicarbonate, HCO_3^-. Hydrostatic pressure also increases the boiling point of water, thereby maintaining water in its liquid state at high temperatures and pressures. By definition hydrostatic pressure increases with depth at the rate of 1 atm per 10 m. **Barophilic** microorganisms, organisms which cannot grow at normal atmospheric pressures, have been isolated from Pacific trenches (depth 1000 to 10,000 m), where enormous hydro-

Table 26-1. Composition of Some Typical Natural Waters, g/liter

Water	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	SO_4^{2-}	CO_3^{2-}
Seawater	10.7	0.39	0.42	1.34	19.3	2.69	0.073
Freshwater:							
Hard	0.021	0.016	0.065	0.014	0.041	0.025	0.119
Soft	0.016		0.010	0.00053	0.019	0.007	0.012

SOURCE: Data from E. Baldwin, *An Introduction to Comparative Biochemistry*, Cambridge, London, 1948.

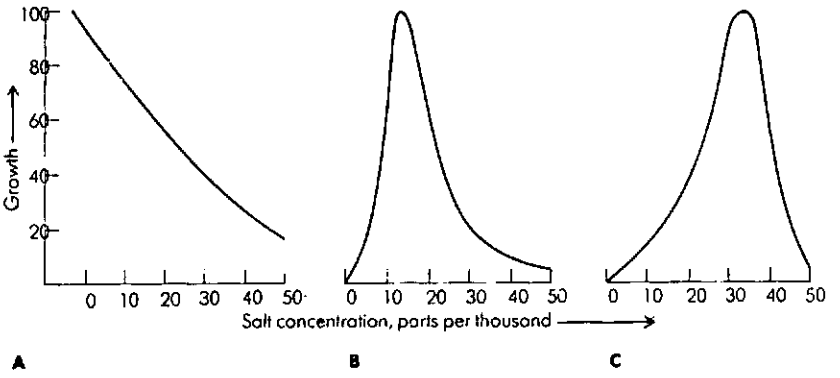


Figure 26-2. Growth curves in salt solutions for (A) a halotolerant freshwater bacterium, (B) an obligatory halophilic bacterium of brackish water, and (C) an obligatory halophilic marine bacterium, all isolated from the Baltic Sea. (*Courtesy of G. Rheinheimer, Aquatic Microbiology, Wiley, New York, 1974.*)

static pressures exist (>100 atm). Hydrostatic pressure of the deep sea is an important factor in the occurrence and growth of marine microorganisms in this environment. Deep sea bacteria have now been isolated from sediment, water, and deep sea animals by using special pressure-retaining sampling devices. In general, barophilic bacteria grow best at pressures slightly less than the pressure of the site from which they were isolated, and almost all must be grown under psychrophilic conditions (about 2°C).

Light

Most forms of aquatic life depend, directly or indirectly, upon the metabolic products of photosynthetic organisms. In most aquatic habitats these primary producers are algae, and their growth is restricted to the upper layers of waters through which light can penetrate. The depth of thephotic zone varies depending on such local conditions as latitude, season, and particularly the turbidity of the water. Generally, the photosynthetic activity is confined to the upper 50 to 125 m. Carbon dioxide is available largely from HCO_3^-, although some gaseous CO_2 is available.

Salinity

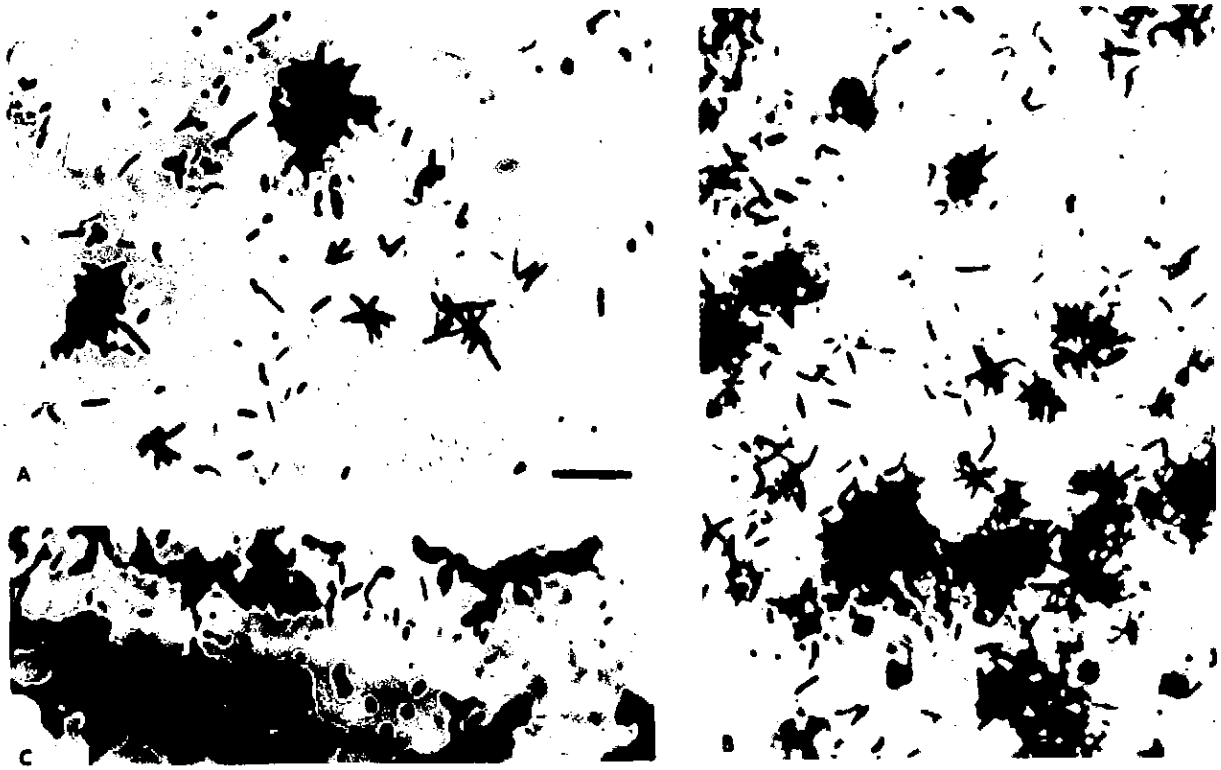
The degree of salinity in natural waters ranges from near zero in freshwater to saturation in salt lakes. A distinctive characteristic of sea water is its high salt content, which is remarkably constant. The concentration of dissolved salts varies between 33 and 37 g/kg water. The major mineral constituents of sea water are listed in Table 26-1. The principal salts are the chlorides, sulfates, and carbonates of sodium, potassium, calcium, and magnesium. The concentration of salts is usually less in shallow offshore regions and near river mouths. Most marine microorganisms are halophilic; they grow best at salt concentra-

tions of 2.5 to 4.0 percent, whereas those from lakes and rivers are salt sensitive and do not grow at a salt concentration of more than 1 percent. The growth response of three bacterial types to different levels of salinity is shown in Fig. 26-2.

Turbidity

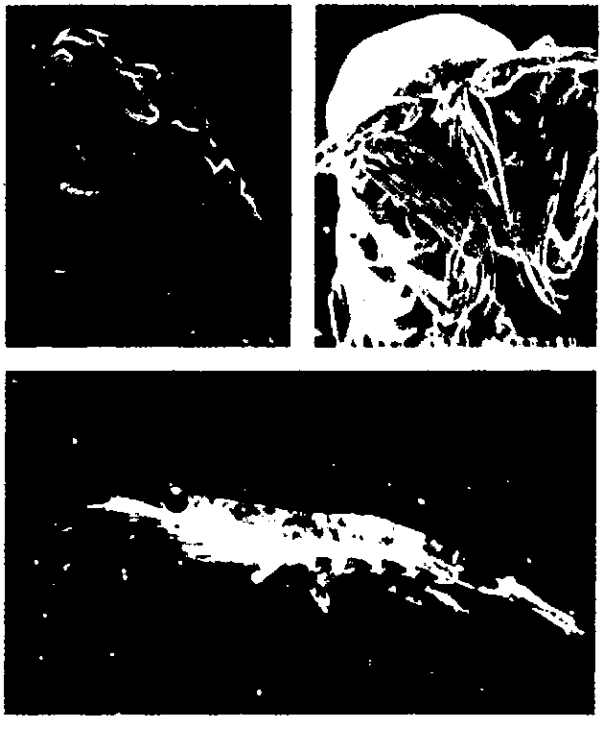
There is marked variation in the clarity of surface waters. The Adriatic Sea is sparkling clear at great depths, whereas some near-shore rivers are often turbid. The suspended material responsible for the turbidity includes (1) particles of mineral material which originate from land; (2) detritus, predominantly particulate organic material, such as cellulose, hemicellulose, and chitin fragments; and (3) suspended microorganisms. As previously mentioned, turbidity of the water influences the penetration of light, which in turn affects the photosynthetic zone. Particulate matter also serves as a substrate to which microorganisms adhere or as substrates that are metabolized. Many species of marine bacteria characteristically grow while attached to a solid surface (see Fig. 26-3) and are called epibacteria or periphytes.

Figure 26-3. Marine bacteria attached to particulate materials are referred to as epibacteria. Shown here are marine bacteria attached to (A) agar particles, (B) chitin particles, and (C) cellulose fragments. (*Courtesy of W. A. Corpe, L. Matsuuchi and B. Armbruster and Proceedings of Third International Biodegradation Symposium, 1976.*)



Protozoa
Left: The dinoflagellate protozoan *Noctiluca* that causes nontoxic red tides in Puget Sound. Note the flagellum. Actual diameter approximately 100 micrometers.

Right: the lorica of the ciliate protozoan *Tintinnopsis*. Actual length approximately 100 micrometers. (Photos courtesy Alexander J. Chester)



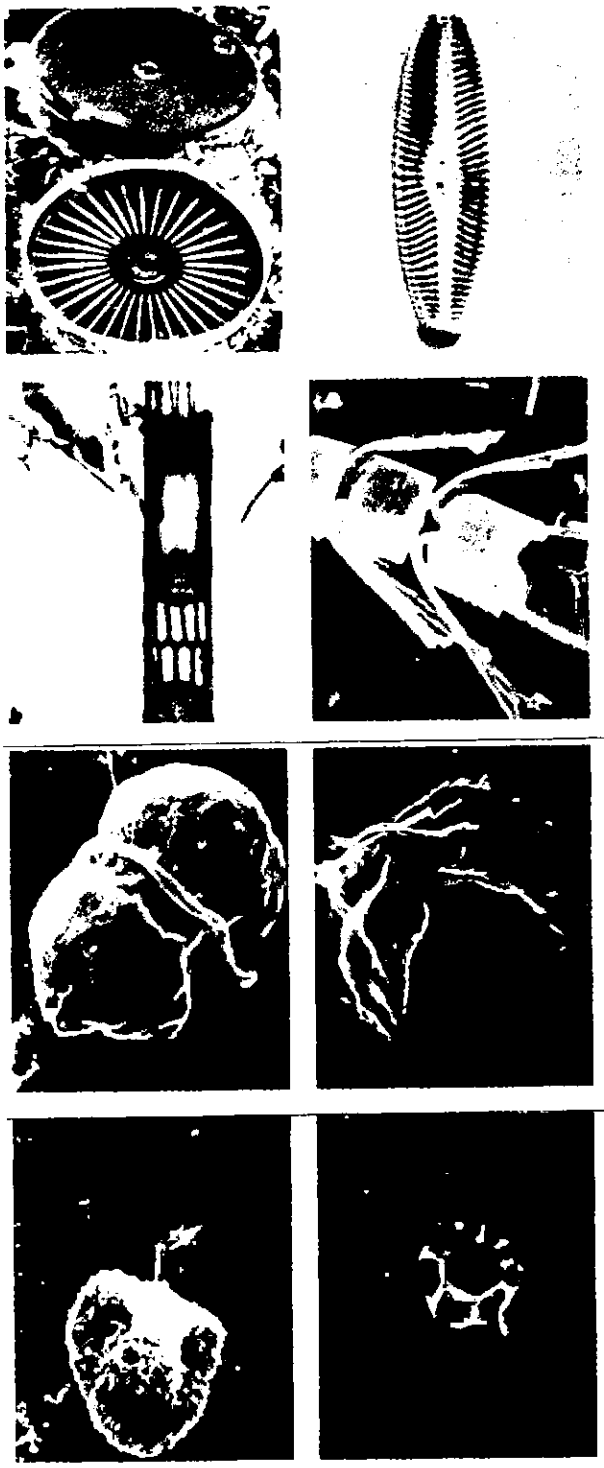
Crustaceans
Left: The herbivorous copepod *Calanus* showing the antennae and feeding (upper) and swimming (lower) appendages. Actual length approximately 3 millimeters.

Right: The carnivorous copepod *Euchaeta*, with coarse feeding appendages adapted for grasping, in contrast to the filtering apparatus of *Calanus*. Actual length approximately 1 centimeter. (Photos courtesy Charles H. Greene)

The euphausiid "krill" *Euphausia*, which is mostly herbivorous in Puget Sound. Actual length approximately 2 centimeters. (Courtesy Mark D. Ohman)

Rotifers
The brackish-water rotifer *Brachionus*. The blur is caused by beating cilia. Actual length approximately 200 micrometers. (Courtesy Richard Kaiser)

Figure 26-4. (A) Zooplankton comprises an extremely diverse animal population ranging from microscopic unicellular organisms (protozoans) to multicellular metazoans as shown in this figure. Several species found on Puget Sound on the Pacific Coast are shown here.



Diatoms
Top left: Separated pieces of the frustule of the solitary centric diatom *Coscinodiscus*. Actual diameter approximately 100 micrometers. (Courtesy National Marine Fisheries Service (NMFS), NOAA, micrograph by Michael Eng)

Top right: The solitary pennate diatom *Navicula*. Actual length approximately 100 mic ometers. (Courtesy School of Oceanography, University of Washington)

Bottom left: A portion of a chain of the centric diatom *Skeletonema* Actual cell diameter approximately 25 micrometers. (Courtesy B. Dumbauld)

Bottom right: A segment of the centric diatom *Chaetoceros*. Actual cell diameter approximately 35 micrometers. (Courtesy Beatrice C. Booth)

Dinoflagellates
Left: The unarmored dinoflagellate *Gymnodinium*. Note the flagellum in the transverse groove. Actual cell length approximately 6 micrometers.

Right: The armored dinoflagellate *Gonyaulax*, cause of paralytic shellfish poisoning. Actual size approximately 30 micrometers. (Photos courtesy Susan B. Stanton)

Phytoflagellates
Left: A green flagellate (probably *Pyramimonas*). Note flagella and surface scales. Actual size approximately 8 micrometers.

Right: The skeleton of the golden-brown silicoflagellate *Dichtyocha*. Actual size including spines approximately 50 micrometers. (Photos courtesy Beatrice C. Booth)

Figure 26-4. (B) Phytoplankton is comprised of algae which are uniquely adapted to the marine environment. Several species found in Puget Sound on the Pacific Ocean are illustrated here. (*Courtesy of R. M. Strickland, The Fertile Fjord, Plankton in Puget Sound, Puget Sound Books, University of Washington Press, Seattle, 1983.*)

Hydrogen-Ion Concentration (pH)

Aquatic microorganisms, in general, can be grown at pH 6.5 to 8.5. The pH of the sea is 7.5 to 8.5. Optimum growth of most marine species is obtained on media adjusted to pH 7.2 to 7.6. Lakes and rivers may show a wider range in pH depending upon local conditions.

Inorganic and Organic Constituents

The quantity and type of inorganic and organic materials present in the aquatic environment are important in determining the microbial flora. Nitrates and phosphates are important inorganic constituents, particularly for the growth of algae. Organic compounds are required for the growth of saprophytic bacteria and fungi. Near-shore waters, which receive domestic wastewater, are subject to intermittent variations in their nutrient load, whereas the nutrient load of the open sea is very low and stable. Industrial wastes may contribute antimicrobial substances to estuaries and coastal waters. Mercury and other heavy metals in small concentrations may inhibit growth of some microorganisms while simultaneously permitting the growth of resistant forms. Resistance is usually coded for by genes associated with R (resistance) plasmids. For example, many pseudomonads and staphylococci are capable of volatilizing mercury, thereby removing its toxic effects from their immediate environment.

DISTRIBUTION OF MICROORGANISMS IN THE AQUATIC ENVIRONMENT

Microorganisms in the aquatic environment may occur at all depths ranging from the surface region to the very bottom of ocean trenches. The top "layers," especially the surface film, and the bottom sediments harbor the higher concentrations of microorganisms, particularly in deep waters.

Plankton (Phytoplankton, Zooplankton)

The aggregation of floating and drifting microbial life in the surface region of the aquatic ecosystem is called *plankton*. Plankton may be composed primarily of algae (*phytoplankton*), or it may be predominantly protozoa and other minute animal life (*zooplankton*). Phototrophic microorganisms are regarded as the most important plankton since they are the primary producers of organic matter via photosynthesis. Most phytoplanktonic organisms are motile, possess some structural feature, or contain oil droplets which give them buoyancy; all these features aid the organisms in maintaining their location in the photosynthetic zone (see Figs. 26-4A and B). The multitude of physical conditions which influence the plankton population quantitatively and qualitatively are shown in Fig. 26-5.

Benthic Microorganisms

Microbial inhabitants of the bottom region of a body of water are referred to as the *benthic organisms*. The richest region of an aquatic system in terms of numbers and kinds of organisms is the *benthic region*. Many aquatic microorganisms inhabit the gut of marine animals, an even richer habitat.

Mixing of Waters (Upwelling)

The movement of water by wind, tide, or currents accomplishes some redistribution of the microbial flora. A phenomenon called *upwelling* occurs in an ocean when water rises from a deeper to a shallower depth, usually as a result of divergence of offshore currents or winds. In this process the bottom water carries with it a rich supply of nutrients that are delivered to the surface region. Upwelling occurs off the coasts of California and Peru and is responsible for

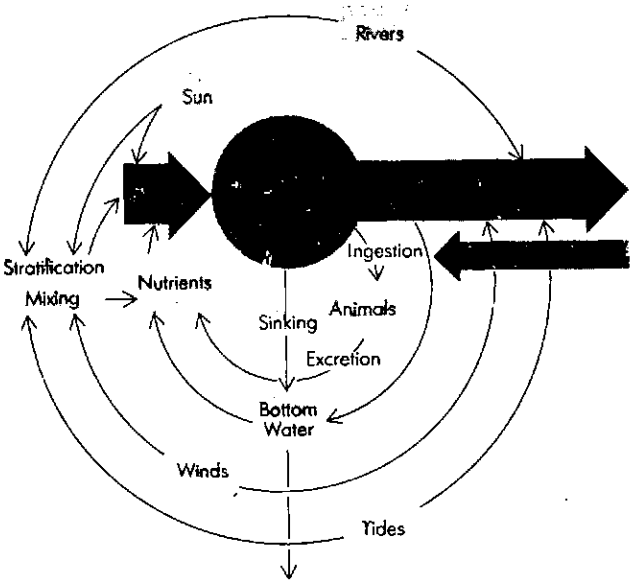


Figure 26-5. Physical forces produce both positive and negative effects on phytoplankton growth, and blooms occur when the forces are balanced. Phytoplankton standing stock is increased by photosynthesis and nutrient uptake, which are regulated by sunshine, stratification, and mixing. Standing stock is decreased by animal consumption, sinking, and flushing by winds, tides, and runoff. (*Courtesy of R. M. Strickland, The Fertile Fjord, Plankton in Puget Sound, Puget Sound Books, University of Washington Press, Seattle, 1983.*)

the high productivity of these regions. Geothermal vents also contribute to the total nutrient budget of the ocean. It has been calculated that vents such as the one near the Galapagos Islands account for most of the nutrients dissolved in the oceans of the world. Prior to discovery of these vents, oceanographers were unable to account for nutrients on the basis of precipitation, input from rivers and streams, and other obvious sources. Another interesting feature of the oceans is the gyre, large spiraling surface currents in the ocean that tend to aggregate and retain nutrients, wastes, and microorganisms. Gyres have only been appreciated in recent years, through the use of satellite imagery (Fig. 26-6).

TECHNIQUES FOR THE STUDY OF AQUATIC MICROORGANISMS

Numerous problems are associated with attempts to characterize the microbial flora of aquatic evironments. This is particularly true of samples from the open sea. Among these problems are the following:

1 Many aquatic microorganisms will not grow on the usual laboratory media such as nutrient agar or nutrient broth and consequently cannot be isolated. It is generally acknowledged that the estuaries and oceans contain a large number of microbial species that await discovery.
2 A high percentage of aquatic bacteria have a natural affinity to grow attached to solid surfaces, either on particulate material or on large organisms. See Fig. 26-3.
3 During the time which elapses between sample collection and transport back to the home-based laboratory, there is a loss of viability of many organisms. Accordingly, a laboratory-equipped ship is desirable for on-location culturing of specimens. This is a very costly facility.
4 The collection of samples from the depth of the estuary or ocean requires specialized sampling equipment (Fig. 26-7).

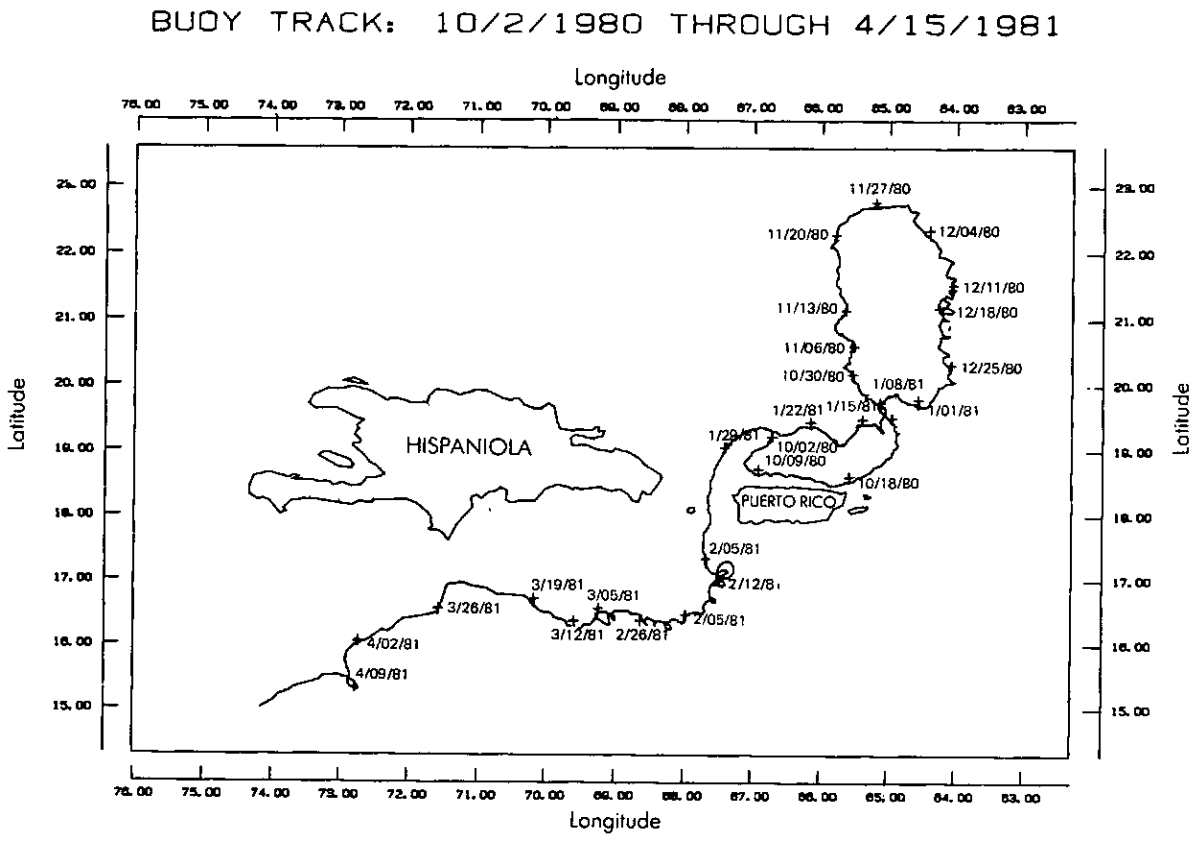


Figure 26-6. Meandering course taken by a buoy released at an ocean waste disposal site, 64 km north of Arecibo, Puerto Rico, on 2 October 1980. The buoy was carried by large surface gyres north toward Bermuda, then back through the original point of release and, finally, into the Caribbean Sea. Gyres can concentrate nutrients, wastes, and microorganisms in the aquatic environment. (*Courtesy of D. J. Grimes, University of Maryland, and W. G. Williams, Clearwater Consultants Inc.*)

5 Routine dependable techniques are not available for isolation of aquatic viruses.

A variety of procedures is used for the microbiological examination of aquatic specimens. The choice of method is determined by the purpose of the examination, e.g.:

1 Microsopic examination for identification and enumeration of algae, bacteria, protozoa, and many fungi.
   (a) Direct viable counting of physiologically responsive bacteria (see Fig. 26-8).
   (b) Detection of specific bacteria by means of epifluorescent microscopy and fluorescent antibody techniques (see Fig. 26-9).

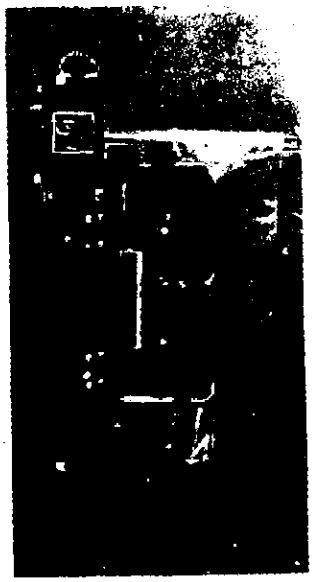


Figure 26-7. Special equipment like the "chopstick" apparatus shown here is used to collect water samples aseptically from the marine environment. Shown here is a sterile plastic bag sealed and ready to be lowered to a desired depth, at which point it is tripped (opened) with a messenger. The sample of water is collected, after which the bag is again sealed and the sample is raised to the surface. (*Courtesy of General Oceanics.*)

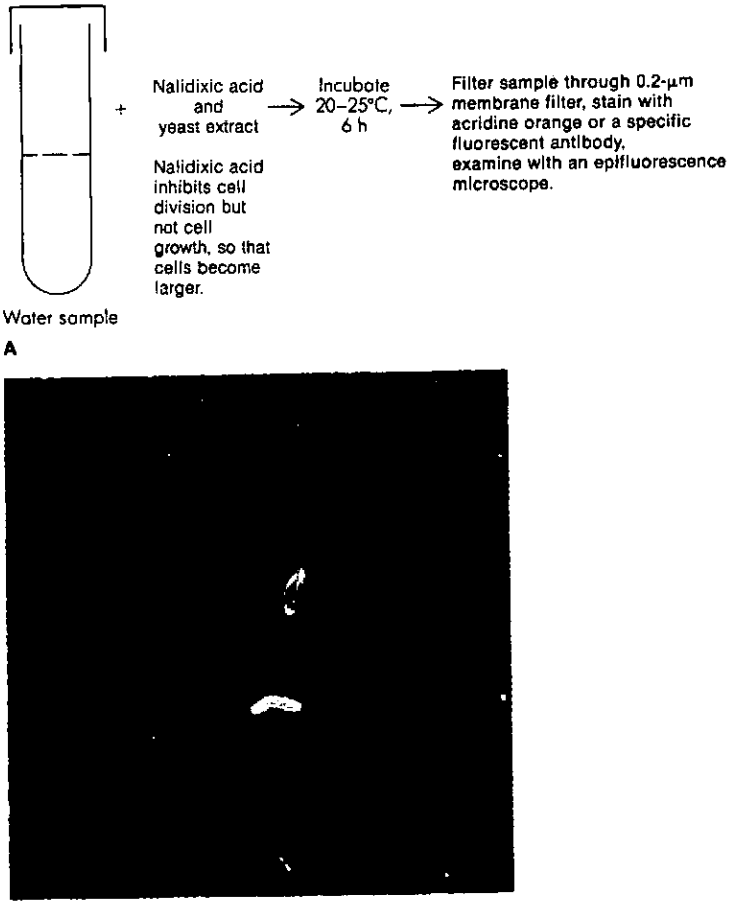


Figure 26-8. The isolation of physiologically responsive bacteria from marine samples. (A) Outline of technique. (B) Photomicrograph of physiologically responsive *Vibrio cholerae* stained with specific fluorescent antiserum (X1,000). Physiologically responsive bacteria are those which have an increased size; compare these cells to the small *V. cholerae* cells in Fig. 26-9 that have been stained with fluorescent antibody without a preincubation with nalidixic acid and yeast extract. (*Courtesy of P. Brayton and D. J. Grimes, University of Maryland.*)

(c) Detection of epibacteria by the submerged-slide technique (see Fig. 26-10).

2 Isolation and/or enumeration of certain groups of bacteria, e.g., *Escherichia, Pseudomonas, Flavobacterium, Proteus,* and *Vibrio.* Many species of bacteria can be cultured by the plate technique on the usual bacteriological media. The membrane-filter technique (see Chap. 27) is applicable for the examination and culti-

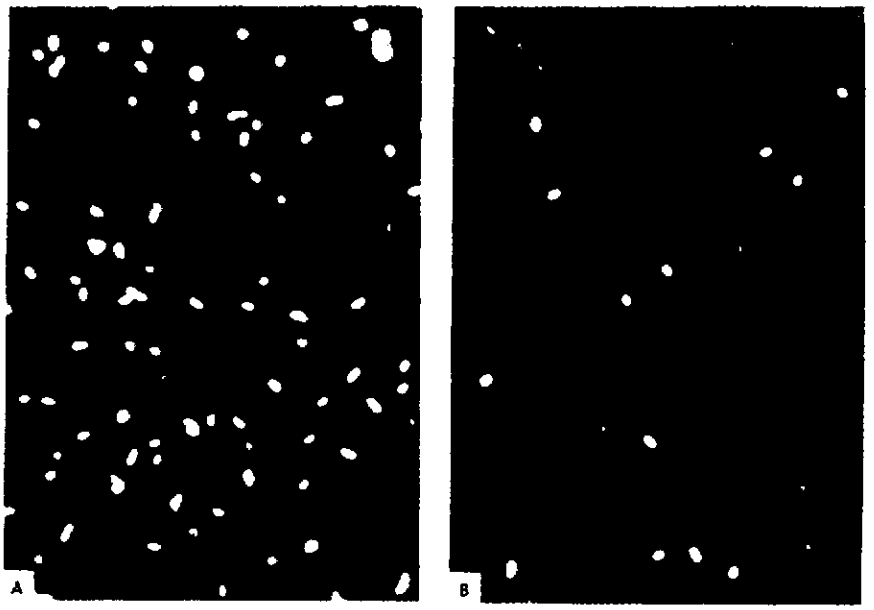


Figure 26-9. Demonstration of marine bacteria (*Vibrio cholerae*) by epifluorescence microscopy (X1,000). (A) Cells stained with acridine orange. (B) Cells stained with fluorescent antibody. (*Courtesy of P. Brayton and D. J. Grimes, University of Maryland.*)

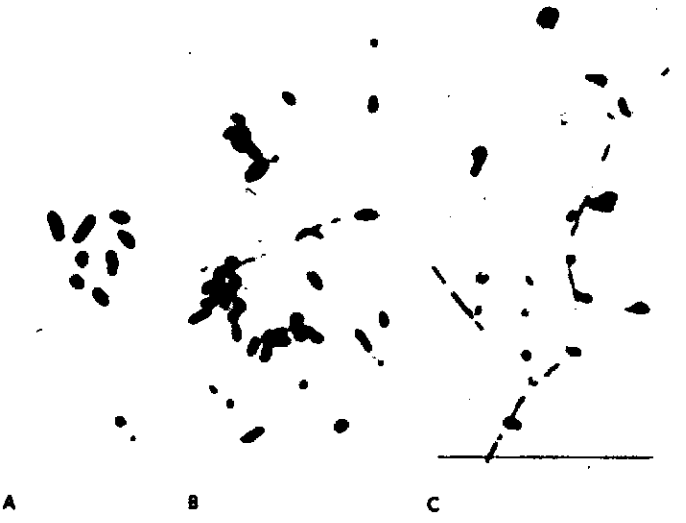


Figure 26-10. The attachment of bacteria to glass slides. Slides were submerged in a regulated marine aquarium, fixed with 2% acetic acid, and stained with Hucker's cyrstal violet solution. (A), (B), and (C) are from slides submerged for 24 h, 48 h, and 72 h, respectively. Note the variety of morophological forms. The scale shown is 5 μm. (*Courtesy of W. A. Corpe, "Attachment of Marine Bacteria to Solid Surfaces," in Adhesion in Biological Systems, Academic, New York, 1970.*)

vation of many bacteria from the aquatic environments. It can also be used to separate different-size fractions of the aquatic microbial community.

3 Enrichment-culture technique for isolation of specific physiological or metabolic types of microorganisms as described in Chap. 6.

4 Measurement of total mass or biochemical activity.

(*a*) ***Biomass determination.*** Dry weight determination of cell mass.

(b) ***Carbon-14 uptake.*** Supplementation of the CO_2 in water with radioactive $^{14}CO_2$ supplied as radioactive sodium bicarbonate ($NaH^{14}CO_3$) and measurement of ^{14}C assimilated by cells.
(c) ***ATP synthesis.*** Measurement of amount of ATP as a function of the rate of microbial activity or total biomass.
(d) ***Chlorophyll determination.*** In the case of algae one can make a measure of chlorophyll.

AQUATIC MICROORGANISMS

As previously stated, aquatic microbiology is the study of microbial life in fresh, estuarine, and marine waters. It includes the microbiology of lakes, ponds, streams, estuaries, and the sea. it is an all-inclusive term for the study of microorganisms in natural waters. The microbiology of freshwaters constitutes a part of the science of limnology, which is the study of the flora and conditions for life in lakes, ponds, and streams.

Lakes and Ponds

Lakes and ponds have a characteristic zonation and stratification (see Fig. 26-11). There is usually a fairly large littoral zone along the shore, which has considerable rooted vegetation and includes regions where light penetrates to the bottom. In open areas, the limnetic zone is determined by the light-compensation level (depth of effective light penetration). Photosynthetic activity decreases progressively in the deeper regions of the open water (profundal

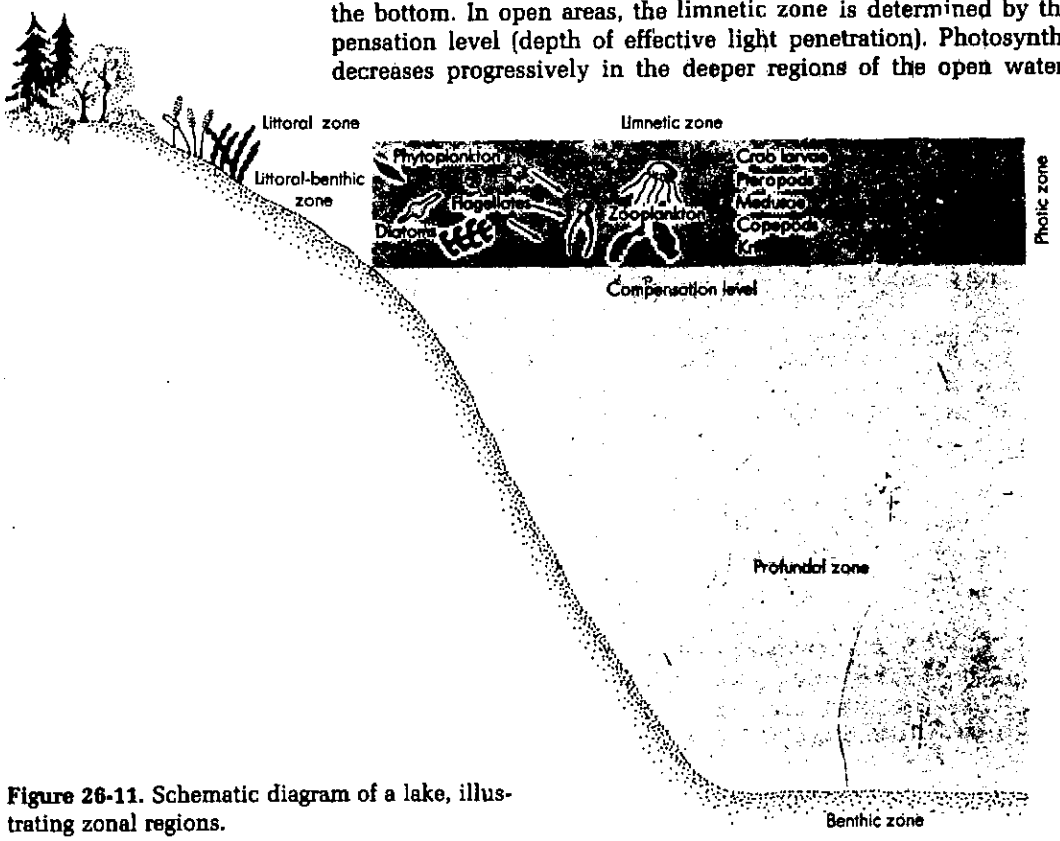


Figure 26-11. Schematic diagram of a lake, illustrating zonal regions.

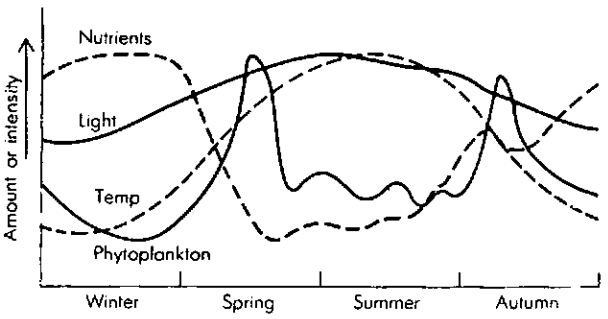


Figure 26-12. Occurrence of "blooms" or phytoplankton "pulses" in northern temperate lakes in spring and autumn. The combination of conditions—nutrient concentration, light, and temperature—accounts for this phenomenon. *(Courtesy of E. P. Odum, Fundamentals of Ecology, 3d ed., Saunders, Philadelphia, 1971.)*

zone). The **benthic zone** is composed of soft mud or ooze at the bottom. Together with the profundal zone, the benthic region is largely populated by heterotrophic organisms. When the benthic zone is composed primarily of organic material, the majority of organisms will be anaerobic decomposers. The greatest variety of physiological types is found in the limnetic and littoral zones, and in addition they constitute the most productive regions. Productivity, of course, is affected by the chemical nature of the basin and the nature of imported materials from streams and rivers. Lakes and ponds of the temperate region exhibit interesting seasonal changes in their microbial populations due to stratification of the water as a result of temperature differences. Such stratification acts as a barrier to nutrient and oxygen exchange, especially in still water. In the summer, the top layers tend to be warmer than the lower regions, but in the winter, ice, which is less dense, collects on the top; therefore, a reversal of temperature and mixing occurs in the spring and in the fall, often resulting in massive growth of algae (**bloom**) (see Fig. 26-12). Lakes or ponds enriched with nutrients, particularly nitrogen and phosphorus, a process referred to as **eutrophication**, are likely to support excessive algal growth.

Streams

Streams obtain a majority of nutrients from the flow of inorganic and organic materials from the surrounding terrestrial system or lakes or ponds. To a major extent, the microbial flora reflects the immediate terrestrial conditions, including the effects of agricultural and industrial practices. The drastic environmental changes in streams and rivers created by rapidly expanding urbanization on the one hand and changes in farming practices on the other make it impossible to generalize upon typical or characteristic microbial flora.

Estuaries

An **estuary** is a semienclosed coastal body of water which has a free connection with the open sea. Stated differently, it is the coastal adjunct of the marine ecosystem. Compared with ocean waters the estuary lacks constancy in many characteristics. Estuaries are complex systems which receive inputs from a variety of sources. Temperature, salinity, turbidity, nutrient load, and other conditions fluctuate over a wide gradient in space and time. Some characteristics of the Chesapeake Bay, one of the world's major estuarine systems, serve to illustrate the variations in an estuary.

1 The Bay serves as the receiving basin for nine major rivers, draining much of southern New York State, Pennsylvania, Maryland, and Virginia.

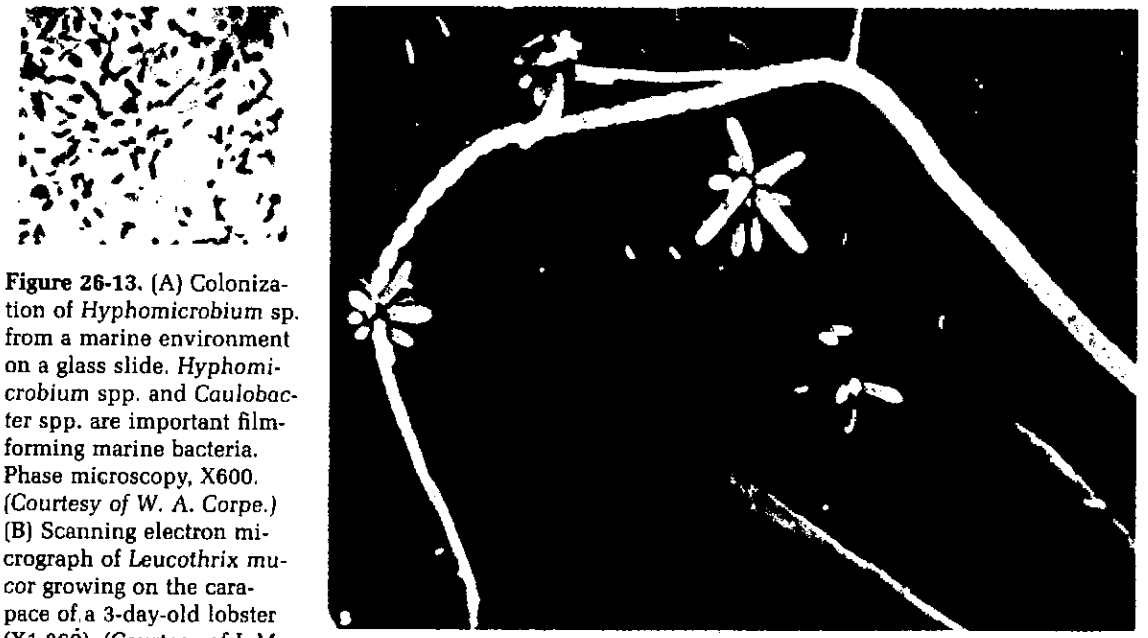


Figure 26-13. (A) Colonization of *Hyphomicrobium* sp. from a marine environment on a glass slide. *Hyphomicrobium* spp. and *Caulobacter* spp. are important film-forming marine bacteria. Phase microscopy, X600. *(Courtesy of W. A. Corpe.)* (B) Scanning electron micrograph of *Leucothrix mucor* growing on the carapace of a 3-day-old lobster (X1,860). *(Courtesy of J. M. Sieburth, Microbial Seascapes, University Park Press, Baltimore, 1975.)*

2 It has a shoreline of 4,600 miles, which includes highly industrialized areas, residential areas, farmlands, and uninhabited marshlands.
3 The salinity varies from less than 1 percent in the tributaries to 3.5 percent (normal seawater) at the mouth of the Bay. It is estimated that an estuary system is filled approximately half by seawater and half by river water. Unlike rivers, estuaries are subject to tidal changes.
4 The Bay, directly or indirectly, is subject to the activities of a multimillion population of humans who reside within the region. Practices associated with agriculture, commerce, industry, and recreation influence the conditions of the Bay.

From these observations it is apparent that the microbial flora of the estuary is subject to considerable fluctuation. Some species are indigenous to specific ecological niches of the estuary; others are transient, having been added from domestic, industrial, agricultural, or atmospheric sources. In areas receiving domestic pollution rich with organic nutrients, predominant bacteria include coliforms, fecal streptococci, and species of *Bacillus*, *Proteus*, *Clostridium*, *Sphaerotilus*, *Beggiatoa*, *Thiothrix*, *Thiobacillus*, and many others. Viruses of the enteric group are also likely to be found. In regions of the estuary that are nutritionally poor, one is likely to find the budding and/or the appendaged bacteria, e.g., *Hyphomicrobium*, *Caulobacter*, and *Gallionella*, in addition to pseudomonads (see Fig. 26-13). Soil bacteria, e.g., *Azotobacter*, *Nitrosomonas*, and *Nitrobacter*, are also likely to be present. Numerous fungi (*Ascomycetes*, *Phycomycetes*, and *Fungi Imperfecti*) occur in various regions of the estuary.

The Sea

Microorganisms are found at all depths and at all latitudes in seawater. They

occur in plankton and in the sediment of the ocean floor. The great volume of the open sea provides an environment with less variations in conditions than the other aquatic waters discussed. By the same token, the process of obtaining samples for study from the sea, including bottom sediments, presents significant technical problems.

Marine Plankton

The phytoplankton population comprises numerous species of diatoms, cyanobacteria, dinoflagellates, coccolithophores, silicoflagellates, chrysomonads, cryptomonads, and chlamydomonads. This group of microorganisms is chiefly responsible for the conversion of radiant energy to chemical energy—energy stored in chemical substances that accumulate in the sea. The magnitude of this accomplishment is revealed by a calculation that suggests a requirement of 50 billion metric tons of phytoplankton to support the growth of the potential world fish catch, estimated at 50 million metric tons.

Planktonic algae, under certain environmental conditions, may grow into enormous populations with resultant discoloration of the water—a condition referred to as **bloom** (see Chap. 18). The characteristic color of the Red Sea is associated with heavy blooms of a cyanobacterium, *Oscillatoria erythraea*, which contains the pigments phycoerythrin and phycocyanin. "Red tides" are likewise due to the explosive growth of certain planktonic species. Brown, amber, or greenish-yellow discoloration of extensive areas of water occur as a result of blooms by other microorganisms.

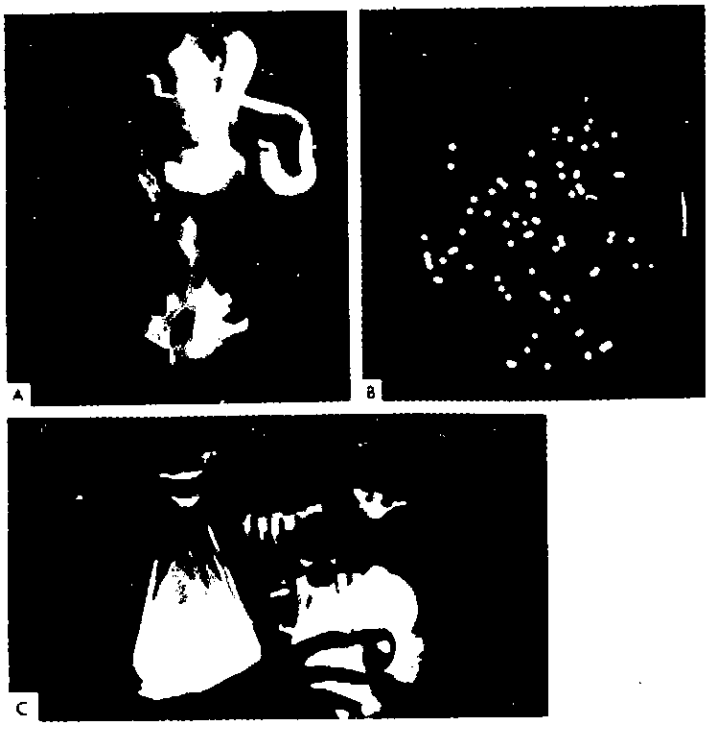


Figure 26-14. Bioluminescence, the chemical emission of light by organisms, is characteristic of many marine forms of life. (A) A saltwater squid is visible in the dark as a result of the development of luminous bacteria on its surface. (B) Colonies of luminous bacteria as they appear in the dark. (*From W. D. McElroy and H. H. Seliger, "Biological Luminescence," Sci Am, 207:76–89, December 1962. Reprinted with permission. Copyright © 1962 by Scientific American, Inc. All rights reserved.*) (C) A photograph taken by the light of the luminous bacterium *Photobacterium phosphoreum*. This bacterium is found in seawater, on the surface and in the alimentary tract of some marine fishes, and in the luminous organs of some fish and cephalopods. (*Courtesy of F. H. Johnson, Princeton University.*)

The bacterial population throughout the photosynthetic zone is closely related to the distribution of phytoplankton. The beneficial effect of the plankton may be attributed to both the organic substances they elaborate and the solid surfaces provided for bacterial aggregation. Bacterial populations differ widely with prevailing conditions. The temperature of the marine environment and the degree of salinity would be suitable for the growth of psychrophilic and halophilic physiological types. Most marine bacteria, however, are halotolerant or slightly to moderately halophilic. Among the psychrophilic forms are certain luminous bacteria which can produce light in the presence of oxygen. These bacteria may exist in symbiotic association with certain species of marine animals (see Fig. 26-14). In general, aquatic bacteria tend to be Gram-negative. It is thought that the Gram-negative envelope provides a structure better suited to support life in nutritionally dilute aquatic environments than the Gram-positive cell wall. Important hydrolytic enzymes are retained in the periplasmic space, rather than being excreted and lost to the aquatic environment, as would be the case for Gram-positive bacteria. In addition, lipopolysaccharide (LPS) of the outer membrane affords Gram-negative bacteria protection from certain toxic molecules, e.g., fatty acids and antibiotics, and it may serve to sequester important nutrients from the water. Species of the following genera are commonly found in freshwater: *Pseudomonas, Flavobacterium, Aeromonas*, and *Alcaligenes*. Common to marine and estuarine waters are *Vibrio, Acinetobacter, Pseudomonas, Flavobacterium, Alteromonas*, and *Staphylococcus*. Bacteria in the surface region of the marine environment are often pigmented, a characteristic which may afford protection from the lethal portion of solar radiation. Mold spores and mycelium fragments are present in seawater throughout the photosynthetic zone. Species of *Deuteromycetes, Phycomycetes*, and *Myxomycetes* have been isolated from marine environments. Although the yeasts have not been extensively studied, they probably have a role similar to bacteria.

Protozoa (species of *Foraminifera* and *Radiolaria*, as well as many flagellated and ciliated species) are present in large numbers in the region inhabited by the phytoplankton. These zooplankton animals feed ("graze") upon phytoplankton organisms, bacteria, or detritus. Observations indicate that many zooplankton avoid light, exhibiting diurnal migrations. At night the animals graze on phytoplankton at the surface, and during the day they sink below the photic zone.

The microbial population is sparse near the surface of the sea because the intensity of illumination is inhibitory. Beneath the region of photic activity there exists another region inhabited during the day by vertically migrating zooplankton. The bacterial population is distributed more or less uniformly throughout and below these layers, feeding on descending organic material and other nutrients.

The area between these upper strata and the area just above the sea floor is relatively barren, a vast microbiological oceanic desert region. The bottom of the sea is populated by a variety of microorganisms described below.

The Benthic Population

Offshore sediments are inhabited by bacteria and protozoa. Large numbers are present at the mud-water interface; the bacterial population may range from a few hundred to millions per milliliter. The counts in sediments are as high as

Table 26-2. Bacteria Isolated from the Pacific Ocean, Grouped into Physiological Types

Bacteria per Gram of Sediment (Wet Basis)	Sample 8160 at 32°51.2′ N, 117°28.3′ W and depth of 780 m	Sample 8330 at 33°25.9′ N, 118°06.5′ W and depth of 505 m	Sample 9309 at 33°44.2′ N, 118°46.1′ W and depth of 1,322 m
Total aerobes, plant count	930,000	31,000,000	8,800,000
Total anaerobes, oval-tube count	190,000	2,600,000	1,070,000
Ammonification:			
Peptone → NH_4	100,000	1,000,000	1,000,000
Nutrose → NH_4	10,000	1,000,000	100,000
Urea fermentation, urea → NH_4	100	+	1,000
Proteolysis:			
Gelatin liquefaction	100,000	10,000,000	1,000,000
Peptone → H_2S	10,000	1,000,000	100,000
Denitrification, $NO_3 \rightarrow N_2$	100	10,000	10,000
Nitrate reduction, $NO_3 \rightarrow NO_2$	100,000	10,000,000	10,000
Nitrogen fixation	0	0	0
Nitrification, $NH_4 \rightarrow NO_2$	0	0	0
Sulfate reduction, $SO_4 \rightarrow H_2S$	1,000	1,000	10,000
Dextrose fermentation	10,000	100,000	1,000
Xylose fermentation	10,000	+	10,000
Starch hydrolysis	10,000	100,000	10,000
Cellulose decomposition	1,000	+	1,000
Fat hydrolysis (lipoclastic)	1,000	+	+
Chitin digestion	100	+	+

SOURCE: C. E. Zobell, *J Sediment Petrol* 8:10, 1938.

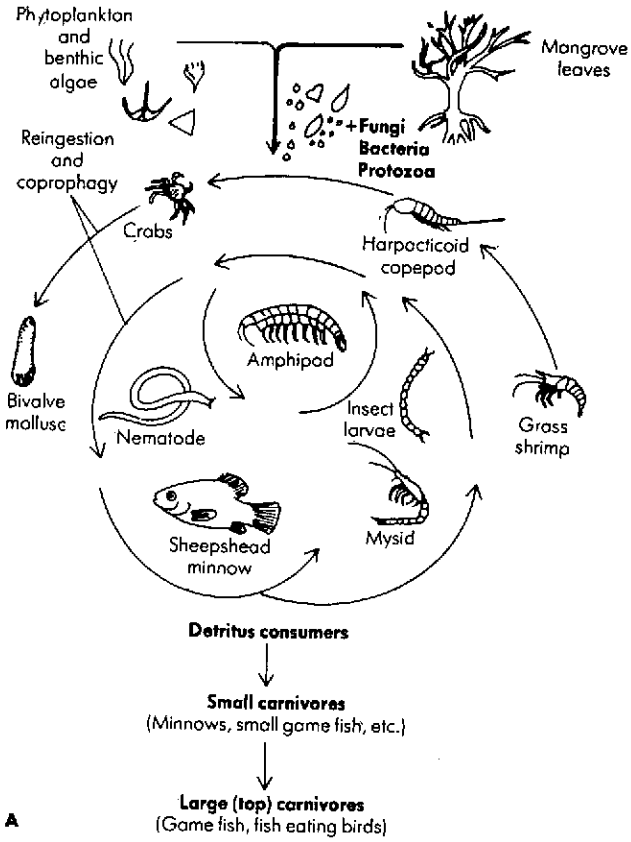
10^8 bacteria per gram. Bacteria from marine bottom deposits represent a variety of physiological types, as indicated by the data in Table 26-2. Many are facultative or strict anaerobes.

THE ROLE AND IMPORTANCE OF AQUATIC MICROBIAL ECOSYSTEMS

Aquatic life exhibits a vast complex of interactions among microorganisms and between microorganisms and macroorganisms—both plant and animal. Microorganisms, particularly algae and protozoa, occupy a key role in the food chain of the aquatic environment. Numerous bacterial species perform a variety of biochemical changes in various substrates that allow the recycling of elements and nutrients.

PRODUCTIVITY OF AQUATIC ECOSYSTEMS

The primary producers in the marine system are cyanobacteria and eucaryotic algae which predominate in the phytoplankton. Through photosynthesis they are capable of transforming radiant energy into chemical energy (organic compounds). The biological activity of an aquatic ecosystem is indeed dependent upon the rate of primary production performed by the photosynthetic organisms. In shallow estuaries the role of photosynthetic organisms as primary producers is considerably reduced. Plant growth from the shoreline contributes leaves, stems, and roots of vegetation and other organic detritus.



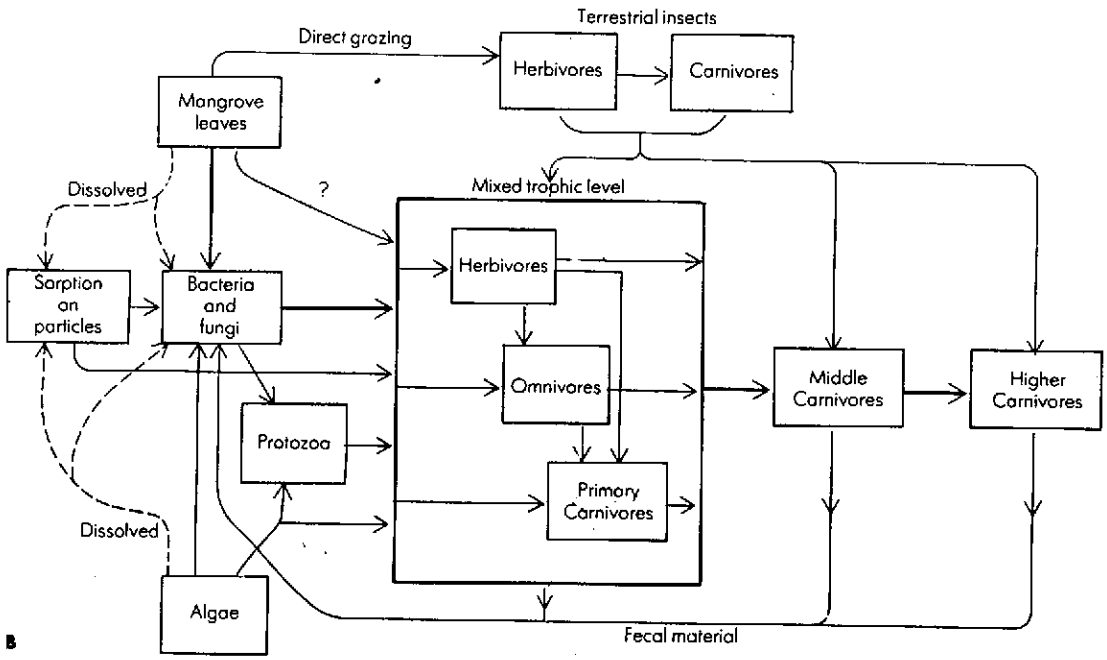


Figure 26-15. (A) A schematic diagram of the detritus-consuming omnivorous organisms of the North River estuary. The cyclical nature of the diagram depicts the utilization and reutilization of detritus particles in the form of fecal material. (B) A conceptual model of the North River food web showing the most important flow of energy as a broad arrow, less important food chains as narrow arrows, and the pathway of dissolved-leaf material as a dashed line. [*Courtesy of W. E. Odum and E. J. Heald, "Sources and Fates of Nutrients of the Pamlico River Estuary, North Carolina," in L. E. Cronin (ed.), Estuarine Research, vol. 1, Academic, New York, 1975.*]

Food Web in a Shallow Estuary

The role of microorganisms in a shallow-estuary food web is shown in Fig. 26-15. Phytoplankton and benthic algae make a small contribution to the food supply. The organic vegetation is degraded by bacteria and fungi and converted to microbial protein which may serve as nutrients for protozoa. However, the estuary contains many detritus consumers (herbivorous and omnivorous crustaceans, mollusks, insect larvae, nematodes, polychaetas, and a few fishes). They derive their energy from vascular plant material. The food web in a deep estuary is more like that in the marine environment.

Fertility of the Ocean

Plankton, particularly phytoplankton, has been referred to as the "pasture of the sea." Fish, whales, and squids feed directly on plankton or on larger plankton-feeding animals. The term **fertility of oceans** is used to express the capacity for the production of organic matter by the organisms present in these waters. The terrestrial environment produces 1 to 10 g of dry organic matter per square meter per day compared to 0.5 g for the deep ocean areas. Nevertheless, the oceanic area is so much larger than the productive land area that this difference is inconsequential and the total productivity of the oceans vastly exceeds that of the land. This fertility depends primarily upon the production of phytoplankton. Growth of the phytoplanktonic organisms is dependent upon radiant energy, carbon dioxide, water, inorganic nitrogen and phosphorus compounds, and several elements in trace amounts. The factors generally limiting growth are radiant energy, nitrogen and phosphorus, and the trace elements. The nitrogen and phosphorus and trace elements are made available through the mineralization reactions of microorganisms, particularly bacteria. As previously mentioned, this involves the dissimilation of organic substrates contributed by the metabolism of marine organisms or the tissues of these organisms following their death. Thus we note that there is a cycle of events in meeting the food

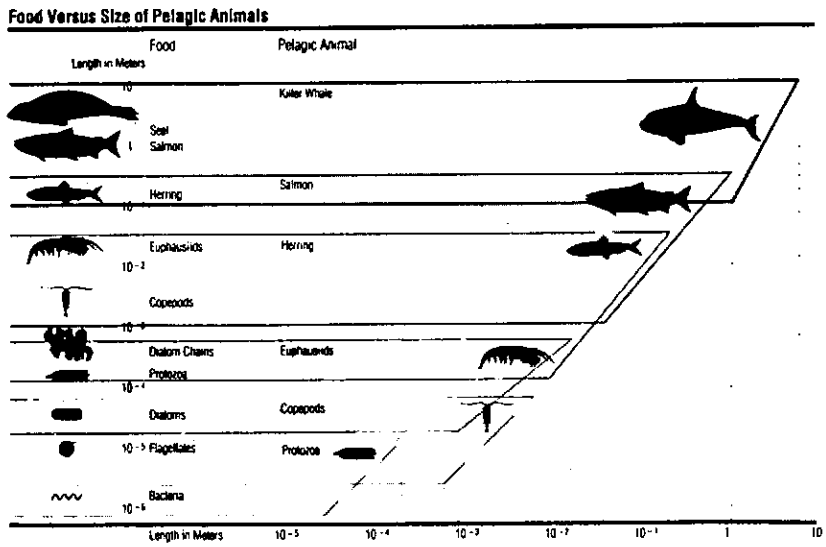


Figure 26-16. Size ranges of pelagic animals from immaturity to adulthood, and of their prey. Sizes are expressed in powers of ten on a logarithmic length scale. Length is a good measure of size in this example because predator-prey relationships often depend on length-related physical properties, such as jaw size or swimming speed. *(After Dexter et al., 1981.) (Courtesy of R. M. Strickland, The Fertile Fjord, Plankton in Puget Sound, Puget Sound Books, University of Washington Press, Seattle, 1983.)*

requirements of various levels of life in the sea and that microorganisms are indispensable to the process.

The Antarctic Ocean is described as being richer in life than any other major oceanic area. The richness of nutrients in this region is attributable to the mixing of the waters of the Atlantic, Pacific, and Indian Oceans. This mixing action is brought about by the movement of the bottom current of cold water that runs outward from the continental shelf of the Antarctica.

An abundance of nutrients in the upper layers results in a large phytoplankton crop. The red shrimplike crustacean *Euphausia superba* (commonly called krill) feeds upon the phytoplankton; in turn, this organism serves as food for fish, penguins, sea birds, seals, and whales. In this environment the krill represents the key organism in the food chain; it is the link between photosynthetic planktonic life and higher forms of life characteristic of the region. The sequence of organisms which serve as food for prey in the food chain is shown in Fig. 26-16, where the food organism is identified and the pelagic animal that feeds upon it. Although coastal and estuarine regions provide a less stable physical environment, their fertility exceeds that of the open sea because of the large amounts of nutrients available.

BIOGEOCHEMICAL TRANSFORMATIONS

A major biochemical activity of bacterial flora is the dissimilation and mineralization of organic matter, i.e., the dissimilation of organic compounds to carbon dioxide, water, and various inorganic salts. All classes of organic substrates are available; they originate from the metabolic processes of the plant and animal life and from the cells of the dead organisms. Under aerobic conditions the principal products resulting from dissimilation of organic compounds are ammonium, carbon dioxide, sulfate, and phosphate. These can be recognized as nutrients for plant growth, including the phytoplankton. Anaerobic degradation yields more reduced products such as methane, hydrogen, and hydrogen sulfide in addition to ammonia, carbon dioxide, and phosphate.

Biochemical Cycles

The transformation of elements to organic compounds and the subsequent release of elements for conversion to inorganic compounds and reutilization occurs in the aquatic environment. Among the better understood cyclic processes are:

The nitrogen cycle
The carbon cycle
The sulfur cycle
The phosphorus cycle

The principal microbial reactions that occur in these cyclic processes were described in Chap. 25. In each instance, microorganisms are responsible for releasing the element (e.g., nitrogen) from an organic compound through a series of reactions mediated by various species of microorganisms, particularly bacteria. The element, in the form of an inorganic compound, becomes available again as a plant nutrient.

In the biogeochemical cycles referred to above, many complex materials are dissimilated by microorganisms. For example, cellulosic material is abundant

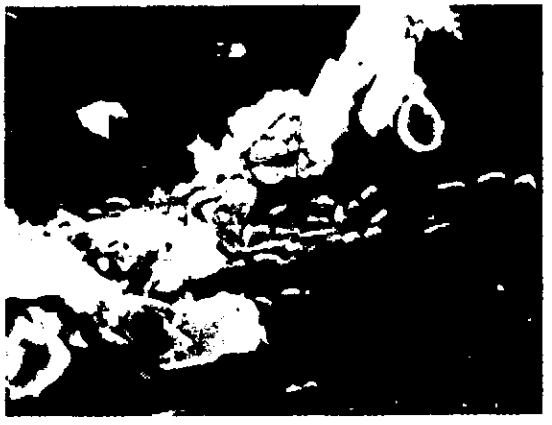


Figure 26-17. Marine bacteria attacking cellulose. The bacteria shown here by scanning electron microscopy are degrading a cellulose (dialysis) membrane (X 1,700). *(Courtesy of J. M. Sieburth, Microbial Seascapes, University Park Press, Baltimore, 1975.)*

Figure 26-18. Marine bacteria (bacilli and spirochetes) growing on a salmon tail and degrading the tissue. *(Courtesy of J. M. Sieburth, Microbial Seascapes, University Park Press, Baltimore, 1975.)*

in waters and is also important in the sea. Cellulolytic aerobic bacteria degrade the cellulose to the sugars cellobiose or even glucose. These sugars are readily metabolized by many other bacteria. An illustration of aquatic microorganisms attacking cellulose is shown in Fig. 26-17. Similarly, proteolytic microorganisms degrade proteins to peptides and amino acids; further dissimilation results in liberation of nitrogen as ammonia. Figure 26-18 illustrates the microbial growth on the flesh of a salmon tail.

Marine Sediments

Large amounts of diatomaceous materials settle to the bottom of marine environments. Diatoms are characterized by a cell wall of silica. The thickness of the siliceous wall may vary greatly from very thin to very thick depending on the benthic species. Radiolaria and silicoflagellates also have siliceous skeletons. Foraminifera and coccolithophores produce calcareous skeletons. These organisms are important members of the plankton. When they die, they gradually sink to the bottom, where the undissolved calcareous and siliceous skeletons accumulate. Vast beds of these materials, sometimes thousands of feet thick, occur in various regions of the sea. The chalk beds of England and France are chiefly the remains of foraminifera, and a large part of the Monterey series at Lompoc, California, consists of the remains of diatoms. Bacteria, particularly

cyanobacteria, are involved in the precipitation of calcium carbonate, which also leads to limestone formation.

The deposition and transformation of iron and manganese in sediments and sulfur deposits, like those found in the Gulf Coast areas of Texas and Louisiana, are closely linked to microbial activity. Petroleum deposits are formed from accumulated and buried organic materials. Microorganisms are believed to play the major role in these processes.

QUESTIONS

1 What is plankton? Phytoplankton? Zooplankton? Name some organisms that belong to each category.
2 What is the Gram reaction of most aquatic bacteria? What explanation is suggested to account for this?
3 Define an estuary.
4 Discuss some of the physical and chemical properties of the aquatic environment in terms of their effects upon microbial growth and survival.
5 What is meant by the term *upwelling*? What effect does upwelling have on productivity of an ocean area? What is a gyre?
6 Describe several techniques that are particularly useful for enumeration and identification of microorganisms from aquatic sources.
7 What occurs when a lake becomes eutrophic?
8 What comparisons can be made between the open sea and offshore oceanic areas with respect to microbial flora?
9 Describe several types of biochemical changes brought about by microorganisms in marine environments. What occurs during the process of mineralization?
10 What contributes to the fertility of the ocean? What does the term *pasture of the sea* refer to?
11 What is referred to by the term *primary producers*?
12 Compare and contrast the fertility of the oceans and inland lakes and rivers.
13 Blooms in lakes usually occur in the spring and fall. Why?
14 Name several geologically important transformations caused by marine microorganisms.

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Chapter 27 Microbiology of Domestic Water and Wastewater

OUTLINE

The drinking water of most communities and municipalities is obtained from surface sources—rivers, streams, and lakes. Such natural water supplies, particularly streams and rivers, are likely to be polluted with domestic and industrial wastes, i.e., the used water of a community (wastewater). Many city dwellers (whose water comes from the rivers) are not aware that a considerable portion of their drinking water may have been used earlier for domestic or industrial purposes. Municipal water-purification systems have been very effective in protecting the inhabitants against polluted water. At the same time, as population centers grow, pollution problems become more serious. A greater quantity of water is required, and the used water must be disposed of, generally by returning it to a natural body of water in the vicinity, which in turn may be the water supply source of another community or municipality.

As a potential carrier of pathogenic microorganisms, water can endanger health and life. The pathogens most frequently transmitted through water are

those which cause infections of the intestinal tract, namely, typhoid and paratyphoid bacteria, dysentery (bacillary and amoebic) and cholera bacteria, and enteric viruses. The causative organisms of these diseases are present in the feces or urine of an infected person, and when discharged may gain entrance into a body of water that ultimately serves as a source of drinking water. The incidence of waterborne diseases is discussed in Chap. 35.

It is therefore necessary to employ (1) treatment facilities that purify wastewater prior to its disposal and (2) water-purification methods that provide safe drinking water.

In the first part of this chapter we shall discuss water purification, i.e., ways of providing safe drinking water, the bacteriological examination of water, and methods of determining whether drinking water is free of infectious microorganisms. The second part deals with wastewater disposal systems.

Wastewater treatment procedures, both natural and artificial, are largely dependent upon microbial activity to eliminate or greatly reduce the development of hazardous or objectionable situations.

WATER PURIFICATION

Water that is free of disease-producing microorganisms and chemical substances deleterious to health is called **potable** water. Water contaminated with either domestic or industrial wastes is called **nonpotable** or **polluted** water. The objectives of primary concern in providing potable water are freedom from harmful microorganisms and freedom from undesirable or harmful chemicals. These standards apply both to wells or springs serving single families and to water systems serving hundreds of thousands of persons. However, the processes of purifying water are quite different in these two extreme situations.

Individual Water Supplies

Underground sources—wells and springs—provide most of the water for individual homes in rural areas. Rainwater caught and stored in cisterns is also used to a limited extent; surface water, however, should not be used for drinking purposes unless it is subjected to purification, since there is constant danger of contamination and consequent transmission of disease. Since it is generally impractical to treat surface waters so as to ensure a continuous safe supply for rural domestic purposes, our discussion is limited to water obtained from wells and springs.

As it penetrates through the layers of soil, water from wells and springs undergoes filtration, which removes suspended particles, including microorganisms. It is of prime importance that the supply of groundwater selected be located a safe distance from possible sources of contamination, e.g., pit privies, cesspools, septic tanks, and barnyards.

Municipal Water Supplies

The following information about American public water systems, taken from an article by H. E. Jordan which appeared in the 1955 Yearbook of Agriculture, "Water," illustrates the progress that has been made in this area:

At Bethlehem, Pa., in 1754, Hans Christiansen, who was an immigrant millwright, began work on the first pumped public water works, which was put into regular operation in 1755. The supply provided at Winchester, Va., in 1799 appears to

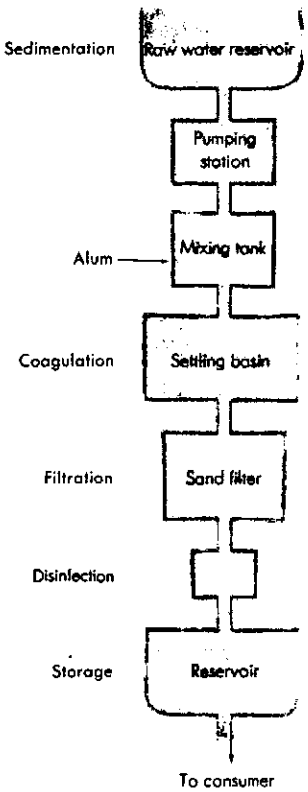


Figure 27-1. Flow diagram of usual procedures in municipal water-purification plant.

have been the first complete works developed as a public enterprise. Seventeen cities were served by public water works in 1800 and 83 at the end of 1850.

In operation in 1900 were 3,300 water works. In 1950 approximately 17,000 communities were served by public systems. . . .

Two-thirds of the nation's population is now served by public water works systems. . . . That service, which involves well-supervised production and distribution of safe water, must be given some of the credit for all but wiping out the scourge of typhoid fever of 50 years ago and for virtually eliminating epidemics of water-borne dysentery.

The principal operations employed in a municipal water-purification plant to produce water of a quality safe for human consumption are sedimentation, filtration, and disinfection (see Fig. 27-1). Sedimentation occurs in large reservoirs, where the water remains for a holding period; large particulate matter settles to the bottom. Sedimentation is enhanced by the addition of alum (aluminum sulfate) at the treatment plant, which produces a sticky flocculent precipitate. Many microorganisms, as well as finely suspended matter, are removed as this precipitate descends through the water in the settling basins. The water is next passed through sand filter beds, a process which removes 99 percent of the bacteria. Subsequently, the water is disinfected to ensure its potability. The majority of municipal water-treatment facilities employ chlorination for disinfection. The chlorine dosage must be sufficient to leave a residual of 0.2 to 2.0 mg/liter free chlorine. Other disinfection methods include ozonation and irradiation with ultraviolet light.

The purification process may include procedures for removing minerals that cause the water to be hard, adjusting the pH if the water is too acid or alkaline, removing undesirable colors or tastes, and adding fluoride for the prevention of dental caries.

DETERMINING SANITARY QUALITY

Water can be perfectly clear in appearance, free from peculiarities of odor and taste, and yet be contaminated. Obviously special procedures are necessary to determine its sanitary quality.

Sanitary Surveys

Inspection of a water-producing system by a qualified sanitarian or engineer is called a sanitary survey. It includes inspection of (1) the source of the raw water and the conditions that may influence its quality, (2) the operation of the water-purification plant or the construction of the well, and (3) the mechanism for distributing the water to the consumers. Conditions in a community or municipality that may influence the quality of water are not static. There may be changes in population, types of industry, and the quantity of sewage and the manner in which it is disposed; consequently, periodic and comprehensive sanitary surveys are necessary. Data obtained from these surveys are of considerable value, both from the standpoint of indicating any changes in operation that may be necessary to prevent complications from developing and of finding the sources of difficulties that may appear unexpectedly.

Sanitary surveys reveal whether water is being produced under conditions in which potable water would ordinarily be produced. However, potability can be determined only by chemical and bacteriological laboratory tests. Chemical analysis indicates whether water is polluted and provides other useful information as well; however, it is not sensitive or specific enough to detect minor degrees of sewage contamination. On the other hand, bacteriological tests have been designed which are extremely sensitive and specific in revealing evidence of pollution.

Bacteriological Evidence of Pollution

One might assume that the objective in the routine analysis of water would be to search for pathogenic microorganisms. This is not true, however, for the following reasons:

1 Pathogens are likely to gain entrance into water sporadically, and they do not survive for long periods of time; consequently, they could be missed in a sample submitted to the laboratory.
2 If they are present in very small numbers, pathogens are likely to escape detection by laboratory procedures.
3 It takes 24 h or longer to obtain results from a laboratory examination. If pathogens were found to be present, many people would have consumed the water by this time and would be exposed to infection.

It is known that the pathogens that gain entrance into bodies of water arrive there via intestinal discharges of humans or other animals. Furthermore, certain bacterial species, particularly *Escherichia coli* and related organisms designated as coliforms, fecal streptococci (e.g., *Streptococcus faecalis*), and *Clostridium perfringens*, are normal inhabitants of the large intestine of humans and other animals and are consequently present in feces. Thus the presence of any of these bacterial species in water is evidence of fecal pollution of human or animal origin. If these organisms are present in water, the way is also open for intestinal pathogens to gain entrance, since they, too, occur in feces. Since the laboratory examination of water for pathogens is beset with the disadvantages already enumerated, techniques have been developed for the demonstration of bacterial species of known excretal origin, particularly organisms of the coliform group. This approach, which has proved satisfactory in practice, has the following advantages:

1 Coliform organisms, particularly *E. coli*, are constantly present in the human intestine in large numbers. It is estimated that billions of these organisms are excreted by an average person in one day.
2 These organisms generally live longer in water than intestinal pathogens.
3 A healthy person would not normally excrete pathogenic organisms, but should an intestinal-tract infection develop, the pathogen is likely to appear in the feces. Thus, the presence of coliforms in water is regarded as a warning signal: the water is subject to potentially dangerous pollution.

The Coliform Group

The coliform group of bacteria includes all the aerobic and facultatively anaerobic, Gram-negative, nonsporulating bacilli that produce acid and gas from the fermentation of lactose. The classical species of this group are *Escherichia coli* and *Enterobacter aerogenes*. The relationship of these organisms to others of

Table 27-1. Differentiation of Typical Strains of *Escherichia coli* from *Enterobacter aerogenes* on the Basis of IMViC Reactions

Organism	Test			
	Indole	Methyl Red	Voges-Proskauer	Citrate
Escherichia coli	+	+	−	−
Enterobacter aerogenes	−	−	+	+

the enteric group—*Salmonella, Shigella, Klebsiella, Proteus, Serratia* and other genera—all of which are Gram-negative, nonsporulating bacilli, is discussed in Chap. 13. *E. coli*, as we have already pointed out, is a normal inhabitant of the intestinal tract of humans and other animals. *Ent. aerogenes* is most frequently found on grains and plants but may occur in human and animal feces. These species bear a very close resemblance to each other in their morphological and cultural characteristics. Consequently, it is necessary to resort to biochemical tests for differentiation. Tests with the following four characteristics are especially important for this purpose:

1 Ability to produce indole from tryptophan. *E. coli* does, and *Ent. aerogenes* does not.
2 Amount of acidity produced in a special glucose-broth medium and detected by the pH indicator methyl red. Both organisms produce acid from glucose. However, *E. coli* produces a lower pH, which turns the indicator red, whereas *Ent. aerogenes* cultures do not produce as large an amount of acid and thus do not produce the color change.
3 Ability to produce the compound acetylmethylcarbinol in a glucose-peptone medium. This chemical is detected by the Voges-Proskauer test procedure. *E. coli* does not produce acetylmethylcarbinol, but *Ent. aerogenes* does.
4 Utilization of sodium citrate. *Ent. aerogenes* is capable of utilizing sodium citrate as its sole source of carbon; i.e., it will grow in a chemically defined medium in which sodium citrate is the only carbon compound. *E. coli* does not grow under the same circumstances.

For convenience, these tests collectively are designated as the **IMViC reactions** (I = indole, M = methyl red, Vi = Voges-Proskauer reaction, and C = citrate). The reactions for a typical strain of each species are shown in Table 27-1. The reactions for all coli-aerogenes organisms are unfortunately not as clear-cut as those described here. Some cultures give other combinations of reactions to this scheme of testing and are usually referred to as intermediate types. Furthermore, there are additional coliform species in the genera *Klebsiella* and *Citrobacter* for which more detailed biochemical, genetic, and immunologic data are needed for identification.

Bacteriological Techniques

Methods of examining water bacteriologically are set forth in the book *Standard Methods for the Examination of Water and Wastewater*, prepared and published jointly by the American Public Health Association, the American Water Works Association, and the Water Pollution Control Federation. The Environmental Protection Agency (EPA) has also developed a manual for this purpose under the title *Microbiological Methods for Monitoring the Environment—Water and Wastewater*. It is essential that strict attention be given to the following details when water samples are submitted for bacteriological analysis:

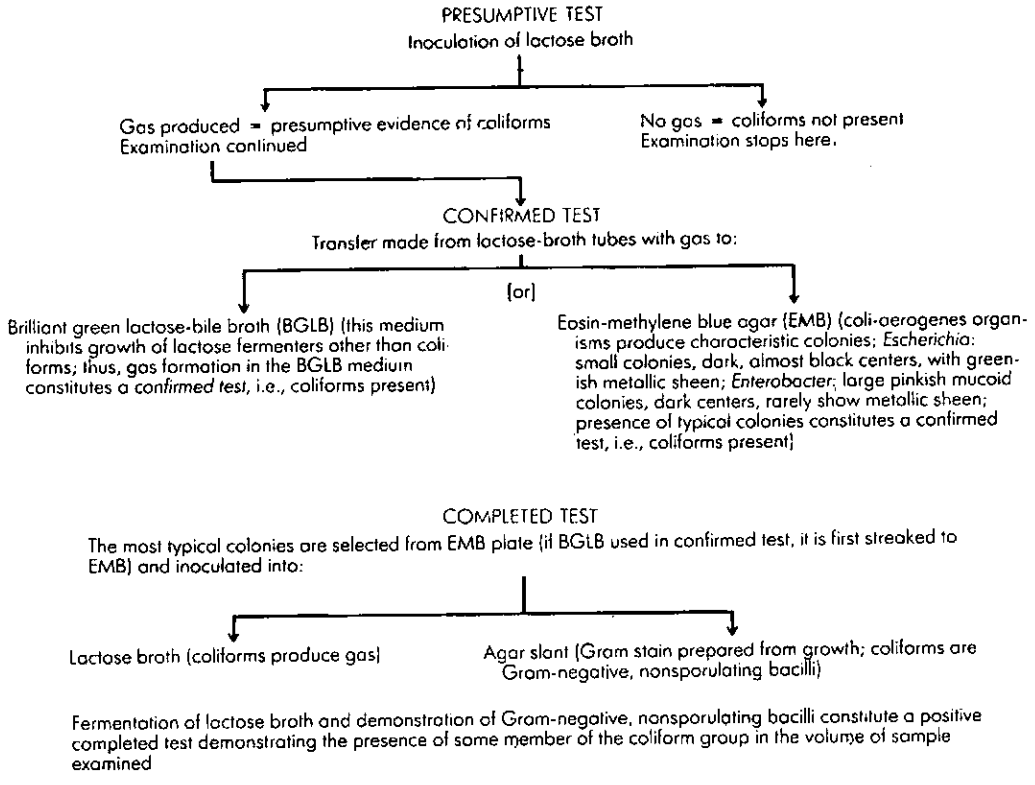


Figure 27-2. General scheme of laboratory testing for detection of coliform group in water.

1 The sample must be collected in a sterile bottle.
2 The sample must be representative of the supply from which it is taken.
3 Contamination of the sample must be avoided during and after sampling.
4 The sample should be tested as promptly as possible after collection.
5 If there is a delay in examination of the sample, it should be stored at a temperature between 0 and 10°C.

The routine bacteriological procedures consist of (1) a plate count to determine the number of bacteria present and (2) tests to reveal the presence of coliform bacteria.

Standard Plate Count

Colony counts are performed after plating aliquots of the water sample. The interpretation of the results of the **standard plate count** must take into account the fact that the presence of a few pathogenic microorganisms is more significant than water containing many saprophytic bacteria. However, water of good quality is expected to give a low count, less than 100 per milliliter. Plate counts are useful in determining the efficiency of operations for removing or destroying organisms, e.g., sedimentation, filtration, and chlorination. A count can be made before and after specific treatment. The results indicate the extent to which the microbial population has been reduced.

Tests for the Presence of Coliform Bacteria

Several selective and differential media greatly expedite the process of examining water for coliform organisms. The **standard microbiological** technique

involves three successive steps: (1) the **presumptive** test, (2) the **confirmed** test, and (3) the **completed** test (see Fig. 27-2).

Membrane-Filter Technique

The membrane-filter technique for the bacteriological examination of water is illustrated in Fig. 27-3 and consists of the following steps:

1 A sterile disk is placed in a filtration unit.
2 A volume of the water to be tested is drawn through this filter disk; the bacteria are retained on the surface of the membrane.
3 The filter disk is removed and placed upon an absorbent pad that has previously been saturated with the appropriate medium. Alternatively, the disk can be placed

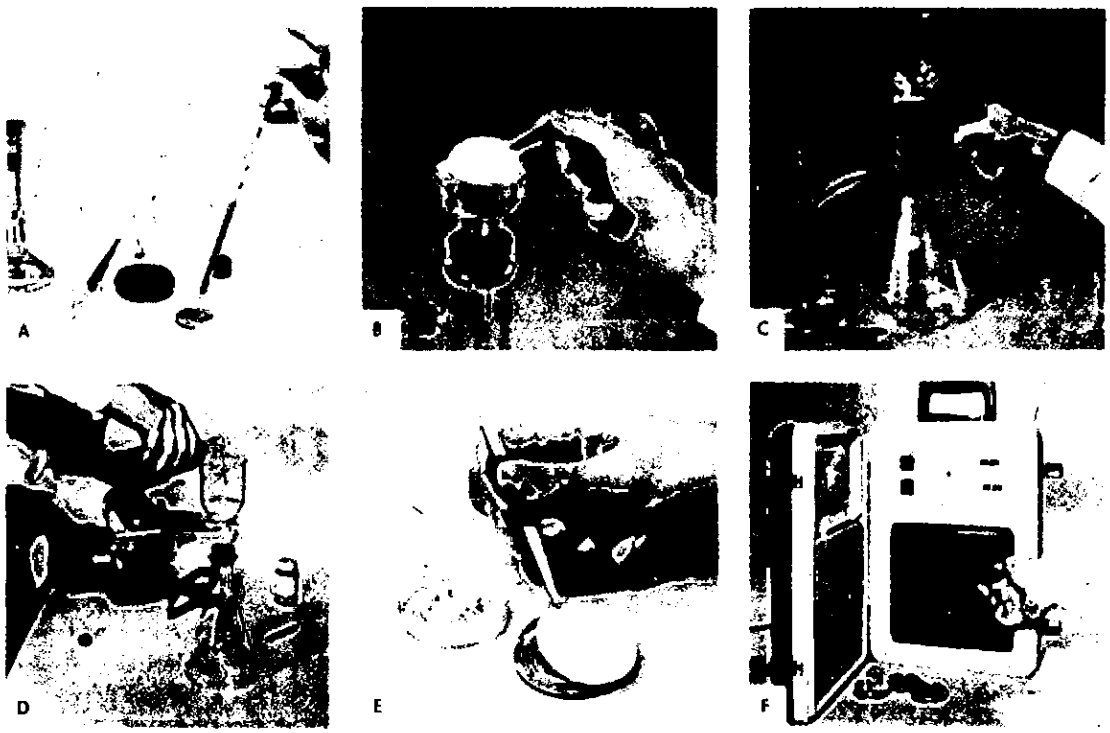
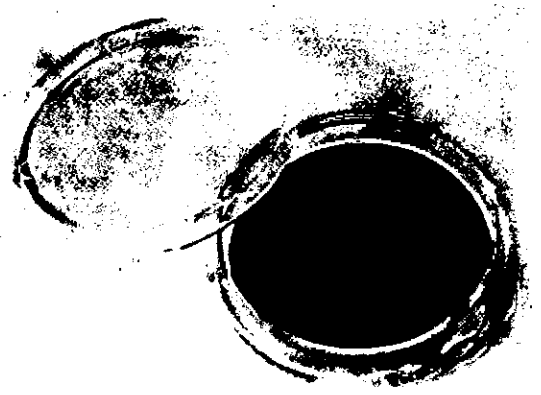
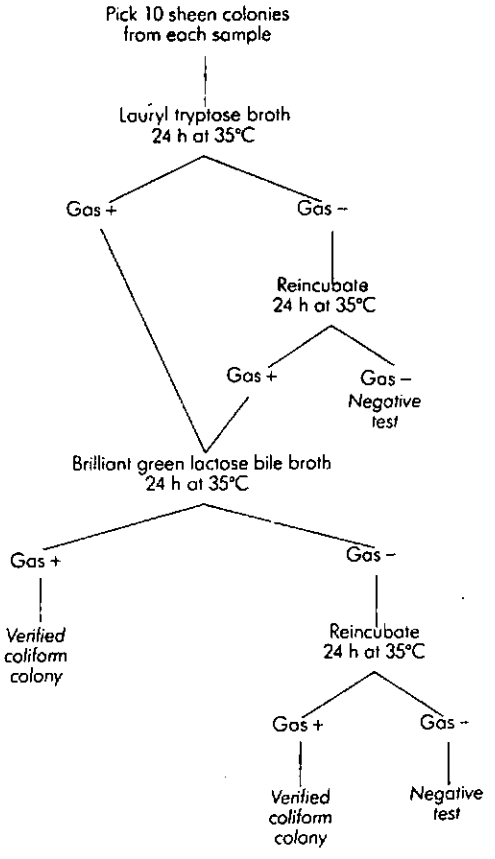


Figure 27-3. Analysis of water with the Millipore filter membrane. (A) Approximately 2 ml of Endo medium is added to the pad contained in the dish. The dish is then covered until the water sample has been filtered through the membrane. (B, C, D) The filter is placed on a filter holder and clamped in position below the funnel, and the water sample (100 ml for testing potability) is poured into the funnel and passed through the Millipore filter by the aid of a vacuum pump. (E) The funnel is removed, and the filter disk, handled with sterile forceps, is placed on the pad previously impregnated with medium (A). (F) The plates are incubated at 35°C for 20 h, at which time the number of coliform colonies can be determined. *(Courtesy of Millipore Filter Corporation.)*

Figure 27-4. (A) Coliform colonies from a water sample grown on a membrane filter with MF-Endo medium acquire an easily distinguishable green metallic "sheen" and are pink to rose red in color. *(Courtesy of Millipore Filter Corporation.)* (B) Verification of total coliform colonies on the membrane filter. *(From Microbiological Methods for Monitoring the Environment: Water and Waste, Environmental Protection Agency, Washington, D.C., 1978.)*



A



B

on the surface of an agar medium in a Petri dish. Special Petri dishes of á size to accommodate the absorbent pad and filtration disk are employed for incubation.

4 Upon incubation, colonies will develop upon the filter disk wherever bacteria were entrapped during the filtration process. (See Fig. 27-4A). Verification of the "sheen" colonies (distinctive appearance of colonies on a differential medium) is done by a procedure shown in Fig. 27-4B.

The membrane filter technique has several desirable features:

1 A large volume of water sample can be examined. Theoretically almost any volume of nonturbid water could be filtered through the disk, the organisms from any given volume being deposited on the disk.
2 The membrane can be transferred from one medium to another for purposes of selection or differentiation of organisms.
3 Results can be obtained more rapidly than by the conventional MPN standard methods.
4 Quantitative estimations of certain bacterial types, e.g., coliforms, can be accomplished when appropriate media are used.

This technique, with modifications, has been adopted for many microbiological procedures other than the examination of water.

Microorganisms Other Than Coliform Bacteria

Some microorganisms besides coliform bacteria are of intestinal origin and could also be used as indicators of fecal contamination of water, such as the fecal streptococci. Other intestinal microorganisms, such as intestinal viruses, are frank pathogens and can cause serious diseases. Still other microorganisms are regarded mainly as **nuisance organisms,** because they create problems of odor, color, and taste, or cause obstruction of water flow.

Fecal Streptococci

Fecal streptococci are enteric bacteria found in the intestines of warm-blooded animals, including humans. *Streptococcus faecalis* is representative of this group; other species are *S. faecium, S. bovis,* and *S. equinus.* Because fecal streptococci, particularly, *S. faecalis,* are abundantly present in the large intestines of humans, their occurrence in water is indicative of fecal pollution.

Slime-Forming Bacteria

Many bacteria are capable of elaborating gummy or mucilaginous materials, either as capsular structures or as extracellular excretion products. The organic and inorganic constituents of the water, which provide nutrients for the bacteria, help to determine whether slime is produced and what organisms are responsible for its production.

Iron Bacteria

The iron bacteria are one of the most important types of nuisance organisms in water. They transform soluble compounds of iron to insoluble compounds of iron (ferric hydroxide) which may be deposited in a sheath around the organism (*Sphaerotilus*) (Fig. 27-5) or secreted so as to form stalks or ribbons attached to the cell (*Gallionella*). This deposition and accumulation of insoluble material in the piping system may eventually have a significant effect on the rate of water flow. Iron bacteria can also produce slime, discolor water, and cause undesirable odors and tastes.

Sulfur Bacteria

Some of the sulfur bacteria are capable of producing and tolerating extreme acidity. Organisms of the genus *Thiobacillus* oxidize elemental sulfur to sulfuric acid and can produce an acidity in the range of pH 1; thus they may be

Figure 27-5. Long trichomes of *Sphaerotilus* appear in this photomicrograph of a slime mass. This organism causes many difficulties in industrial water systems because it forms large slime masses and iron deposits. Dark phase contrast (X640). *(Courtesy of J. M. Sharpley, Applied Petroleum Microbiology, Buckman Laboratories, Inc., Memphis, Tenn., 1961.)*

Figure 27-6. Sulfate-reducing bacteria *(Desulfovibrio)* are visible as white curved lines in this photomicrograph from a mine-water specimen. Dark phase contrast (X1,700). *(Courtesy of J. M. Sharpley, Applied Petroleum Microbiology, Buckman Laboratories, Inc., Memphis, Tenn., 1961.)*

responsible for the corrosion of pipes. *Desulfovibrio desulfuricans* (Fig. 27-6) reduces sulfates and other sulfur compounds to hydrogen sulfide.

Algae

When water is exposed to sunlight, algal growth often results; the occurrence of algae in water is much like the growth of weeds in a garden. Algae are present in all natural aquatic environments. Their nuisance characteristics involve production of turbidity, discoloration, odor, and taste in water. Algae are frequently the primary cause for the clogging of filters during water purification. The diatoms are the most important in this respect, although green and yellow algae are also involved. Aside from these nuisance characteristics, some algae are capable of producing substances toxic to humans and animals.

Viruses

Many viruses are known to be excreted from humans through the intestinal tract, and these may find their way via sewage into sources of drinking water. The enteroviruses are the ones most commonly found in sewage; they include the polio, coxsackie, and echo viruses. The virus that causes infectious hepatitis has been isolated from polluted water and shellfish; occurrence of this disease has been traced to these sources. Rotaviruses are also of major importance. The possibility that virus diseases, particularly the enteric virus diseases, may be waterborne indicates that methods for evaluating the potability of a water supply from a virological standpoint should be developed.

Considerable research is underway for the development of a routine test method for the detection of viruses in water and wastewater. At the same time more attention is being given to the assessment of the effectiveness of water-treatment processes for the removal and/or inactivation of viruses.

SWIMMING POOLS

Water in swimming places, and particularly in public swimming pools, may be a health hazard. Swimming pools and surrounding areas may be involved in the transmission of infections of the eye, nose, and throat, infections of the intestinal tract, and the spread of athlete's foot, impetigo, and other dermatoses. These facts, together with the extensive increase in the use of public pools, make it imperative that constant attention be given to the sanitary quality of the water.

Water in swimming pools is disinfected with chlorine, the only disinfectant

that has been approved by all state health departments for use in treating bathing waters. Bromine, which belongs to the same chemical family, the halogens, is also an effective disinfectant for this purpose and has been approved by some states. In using these chemicals for treatment of bathing water, dosage must be carefully regulated. There must be enough *residual* chemical in the water to destroy microorganisms effectively, and in this concentration the agent must be nontoxic and nonirritating to swimmers' eyes, skin, and mucous membranes. Various types of filtration units are also used for the treatment of swimming-pool waters

WATER POLLUTION

The 1972 Federal Water Pollution Control Act and Amendments represents a major national program directed to restoring the quality of the United States streams and lakes as well as the coastal waters. Administered by the Environmental Protection Agency (EPA), the law established a stringent regulatory system stating precise and detailed pollution abatement requirements with heavy penalties for violations. A few of the provisions of the law are as follows:

1 The EPA is required to establish effluent limitations and national performance standards for sources of water pollution, including factories, power plants, wastewater-treatment plants, and animal feedlots.
2 The law requires publicly owned waste-treatment plants to meet treatment effluent limits by July 1988, wherever possible.

WASTEWATER

Wastewater is the used water supply of a community and consists of:

1 Domestic waterborne wastes, wastewater or sewage, including human excrement and wash waters—everything that goes down the drains of a home and into a sewerage system
2 Industrial waterborne wastes such as acids, oil, greases, and animal and vegetable matter discharged by factories
3 Ground, surface, and atmospheric waters that enter the sewerage system

The wastewater of a city is collected through a sewerage system, which carries the used water to its ultimate point of treatment and disposal. There are three kinds of sewerage systems: (1) **sanitary sewers**, which carry domestic and industrial wastewater; (2) **storm sewers**, designed to carry off surface and storm (atmospheric) water; and (3) **combined sewers**, which carry all the wastewater (sewage) through a single system of sewers.

Chemical Characteristics

Domestic wastewater or sewage consists of approximately 99.9 percent water, 0.02 to 0.03 percent suspended solids, and other soluble organic and inorganic substances. On a percentage basis, the amount of solids appears small; however, the tremendous volume of material handled daily by a major municipal plant (e.g., several hundred millions of gallons) contains as much as 100 tons of solids. The chemical constituents, present in low concentrations, nevertheless are extremely important and are subject to variations, between communities as well as within a community, even from hour to hour. Inorganic chemicals initially present in the water supply will likewise be present in the sewage;

organic compounds are contributed through human excrement and other domestic wastes, and both organic and inorganic compounds are added by industrial wastes. For example, slaughterhouses, sugar factories, paper mills, and creameries add organic substances; mines and metal industries contribute acids and salts of metals and other inorganic wastes. The organic compounds in sewage are classified as nitrogenous or nonnitrogenous. The principal nitrogenous compounds are urea, proteins, amines, and amino acids; the nonnitrogenous substances include carbohydrates, fats, and soaps.

Modern technology may produce significant changes in sewage characteristics. The increased use of household garbage-disposal units has increased the total organic load. Some synthetic detergents displacing soaps are resistant to microbial degradation.

Biochemical Oxygen Demand (BOD)

The **biochemical oxygen demand (BOD)** is a measure of the amount of oxygen used in the respiratory processes of microorganisms in oxidizing the organic matter in the sewage and for the further metabolism (oxidation) of cellular components synthesized from the wastes. One of the primary reasons for treating wastewater prior to its being returned to the water resource (e.g., stream or lake) is to reduce the drain on dissolved oxygen supply of the receiving body of water. The magnitude of the BOD is related to the amount of organic material in the wastewater—i.e., the more oxidizable organic material, the higher the BOD. The "strength" of wastewater is expressed in terms of BOD level.

Microbiological Characteristics

Since the composition of wastewater varies, it is to be expected that the types and numbers of organisms will fluctuate. Fungi, protozoa, algae, bacteria, and viruses are present. Raw sewage may contain millions of bacteria per milliliter, including the coliforms, streptococci, anaerobic spore-forming bacilli, the *Proteus* group, and other types originating in the intestinal tract of humans. Sewage is also a potential source of pathogenic protozoa, bacteria, and viruses. The causative agents of dysentery, cholera, and typhoid fever may occur in sewage. The poliomyelitis virus, the virus of infectious hepatitis, and the coxsackie viruses are excreted in the feces of infected hosts and thus may appear in sewage. Certain bacterial viruses are readily isolated from sewage.

Predominant physiological types of bacteria may shift during the course of sewage digestion. In an anaerobic digester, facultative types (*Enterobacter, Alcaligenes, Escherichia, Pseudomonas*, etc.) predominate during initial stages. This is followed by methane producers, which are strict anaerobes, e.g., *Methanobacterium, Methanosarcina*, and *Methanococcus*. Organic acids produced by the facultative bacteria are metabolized by the methane formers; the end products are methane and carbon dioxide. Large amounts of these gases are produced in anaerobic digesters.

The various processes associated with treatment of wastewater bring about pronounced changes in the predominant types of organisms. These changes and their significance will be discussed later.

WASTEWATER TREATMENT AND DISPOSAL

Wastewater treatment is necessary before wastewater can be disposed of without producing significant undesirable or even harmful effects. However, some com-

munities and municipalities still dispose of inadequately treated wastewater into natural bodies of water, either because they are indifferent to the consequences or because it is assumed that the body of water is sufficiently large and so located that dilution prevents hazards. Communities and municipalities can no longer rely on disposal of wastewater by dilution. There is an ever-increasing demand for domestic and industrial water, necessitating more reuse of waters that receive wastewaters. Disposal of inadequately treated wastewater leads to:

1 Greater possibility for dissemination of pathogenic microorganisms
2 Increased danger in using natural bodies of water for drinking supplies
3 Contamination of oysters and other shellfish by the pollution, making them unsafe for human consumption
4 Large losses in the waterfowl population, chargeable to pollution of their winter feeding grounds
5 Increased danger of swimming in the water and diminished value of the water for other recreational purposes
6 Depletion of oxygen supply of the water by unstable organic matter in sewage, killing aquatic life
7 Creation of miscellaneous objectionable conditions such as offensive odors and accumulation of debris, which decrease property values
8 Accumulation and dissemination of toxic chemicals that endanger ecosystems and threaten public health

WASTEWATER-TREATMENT PROCESSES

Wastewater-treatment processes are many and varied. We will discuss the treatment processes as they are applicable to two separate situations: (1) a single dwelling or unit structure, and (2) a community or municipality.

Single Dwelling Units

Treatment and disposal of wastewater and sewage from individual dwellings or other unit structures (e.g., some motels or shopping centers) can be accomplished by anaerobic digestion and/or by aerobic metabolic processes. One of the more common installations used to accomplish this is the septic tank, an anaerobic digesting system.

Septic Tank

A septic tank (see Fig. 27-7) is a sewage-settling tank designed to retain the solids of the sewage entering the tank long enough to permit adequate decomposition of the sludge. Thus the unit accomplishes two processes: sedimentation and biological degradation of the sludge. As sewage enters this type of tank, sedimentation occurs from the upper portion, permitting a liquid with fewer suspended solids to be discharged from the tank. The sedimented solids are subject to degradation by anaerobic bacteria; hence the end products are still very unstable, i.e., high in BOD and odorous. The effluent from the septic tank is distributed under the soil surface through a disposal field, as shown in Fig. 27-7.

Septic tanks are the most satisfactory method for disposing of sewage from small installations, especially individual dwellings and isolated rural buildings where public sewers are not available. They cannot, however, be relied upon to eliminate pathogenic microorganisms carried in the sewage. Consequently, it is imperative that the drainage from the tank be prevented from contaminating the drinking-water supply.

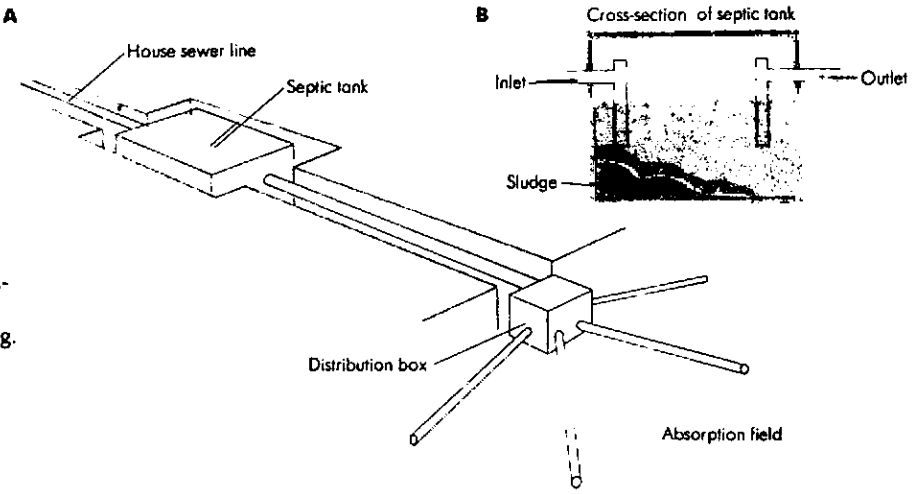


Figure 27-7. Septic-tank installation for sewage disposal from private dwelling. (A) Overall installation including absorption field. *(Redrawn from Public Health Rep, Reprint 2361.)* (B) Cross-sectional view.

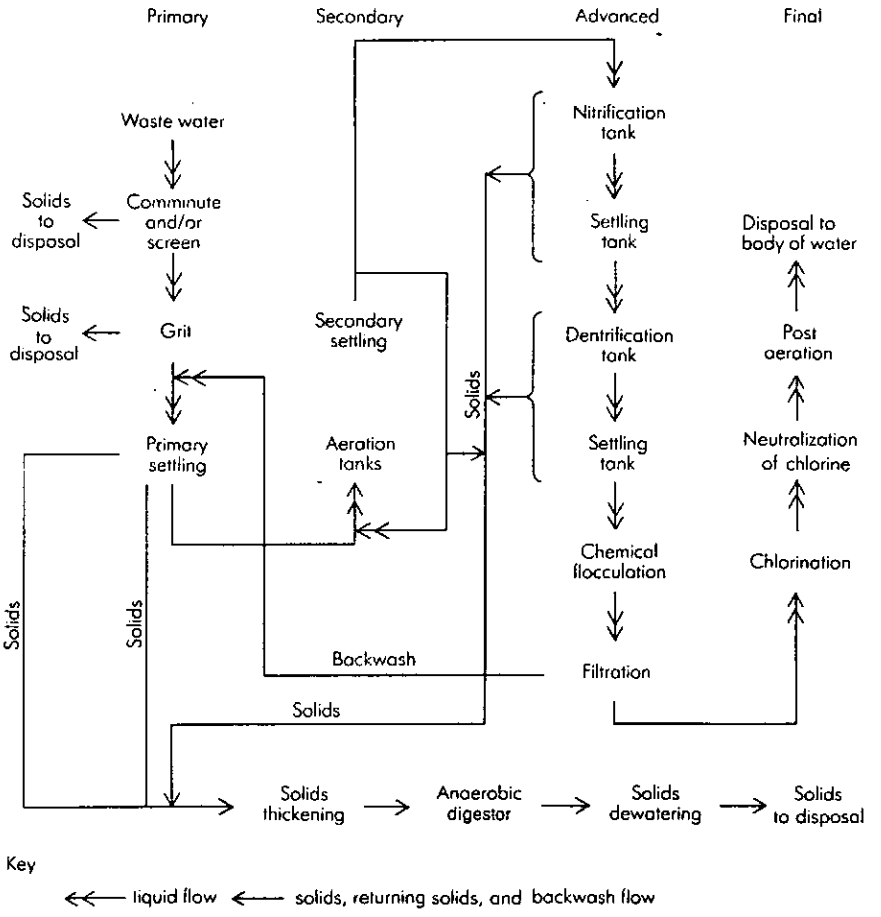


Figure 27-8. Modern advanced wastewater treatment facility.

Municipal Treatment Processes

Municipal wastewater–treatment plants carry out a series of treatment processes (see Fig. 27-8) which may be summarized as follows:

1 ***Primary treatment.*** To remove coarse solids and to accomplish removal of "settleable" solids
2 ***Secondary (biological) treatment.*** To adsorb and ultimately oxidize organic constituents of the wastewater, i.e., to reduce the BOD
3 ***Advanced treatment.*** To remove additional objectionable substances to further reduce BOD; includes removal of nutrients such as phosphorus and nitrogen
4 ***Final treatment.*** To disinfect and dispose of liquid effluent
5 ***Solids processing.*** To stabilize solids removed from liquid processes, to dewater solids, and ultimately to dispose of solids (land application, landfill, incineration)

Each of these processes is briefly described below:

Primary Treatment

Wastewater as it arrives at a wastewater-disposal plant is first treated to remove coarse solid materials by a variety of mechanical techniques including screening, grinding, and grit chambers. Subsequent to this it is treated to remove settleable solids.

Sedimentation. Sedimentation units (tanks, basins, or mechanical devices) provide the means for concentrating and collecting the particulate material referred to as sludge. Following sedimentation, the sludge and the liquid effluent are processed separately.

Secondary (Biological) Treatment

Secondary treatment processes accomplish oxidation of the organic material in the liquid wastewater by microbial activity. The oxidation methods employed are:

1 Filtration by intermittent sand filters, contact filters, and trickling filters
2 Aeration by the activated-sludge process or by contact aerators
3 Oxidation ponds

Only the more commonly used secondary treatment processes will be described.

The Trickling Filter. The trickling filter consists of a bed of crushed stone, gravel, slag, or synthetic material with drains at the bottom of the tank. Trickling filters have been described as "a pile of rocks over which sewage or organic wastes slowly trickle." The liquid sewage is sprayed over the surface of the bed either by a rotating arm or through nozzles. The spraying saturates the liquid with oxygen. Intermittent application of the sewage permits maintenance of aerobic conditions in the bed. The filtering medium of the tank becomes coated with a microbial flora, the **zoogloeal film**, which consists of bacteria, fungi, protozoa, and algae. As the sewage seeps over these surfaces, the microorganisms adsorb and metabolize the organic constituents to more stable end products. This operation may be regarded as a stationary microbial culture (on the stones) fed by a continuous supply of nutrients (organic constituents of the sewage). A newly constructed bed must acquire the zoogloeal film before it can function efficiently. This requires operation over a period of a few weeks. Figures 27-9 and 27-10 illustrate the design and operation of a trickling filter. Examples of zoogloeas from trickling filters are shown in Fig. 27-11.

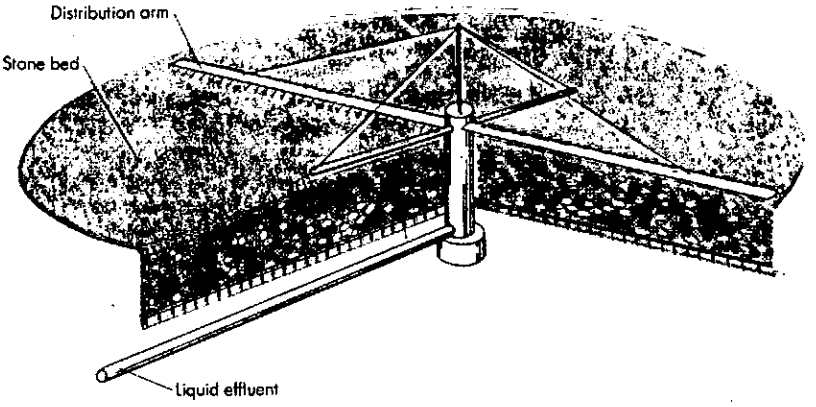


Figure 27-9. Commercial distributor (trickling filter) is shown in this cut-away drawing. Note the distributor arm which applies the liquid sewage, the filter bed through which the liquid travels, and the trough for collection of the effluent. *(Courtesy of Door-Oliver, Inc.)*

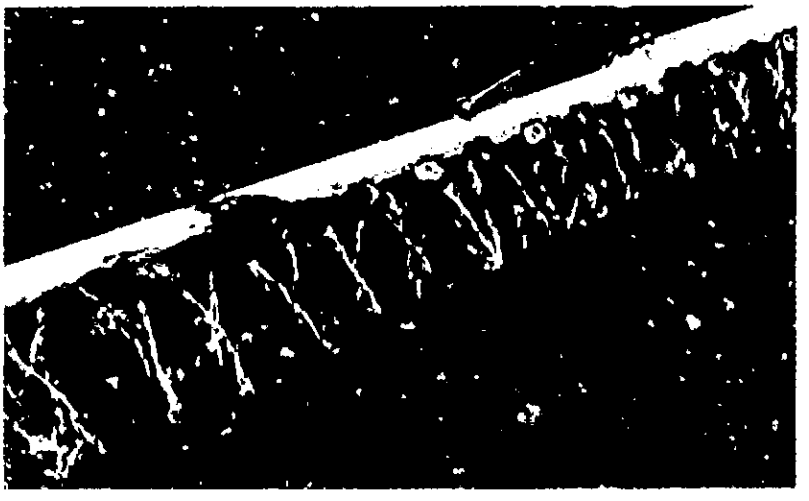


Figure 27-10. Distribution of wastewater onto filter stones from rotary distributor. Filter stones are coated with microbial growth. *(Courtesy of R. E. McKinny, Microbiology for Sanitary Engineers, McGraw-Hill, New York. Copyright 1962. Used by permission.)*

The upper region of the trickling filter is favorable for the growth of algae, and at times their growth may become so extensive that it impairs the operation of the filter. Many species of protozoa and fungi occur throughout the filter; their numbers are influenced in part by the availability of oxygen and nutrients. It is apparent that the microbial activities and interactions are extremely complex in such a heterogeneous environment.

The Activated-Sludge Process. Vigorous aeration of sewage (Fig. 27-12) results in the formation of a floc; the finely suspended and colloidal matter of sewage forms aggregates designated as floccules. If this floc is permitted to settle and then added to fresh sewage that is again vigorously aerated, flocculation occurs in a shorter time than before. By repetition of this process, i.e., addition of sedimented floc to fresh sewage, aeration, sedimentation, addition of sediment

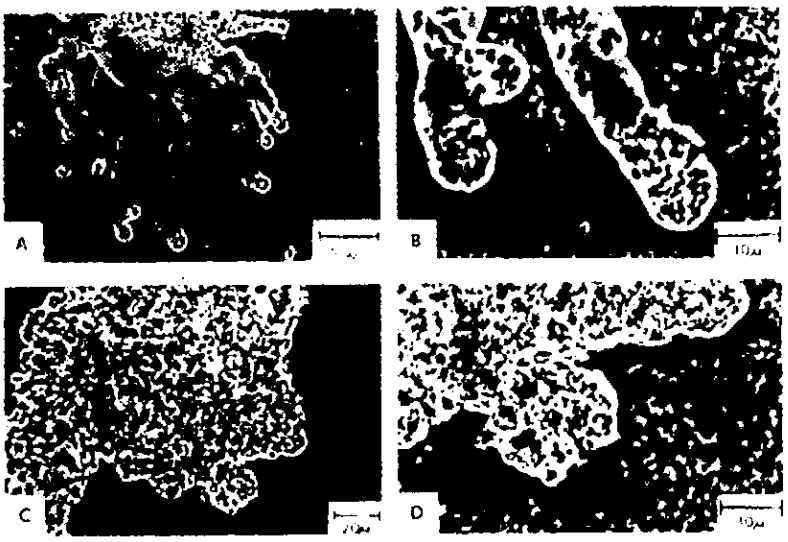


Figure 27-11. Branched and amorphous zoogloeas collected from the surface of trickling filters receiving primarily domestic wastewaters. (A) Natural, branched, trickling-filter zoogloea. (B) Portion of specimen shown in (A) illustrating morphological similarity of bacterial cells and their concentration at anterior points of zoogloeal branches. (C) Natural, amorphous, trickling-filter zoogloea. (D) Portion of specimen shown in (C) illustrating presence of morphologically different bacteria. (All specimens treated with 10% skim milk to accentuate zoogloeal matrix. Phase-contrast microscopy.) (*Courtesy of R. F. Unz and N. C. Dondero, Water Res,* 4:575, 1970.)

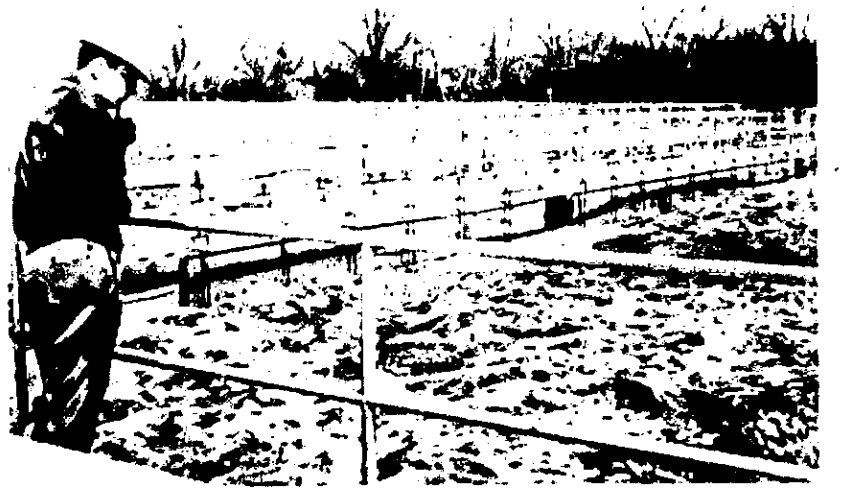


Figure 27-12. An activated-sludge basin with a full load of sewage in the process of aerobic microbiological treatment. (*Courtesy of Washington Suburban Sanitary Commission.*)

to fresh sewage, aeration, etc., a stage is reached where complete flocculation of the fresh sewage occurs very quickly, e.g., a few hours. These particles of floc, i.e., "activated sludge," contain large numbers of very actively metabolizing bacteria, together with yeasts, molds, and protozoa. This combination of microbial growth is very effective in the oxidation of organic compounds. A poor settlement of activated-sludge flocs adversely affects performance of a sewage-treatment plant. The sludge becomes more voluminous and is difficult to con-

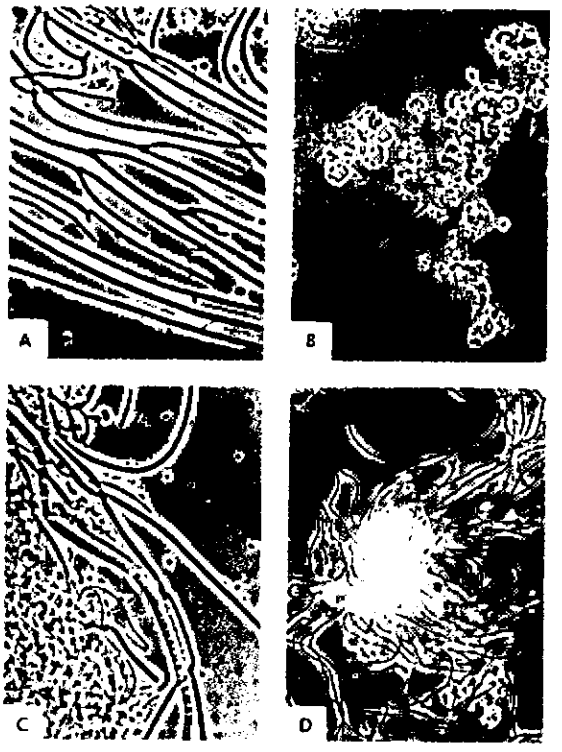


Figure 27-13. Filamentous organisms observed in activated sludge in which bulking has occurred. (A) *Sphaerotilus natans*. The cells occur within sheaths (X825). (B) *Streptothrix hyalina*, thin filaments protruding from the flocs (X406). (C) Unidentified long, unbranched, nonmotile multicellular filaments. Cells have some similarity to *Leucothrix* and *Thiothrix* (X825). (D) *Microthrix parvicella*. Filamentous cells, strongly curled (X310). (*Courtesy of D. H. Eikelboom and Water Res*, 9:365, 1975.)

trol. The principal reason for poor settling (bulking) of activated sludge is the growth of filamentous microorganisms. Many different species of microorganisms have been isolated from sludge in this condition. A few types are shown in Fig. 27-13.

The use of activated sludge is of great importance in wastewater treatment. This process usually employs an aeration period of 4 to 8 h, after which the mixture is piped to a sedimentation tank. The effluent from these tanks represents wastewater treated to secondary levels; there is a considerable reduction of suspended solids, and the BOD is reduced. Some of the advantages of this method of treatment are that relatively little land space is needed and the quality of final effluent is such that it does not require high dilution for disposal.

Oxidation Ponds (Lagoons). Oxidation ponds (also called lagoons or stabilization ponds) are shallow ponds (2 to 4 ft in depth) designed to allow algal growth on the wastewater effluent. Use of oxidation ponds should be preceded by primary treatment. Oxygen for biochemical oxidation of nutrients is supplied from the air, but the release of O_2 during photosynthesis by the alga *Chlorella pyrenoidosa* provides an additional important source of oxygen.

Advanced Treatment

Advanced wastewater treatment is required when removal of substances beyond the limits normally achieved by conventional primary and secondary processes

are necessary. Unit processes have been developed to remove nutrients, simple organic substances, and complex synthetic organic compounds. Processes include biological treatment; however, physical-chemical methods predominate. Common unit processes include biological nitrification-denitrification, filtration, reverse osmosis, carbon adsorption, chemical addition, and ion exchange. The major disadvantage of advanced treatment processes is high cost.

Final Treatment

The liquid effluent, upon completion of other treatments, is disinfected and usually discharged into a body of water. Disinfection of wastewaters is necessary to protect public health when the receiving waters are used for purposes such as downstream water supply, recreation, irrigation, or shellfish harvesting. Most facilities use chlorination for disinfection. Current research has proved the serious impact chlorinated waters have on the aquatic life of the receiving water. This has led to the development of several disinfection alternatives. The use of ozone and ultraviolet light is becoming more prevalent. Many facilities that continue to employ chlorine for disinfection now include dechlorination prior to discharge to a body of water.

Dissolved oxygen may also be added to the treated wastewater prior to final discharge. This process is termed post aeration and is accomplished by mechanical means or a cascading slow technique. Post aeration minimizes the decrease in receiving water-dissolved oxygen that normally occurs where treated wastewater effluent is discharged.

Although disposal into surface waters is widely practiced, land application has been and continues to be a feasible alternative to surface-water discharge. Land application of wastewaters was given substantial recognition in the Federal Water Pollution Control Amendment of 1972 to implement the "national goal that discharge of pollutants into navigable waters be eliminated by 1985." It is a fact that municipal and industrial wastewaters have been used nationwide for crop irrigation. During the next decade we are likely to give more consideration to land application of the treated liquid waste from wastewater-treatment plants.

Solids Processing

A major cost at modern wastewater-treatment facilities is associated with solids processing—the thickening, stabilization, dewatering, and disposal of sludge. Solids are removed from the primary, secondary, and the advanced stages of the treatment process. Thickening is generally employed to further concentrate the solids or sludge prior to stabilization. Thickening may be accomplished by gravity thickening, similar to sedimentation, or by dissolved air flotation.

Many stabilization processes are employed, including aerobic and anaerobic digestion, composting, chemical addition, and heat treatment. The most common process for modern municipal treatment facilities is the anaerobic digestion system.

Dewatering is accomplished by physical methods and is often enhanced by the addition of polymer or other chemical coagulant aids. Equipment used for dewatering includes vacuum filters (Fig. 27-14), belt filter presses, plate and frame presses, and centrifuges. Small treatment facilities and older plants continue to use sand filter beds for dewatering.

Anaerobic Sludge Digestion. The solids which accumulate during sedimentation

Figure 27-14. The dewatering (drying) of sludge can be accomplished in several ways. This illustration shows sludge being dried by a vacuum-filter process; the liquid in the sludge is drawn through steel coils of a rotating drum by suction. The sludge "cake" remains on the outside of the drum and when dry falls off onto a conveyor belt. *(Courtesy of Washington Suburban Sanitary Commission.)*

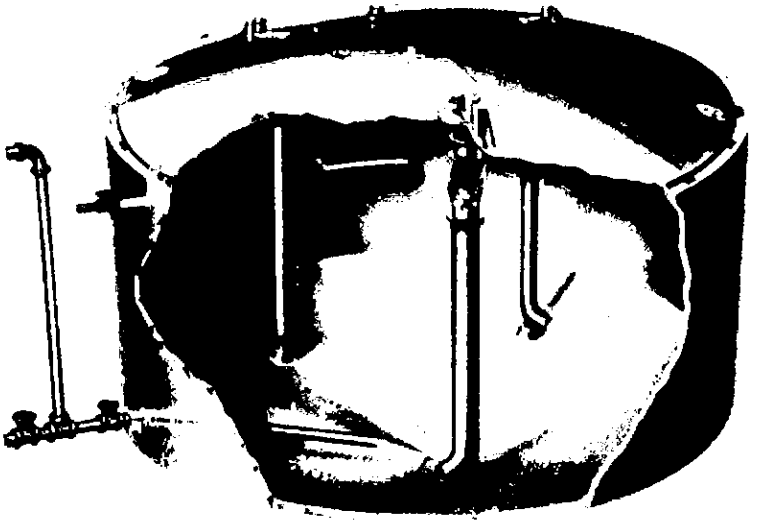


Figure 27-15. Sludge digestor is a special tank designed to process sludge under controlled conditions. *(Courtesy of Door-Oliver, Inc.)*

are pumped into a separate tank designed especially for the digestion of sludge under controlled conditions (Fig. 27-15 and Fig. 27-16). Solids recovered from the aerobic treatment processes may also be returned to the sludge digestor.

The microbial action on the constituents of sludge is termed **sludge digestion.** An anaerobic condition prevails in these tanks; and the anaerobic and facultative types of bacteria are active. These microorganisms bring about a decrease in organic solids by degrading them to soluble substances and gaseous products. Large amounts of gases consisting mainly of methane (60 to 70 percent) and carbon dioxide (20 to 30 percent), with smaller amounts of hydrogen and nitrogen, are produced during sludge digestion. This gas mixture can be used as a fuel for heating purposes and for operating power.

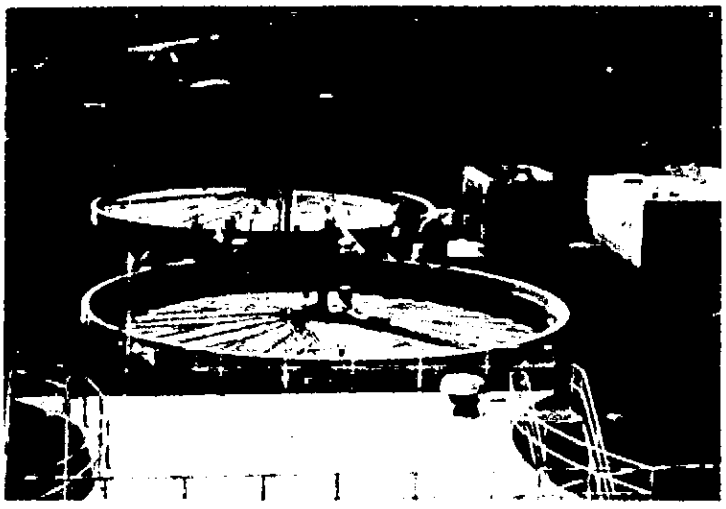


Figure 27-16. Anaerobic sludge digestors. Gases formed in the process of microbiological metabolism can be cycled through the treatment plant to provide fuel for heating. *(Courtesy of Washington Suburban Sanitary Commission.)*

Conditions affecting microbial growth and metabolism will be reflected in the degree of digestion of the sludge, e.g., inoculum, pH, and temperature. Fresh sludge entering the tanks is inoculated, or **seeded**, with a portion of **ripe sludge** (sludge which has already undergone digestion). The ripe sludge may be regarded as an actively growing culture of the types of microorganisms required for rapid digestion of sludge. The proportion of inoculum used and the thoroughness of mixing with the fresh sludge are important. Most rapid digestion of sludge occurs at temperatures which favor growth of thermophilic bacteria (50 to 60°C). A neutral pH (7.0) is optimum for sludge digestion. These conditions can be controlled only in specially designed tanks as shown in Fig. 27-15. The temperature is maintained around 30°C, which is below the optimum stated above for most rapid digestion, because it has not been found practical to operate at thermophilic temperatures. Complete digestion requires 2 to 3 weeks or even longer.

In a modern wastewater treatment plant the sludge remaining in the digestor is removed to a mechanical drying unit (see Fig. 27-14). The sludge dries into "cakes" which are carried by conveyor belts to incinerators. The incinerator reduces the sludge cake to ash for final disposal in a landfill. An alternative is to spread stabilized dewatered sludge on land. Several municipalities are engaged in land-utilization techniques for disposal of treated sludge. However, such practice requires inexpensive and available land. Furthermore, the volume of sludge generated by a large plant can be staggering.

Composting. Composting is a process where dewatered sludge undergoes decomposition, usually within the thermophilic temperature range. Dewatered sludge is mixed with a bulking agent such as wood chips. The bulking material is added to enhance circulation of air throughout the sludge to improve the stabilization process. The mixture of sludge and bulking material is placed in aerated piles. Oxygen is furnished by forced aeration. The mixture is allowed

Table 27-2. Generalized Scheme of Microbial Degradation of the Organic Constituents in Sewage

Substrates	+	Enzymes of Microorganisms	→	Representative End Products: Anaerobic Conditions	Representative End Products: Aerobic Conditions
Proteins and other organic nitrogen compounds				Amino acids Ammonia Nitrogen Hydrogen sulfide Methane Carbon dioxide Hydrogen Alcohols Organic acids Indole	Amino acids Ammonia → nitrites → nitrates Hydrogen sulfide → sulfuric acid Alcohols, Organic acids } → $CO_2 + H_2O$
Carbohydrates				Carbon dioxide Hydrogen Alcohols Fatty acids Neutral compounds	Alcohols, Fatty acids } → $CO_2 + H_2O$
Fats and related substances				Fatty acids + glycerol Carbon dioxide Hydrogen Alcohols Lower fatty acids	Alcohols, Lower fatty acids } → $CO_2 + H_2O$

to "cure" or biologically decompose for a period of time. For effective stabilization this period of time is normally considered to be 21 days.

After 21 days, the bulking agent is separated from the sludge and the sludge is allowed to cure further for several weeks. Upon final curing, the sludge has been transformed to a humus-type material and is suitable for use as a soil conditioner.

MICROORGANISMS AND WASTEWATER-TREATMENT PROCEDURES

The effectiveness of secondary wastewater-treatment processes is almost entirely dependent upon microbial growth and metabolism. The chemical activities of the microorganisms are responsible, to a major degree, for stabilization of the final effluent. The same is true for liquefaction, gasification, and mineralization of the constituents of sludge. From the standpoint of ecology, wastewater represents one of the most complex microbiological environments. Total populations, as well as the distribution of physiological types, are subject to wide fluctuations. The variety of substrates are susceptible to dissimilation by this mixed microbial flora. Interactions may occur among species, producing results that are not characteristic of pure cultures. However, the types of change accomplished by these microorganisms may be summarized as shown in Table 27-2.

From the reactions shown in Table 27-2, it can be seen that anaerobic conditions result in a variety of incompletely oxidized products. Aerobic conditions yield products that are more highly oxidized. The efficiency of aeration, the metabolic capacity of the microorganisms, and the time allowed for treatment

will determine the extent of oxidation; e.g., carbohydrates under suitable conditions can be degraded and oxidized to carbon dioxide and water.

EFFICIENCY OF WASTE-WATER-TREATMENT PROCEDURES

The efficiency of wastewater treatment obtained by the several procedures described is shown in Tables 27-3 and 27-4. It should be noted that the efficiency reported for any single process shows considerable variation.

This variation may be attributed to the design of the unit, the method of operation, the nature of the wastewater, or other differences. Most importantly, however, the data in these tables demonstrate that methods are available for accomplishing a high degree of wastewater purification.

THE POLLUTION PROBLEM

Despite the fact that modern technology has provided effective methods for wastewater treatment, many communities and municipalities do not employ adequate treatment procedures; but, even worse, some perform no treatment: they dump raw wastewater into the waterways. Fortunately significant progress is being made in upgrading wastewater-treatment processes.

Table 27-3. Efficiency of Various Sewage-Treatment Methods

Method	Percentage of Removal of Suspended Solids	Gallons of Sludge per Million Gallons of Sewage	Percentage of Removal of	
			Bacteria	BOD
Plain sedimentation	40–95	1,000–5,000	40–75	30–75
Chemical precipitation	75–95	5,000–10,000	80–90	60–80
Septic tank	40–75	500–1,500	40–75	25–65
Imhoff tank	35–80	250–750	40–75	25–65
Intermittent sand filter	95–98		98–99+	70–96
Contact bed	55–90		50–75	60–80
Trickling filter	0–80	250–750	70–85	60–90
Activated sludge	70–97	10,000–30,000	95–99+	70–96

SOURCE: From P. L. Gainey and P. H. Lord, *Microbiology of Water and Sewage*, Prentice-Hall, Englewood Cliffs, N.J., 1952.

Table 27-4. The Transformation of Pollutants in Sewage after Processing in a Modern Sewage-Treatment Plant

	Influent	→ Primary and Secondary	→ Nitrification	→ Denitrification	→ Filtration and Disinfection	→ Effluent
	Pollutant Concentrations, mg/liter					
BOD	206	35	10	6	4	
Phosphorus, total	8.4	2.0	1.0	0.5	0.2	
Nitrogen:						
Organic	8.6	3.0	1.0	1.0	0.5	
NH_3—N	13.7	14.8	1.5	1.0	1.0	
NO_2 and NO_3—N	0	0.2	11.1	1.0	0.5	
Total N	22.3	18.0	13.6	3.0	2.0	

SOURCE: *Environ Sci Technol*, 8(10), October 1974.

The demand for larger quantities of potable water continues to grow worldwide. Accordingly, there is more need for conservation together with more efficient wastewater treatment processes so that the cycle between "used" water and its reuse can be shortened. The gravity of the water pollution problem is reflected in the following quotation taken from the article written by Nandor Porges, which is included in the references at the end of this chapter.

> Waste treatment is a means of maintaining or recovering man's most precious and most abused natural resource, fresh water. Fresh water supplies were all-important in the establishment and growth of civilizations. Much of man's bitterest fighting has been incited by altercations over water rights, and the course of history may well be written around the theme of primitive and modern man's need for water.

QUESTIONS

1 Define the following terms as they refer to the sanitary quality of water: potable, polluted, contaminated, sanitary survey, wastewater.
2 Why is *Escherichia coli* considered an indicator of pollution? What are coliforms?
3 Name several species of pathogenic organisms which may be present in polluted water.
4 Why isn't the routine bacteriological examination of water directed toward isolation and identification of specific pathogens?
5 Outline the process by which a municipality produces potable water. How is the microbial population of the raw water affected at each step?
6 What is the significance of finding *Streptococcus faecalis* in water? What are fecal streptococci?
7 Is fermentation of lactose with production of acid and gas positive evidence for the presence of *E. coli*? Of the coliforms? Explain.
8 Describe how selective and differential media facilitate the bacteriological analysis of water samples.
9 How can one distinguish among members of the coliform group?
10 What advantages does the membrane filter technique offer for microbiological analysis of water?
11 What are the morphological and cultural characteristics of *Escherichia coli*, *Enterobacter aerogenes*, *Proteus vulgaris*, *Salmonella typhi*, and *Shigella dysenteriae*?
12 Where are septic tanks used? Describe the microbiological activities that take place in a septic tank.
13 Outline the process of wastewater treatment which is followed in most large cities. Which steps in the process depend upon microbial activity for successful performance? Explain.
14 Define the following terms: effluent, sludge, activated sludge, BOD, lagoon.
15 In terms of sewage treatment, what problem has accompanied the wide usage of detergents? The increase in use of home garbage-disposal units?
16 What is activated sludge? Compare the microbial activity in the activated-sludge process with that which occurs in a septic tank.

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Chapter 28 Microbiology of Foods

OUTLINE

Microorganisms are associated, in a variety of ways, with all of the food we eat. They may influence the quality, availability, and quantity of our food. Naturally occurring foods such as fruits and vegetables normally contain some microorganisms and may be contaminated with additional organisms during handling. Food can serve as a medium for the growth of microorganisms, and this growth may cause the food to undergo decomposition and spoilage. Foods may also carry pathogenic microorganisms and as a result transmit disease. Other microorganisms, if allowed to grow in certain food products, produce toxic substances that result in food poisoning when the food is ingested. Still other microorganisms are used in the preparation and preservation of food products, such as yogurt and sauerkraut.

From these general remarks it is clearly evident that the major concern in food microbiology is the control of microorganisms. In this chapter we will discuss the microbiology of foods in terms of the normal flora of foods, their significance, and the manner in which foods may be protected from microbial contamination and microbial spoilage. We will also describe food product manufactured by microbial fermentation. The role of foods in transmissi disease and in food poisoning will be discussed in Chap. 35.

MICROBIAL FLORA OF FRESH FOODS

The inner tissues of healthy plants and animals are free of microorganisms (sterile). However, the surfaces of raw vegetables and meats are contaminated

with a variety of microorganisms. The magnitude of this microbial contamination reflects one or more of the following: the microbial population of the environment from which the food was taken, the condition of the raw product, the method of handling, the time and conditions of storage. It is desirable to maintain a very low microbial level of contamination on raw foods; the presence of extremely large numbers of microorganisms suggests that some undesirable events have occurred and that the food is indeed susceptible to further deterioration.

Meats

The carcass of a healthy animal slaughtered for meat and held in a refrigerated room is likely to have only nominal surface contamination while the inner tissues are sterile. Fresh meat cut from the chilled carcass has its surface contaminated with microorganisms characteristic of the environment and the implements (saws or knives) used to cut the meat. Each new surface of meat, resulting from a new cut, adds more microorganisms to the exposed tissue. The ultimate in providing new surfaces and potential contamination of meat surfaces occurs in the process of making hamburger.

To improve the microbiological quality of meats, particularly ground beef (in addition to cold cuts and frankfurters), most states have adopted standards, or are considering establishing regulations, to require microbiological standards for these products at the time of purchase.

Among the more common species of bacteria occurring on fresh meats are pseudomonads, staphylococci, micrococci, enterococci, and coliforms. The low temperature at which fresh meats are held favors the growth of psychrophilic microorganisms.

Poultry

Freshly dressed eviscerated poultry have a bacterial flora on their surface (skin) that originates from the bacteria normally present on the live birds and from the manipulations during killing, defeathering, and evisceration. Under good sanitary conditions the bacterial count has been reported to be from 100 to 1,000 bacteria per square centimeter of skin surface, whereas under less sanitary conditions the count may increase 100-fold or more. Pseudomonads constitute the major contaminants on the skin of freshly dressed poultry.

Eggs

The interior of a freshly laid egg is usually free of microorganisms; its subsequent microbial content is determined by the sanitary conditions under which it is held, as well as the conditions of storage, i.e., temperature and humidity. Microorganisms, particularly bacteria and molds, may enter the egg through cracks in the shells or penetrate the shells when the "bloom" (thin protein coat) covering the shell deteriorates. The types of microorganisms involved reflect those present in the environment.

Fruits and Vegetables

Fruits and vegetables are normally susceptible to infection by bacteria, fungi, and viruses. Microbial invasion of plant tissue can occur during various stages of fruit and vegetable development, and, hence, to the extent that the tissues are invaded, the likelihood of spoilage is increased. A second factor contributing to the microbial contamination of fruits and vegetables pertains to their post-harvest handling. Mechanical handling is likely to produce breaks in the tissue

which facilitates invasion by microorganisms. The pH of fruits is relatively acid, ranging from 2.3 for lemons to 5.0 for bananas. This restricts bacterial growth but does not retard fungal growth. The pH range for vegetables is slightly higher, pH 5.0 to 7.0, and hence they are more susceptible than fruits to attack by bacteria.

Shellfish and Finfish

The microbial flora of freshly caught oysters, clams, fish, and other aquatic specimens is very largely a reflection of the microbial quality of the waters where they are harvested. Of particular significance is whether the water is sewage-polluted, in which case the seafood is potentially capable of transmitting various pathogenic microorganisms. The marine bacterium *Vibrio parahaemolyticus* has been responsible for a number of gastroenteritis epidemics in the United States due to consumption of raw or inadequately cooked seafood. This organism occurs widely in the Atlantic, Pacific, and Gulf Coast waters and has been isolated from seafood samples including fish, shellfish, and crustaceans. Shellfish that grow in contaminated water can concentrate viruses and may be sources of hepatitis infection. For example, raw oysters and clams from polluted waters have caused numerous epidemics in various parts of the world.

Milk

At the time it is drawn from the udder of a healthy animal, milk contains organisms that have entered the teat canal through the teat opening. They are mechanically flushed out during milking. The number present at the time of milking has been reported to range between several hundred and several thousand per milliliter. The counts vary among cows and among the quarters of the same cow and are highest during the initial stages of milking. From the time the milk leaves the udder until it is dispensed into containers, everything with which it comes into contact is a potential source of more microorganisms. This includes the air in the environment, the milking equipment, and the personnel. Disregard of sanitary practices will result in heavily contaminated milk that spoils rapidly. However, milking performed under hygienic conditions with strict attention to sanitary practices will result in a product with low bacterial content and good keeping quality.

We shall discuss the microorganisms found in milk on the basis of their major characteristics, namely:

1 Biochemical types
2 Temperature response
3 Ability to cause infection and disease

Biochemical Types of Bacteria in Milk

If maintained under conditions that permit bacterial growth, raw milk of a good sanitary quality will develop a clean, sour flavor. This change is brought about mainly by *Streptococcus lactis* and *S. cremoris* (Fig. 28-1A, B) and certain lactobacilli (Fig. 28-1C). The principal change is lactose fermentation to lactic acid; evidence of proteolysis or lipolysis is not detectable by taste or smell. This type of change is sometimes referred to as the **normal fermentation** of milk. However, other organisms may produce changes beyond mere production of acid as shown in Table 28-1.

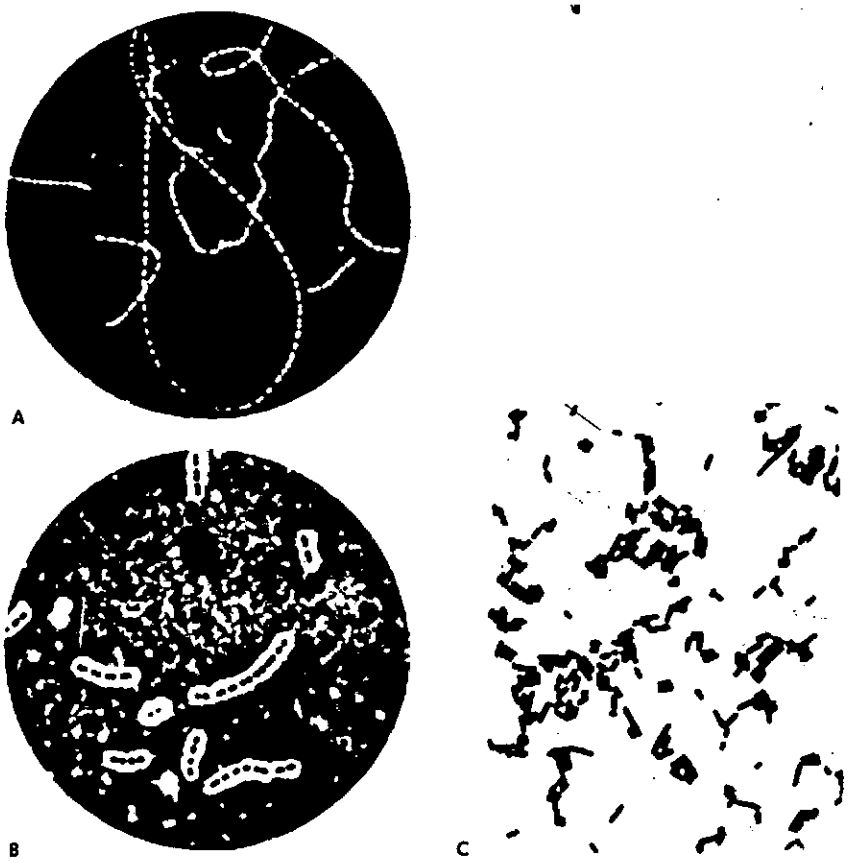

Figure 28-1. *Streptococcus lactis* (A) and *S. cremoris* (B), two important fermentative bacterial species in milk and milk products. These species, along with *Lactobacillus fermenti* (C), cause the so-called normal fermentation of milk; they are not pathogens. *(Courtesy of S. Orla-Jensen, The Lactic Acid Bacteria, Ejnar Munksgarrd, Copenhagen, 1919.)* (C) *Lactobacillus fermenti*, one of the heterofermentative lactobacilli. It produces a mixture of acids and is involved in the normal fermentation of milk; it is not pathogenic. Its cells vary in length and are Gram-positive, nonmotile, and nonsporeforming. *(Courtesy of A. P. Harrison.)*

Temperature Characteristics of Bacteria in Milk

Bacteria that gain entrance into milk may be classified according to their optimum temperature for growth and heat resistance. Temperature is a very practical consideration, since low temperatures are used to prevent changes due to microorganisms and high temperatures (pasteurization) are used to reduce the microbial population, destroy pathogens, and in general improve the keeping quality of the milk. Collectively, the bacteria encountered in milk are of the following four temperature types: psychrophilic, mesophilic, thermophilic, and thermoduric.

Since certain psychrophiles grow at temperatures just above freezing and some thermophiles grow at temperatures in excess of 65°C, it follows that the temperature at which milk is held will determine which species grow and predominate. Pasteurized milk stored in a refrigerator may be satisfactorily preserved for a week or even longer. But eventually microbial deterioration, manifested by "off" flavor or odor, will become evident because of the accumulation of metabolic products of psychrophilic bacteria. Thermophiles present a problem at the other extreme of the temperature scale. The holding method of pasteuri-

Table 28-1. Biochemical Types of Microorganisms in Milk

Biochemical Types	Representative Microorganisms	Source of Microorganisms	Substrate Acted Upon and End Products	Additional Remarks
Acid producers	Streptococci, e.g., *Streptococcus lactis* *S. cremoris*	Dairy utensils, silage, plants	Lactose fermented to lactic acid or lactic acid and other products such as acetic acid, ethyl alcohol, and carbon dioxide	Acid producers that produce only lactic acid are referred to as *homofermentative* types; those which produce a variety of products are called *heterofermentative* types
	Lactobacilli, e.g., *Lactobacillus casei* *L. plantarum* *L. brevis* *L. fermentum*	Feeds, silage, manure	Lactose is fermented to lactic acid and other products. Some species of lactobacilli are homofermentative; others are heterofermentative	
	Microbacteria, e.g., *Microbacterium lacticum*	Manure, dairy utensils, and dairy products	Lactose fermented to lactic acid and other end products; do not produce as much acid as the streptococci or lactobacilli	Some of these bacteria can survive exposure to very high temperatures, e.g., 80–85°C for 10 min
	Coliforms, e.g., *Escherichia coli* *Enterobacter aerogenes*	Manure, polluted water, soil, and plants	Lactose fermented to a mixture of end products, e.g., acids, gases, and neutral products	The number of coliform bacteria present in milk is an indicator of its sanitary quality
	Micrococci, e.g., *Micrococcus luteus* *M. varians* *M. freudenreichii*	Ducts of cow's mammary glands, dairy utensils	Small amounts of acid produced from lactose (weakly fermentative); micrococci are also weakly proteolytic	Moderately heat resistant; some strains capable of surviving 63°C for 30 min
Gas producers	Coliforms *Clostridium butyricum* *Torula cremoris*	Soil, manure, water, feed	Lactose fermented with accumulation of gas; the gas may be a mixture of carbon dioxide and hydrogen, or only carbon dioxide in the case of yeast fermentation	Bulk containers of milk may have their lids lifted by gas pressure in instances where contamination with gas producers is unusually high.
Ropy or stringy fermentation	*Alcaligenes viscolactis* *Enterobacter aerogenes* *Streptococcus cremoris*	Soil, water, plants, feed	Organisms synthesize a viscous polysaccharide material that forms a slime layer or capsule on the cells	Milk favors the formation of capsular material; sterile skim milk is frequently used as the culture medium when capsule formation is sought
Proteolytic	*Bacillus* spp., e.g., *B. subtilis* *B. cereus*	Soil, water, utensils	Proteolytic organisms degrade the casein to peptides which may be further dissimilated to amino acids; proteolysis may be preceded by coagulation of the casein by the enzyme renin	End products of proteolysis may impart abnormal flavor or odor to the milk; *Pseudomonas* spp. may produce coloration of milk.
	Pseudomonas spp. *Proteus* spp. *Streptococcus liquefaciens*			
Lipolytic	*Pseudomonas fluorescens* *Achromobacter lipolyticum* *Candida lipolytica* *Penicillium* spp.	Soil, water, utensils	Lipolytic microorganisms hydrolyze milk fat to glycerol and fatty acids	Some fatty acids impart rancid odor and taste to milk

zation exposes milk to a temperature of 62.8°C for 30 min; the thermophile *Bacillus stearothermophilus*, however, grows at 65°C. Other generalizations relating to bacterial growth and the types of bacteria that predominate in milk held at various temperatures are shown in Table 28-2.

In the dairy industry, thermoduric bacteria are regarded as those which survive pasteurization in considerable numbers but do not grow at pasteurization temperatures. Microorganisms of this category are extremely troublesome from the standpoint of producing raw milk with a low bacterial count. Because they are not killed by pasteurization, the microorganisms may contaminate equipment and accumulate as a result of faulty cleaning. Subsequent batches of milk processed through the same equipment will become heavily contaminated.

Thermoduric bacteria are not restricted to a single species or genus but are found in species of several genera, e.g., *Microbacterium lacticum*, *Micrococcus luteus*, *Streptococcus thermophilus*, and *Bacillus subtilis*.

Pathogenic Types of Bacteria in Milk

In recent years milk has been involved in fewer and fewer outbreaks of illness, to the point that the public and regulatory agencies no longer consider milk a primary source of foodborne illness. Milk and dairy products can now be considered model foods from the standpoint of regulations and surveillance of production, processing, and distribution. Furthermore, there are companion standardized methods for analysis of dairy products. No other food can claim the degree of standardized surveillance and analysis that is practiced for milk and milk products.

A variety of diseases are potentially transmissible through milk. The source of a pathogenic agent occurring in milk may be either a cow or a human, and

Table 28-2. Effect of Holding Temperature of Raw Milk on Numbers and Types of Bacteria

Holding Temperature, °C	Changes in Numbers	Predominant Organisms
1–4	Slow decline first few days followed by gradual increase after 7 to 10 days	True psychrophiles, e.g., species of *Flavobacterium*, *Pseudomonas*, and *Alcaligenes*
4–10	Little change in number during first few days followed by rapid increase in numbers; large populations present after 7 to 10 days or more	As above; changes produced on holding are of the following types: ropiness, sweet curdling, proteolysis, etc.
10–20	Very rapid increase in numbers; excessive populations reached within few days or less	Mainly acid-producing types such as lactic streptococci
20–30	High populations develop within hours	Lactic streptococci, coliforms, and other mesophilic types; in addition to acid there may be gas, off flavors, etc.
30–37	High populations develop within hours	Coliform group favored
Above 37	High populations develop within hours	Some mesophiles, thermophiles, e.g., *Bacillus coagulans* and *B. stearothermophilus*

it may be transmitted to either. The following modes of transmission are possibilities.

1 Pathogen from infected cow → milk → human or cow, e.g., tuberculosis, brucellosis, mastitis
2 Pathogen from human (infected or carrier) → milk → human, e.g., typhoid fever, diphtheria, dysentery, scarlet fever

It is also possible for humans to infect cows. For example, mastitis may be caused by a variety of organisms, including *Staphylococcus aureus*. The infecting organism, in some cases, has been traced to humans.

More specific aspects of disease transmission by milk and other foods are discussed in Part Eight.

MICROBIAL SPOILAGE OF FOODS

Considering the variety of natural food substances and the methods by which each is handled during processing, it is apparent that practically all kinds of microorganisms are potential contaminants. The type of food substance and the method by which it is processed and preserved may favor contamination by certain groups of microorganisms. Most foodstuffs serve as good media for the growth of many different microorganisms. Given a chance to grow, the organisms will produce changes in appearance, flavor, odor, and other qualities of foods. These degradation processes may be described as follows:

Putrefaction:
Protein foods + proteolytic microorganisms → amino acids + amines + ammonia + hydrogen sulfide

Fermentation:
Carbohydrate foods + carbohydrate-fermenting microorganisms → acids + alcohols + gases

Rancidity:
Fatty foods + lipolytic microorganisms → fatty acids + glycerol

The changes that microbes cause in foods are not limited to the results of degradation; they may also be caused by products of microbial synthesis. Some microorganisms discolor foods as a result of pigment production. Slimes may be developed in or on foods by microorganisms capable of synthesizing certain polysaccharides.

Fresh Foods

Examples of types of food spoilage (other than canned-food spoilage), together with some of the microorganisms involved, are shown in Table 28-3.

Fresh Milk

Milk is an excellent bacteriological medium. In fact, sterile skimmed milk is used routinely as a culture medium. Fresh whole milk contains protein (casein), carbohydrate (lactose), and fat. All of these substrates can be degraded enzymatically by microorganisms. If the degradation of these substrates is extensive, the accumulation of end products will impart undesirable characteristics to the milk. Some microorganisms can synthesize compounds like pigments and slimes which also give undesirable characteristics to the milk. A summary of

Table 28-3. Types of Food Spoilage (Other than Canned Foods) with Some Examples of Causative Organisms

Food	Type of Spoilage	Some Microorganisms Involved
Bread	Moldy	*Rhizopus nigricans* *Penicillium* *Aspergillus niger*
	Ropy	*Bacillus subtilis*
Maple sap and syrup	Ropy	*Enterobacter aerogenes*
	Yeasty	*Saccharomyces* *Zygosaccharomyces*
	Pink	*Micrococcus roseus*
	Moldy	*Aspergillus* *Penicillium*
Fresh fruits and vegetables	Soft rot	*Rhizopus* *Erwinia*
	Gray mold rot	*Botrytis*
	Black mold rot	*A. niger*
Pickles, sauerkraut	Film yeasts, pink yeasts	*Rhodotorula*
Fresh meat	Putrefaction	*Alcaligenes* *Clostridium* *Proteus vulgaris* *Pseudomonas fluorescens*
Cured meat	Moldy	*Aspergillus* *Rhizopus* *Penicillium*
	Souring	*Pseudomonas* *Micrococcus*
	Greening, slime	*Lactobacillus* *Leuconostoc*
Fish	Discoloration	*Pseudomonas*
	Putrefaction	*Alcaligenes* *Flavobacterium*
Eggs	Green rot	*P. fluorescens*
	Colorless rots	*Pseudomonas* *Alcaligenes*
	Black rots	*Proteus*
Concentrated orange juice	"Off" flavor	*Lactobacillus* *Leuconostoc* *Acetobacter*
Poultry	Slime, odor	*Pseudomonas* *Alcaligenes*

microbial biochemical types that may occur in milk, their source, and the changes they produce are shown in Table 28-1.

Table 28-4. Microbiology of Canned-Food Spoilage

Types of Product	Type of Spoilage Organisms, with Examples	Signs of Spoilage	
		Can	Contents of Can
Low and medium acid products, pH above 4.6, e.g., corn, peas, spinach, asparagus	Flat sour (*Bacillus stearothermophilus*)	Possible loss of vacuum on storage	Appearance not usually altered; pH markedly lowered; sour; may have slightly abnormal odor; sometimes cloudy liquor
	Thermophilic anaerobe (*Clostridium thermosaccharolyticum*)	Can swells, may burst	Fermented, sour, cheesy, or butyric odor
	Sulfide spoilage (*Clostridium nigrificans*)	Can flat, hydrogen sulfide gas absorbed by product	Usually blackened, "rotten egg" odor
	Putrefactive anaerobe (*Clostridium sporogenes*)	Can swells, may burst	May be partially digested; pH slightly above normal; typical putrid odor; may be toxic
	Aerobic sporeformers (odd types) (*Bacillus* spp.)	Usually no swelling, except in cured meats when nitrate and sugar are present	Coagulated evaporated milk, black beets
Acid products, pH below 4.6, e.g., tomato juice, fruits, and fruit juices	Flat sour (*Bacillus thermoacidurans*)	Can flat, little change in vacuum	Slight pH change; off odor and flavor
	Butyric anaerobes (*Clostridium butyricum*)	Can swells, may burst	Fermented, butyric odor
	Nonsporeformers (mostly lactic acid types of bacteria)	Can swells, usually bursts, but swelling may be arrested	Acid odor
	Yeasts	Can swells, may burst	Fermented; yeasty odor
	Molds	Can flat	Surface growth; musty odor

SOURCE: Data from the National Food Processors Association.

Canned Foods

Because of their heat resistance, sporeformers (species of *Clostridium* and *Bacillus*) constitute the most important group of microorganisms in the canning industry. The three most important types of microbiological spoilage of commercially canned foods are (1) flat sour spoilage, (2) thermophilic anaerobe (TA) spoilage, and (3) putrefaction. Table 28-4 presents a summary of organisms involved in spoilage of canned food, together with the changes they produce.

MICROBIOLOGICAL EXAMINATION OF FOODS

Microbiological examination of foods may provide information concerning the quality of the raw food and the sanitary conditions under which the food was processed as well as the effectiveness of the method of preservation. In the case of spoiled foods, it is possible to identify the agent responsible for the spoilage; having discovered the agent, it may be possible to trace the source of contamination and the conditions which permitted spoilage to occur. Corrective measures can then be instituted to prevent further spoilage.

Microbiological food examination takes advantage of special microscopic techniques and cultural procedures. Extensive use is made of selective and differential media to facilitate the enumeration and isolation of certain types of microorganisms. The particular procedure used is determined by the type of food product being examined as well as by the specific purpose of the examination. For example, a food sample being investigated for possible contamination by *Clostridium botulinum* would be subject to different laboratory tests than one being examined for coliform organisms. The increasing significance of salmonellas in foodborne disease has made it mandatory to develop more rapid, reliable, and reproducible methods for the detection of salmonellas in foods.

A schematic summary of the various approaches to consider in the microbiological examination of a food sample is shown in Fig. 28-2. The procedures selected for examination of a particular sample are, of course, determined by the facts relating to that sample and the purpose of the examination.

Microscopic Techniques

Standard microscopic techniques are available for the examination of some food products. For example, a procedure known as the Breed smear is used to make a direct microscopic count of microorganisms in milk. The essential procedures of this technique are: (1) spreading a measured amount of milk over a known area on a glass slide, (2) staining the film of milk with methylene blue, (3) making a microscopic count of organisms or clumps of organisms in several microscopic fields, and (4) calculating the total number of bacteria per unit volume.

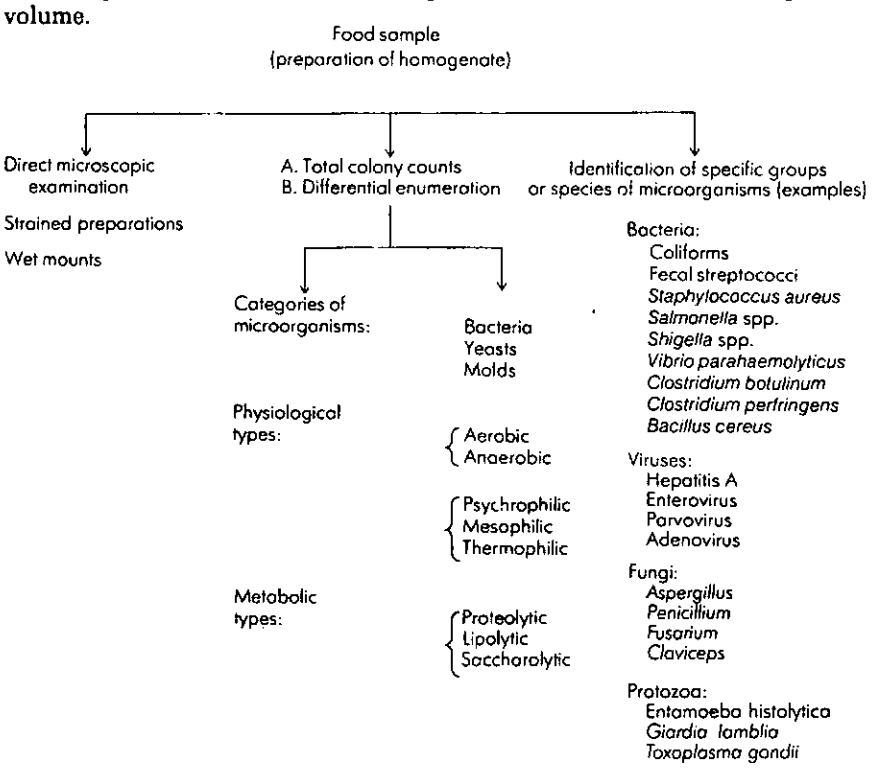

Figure 28-2. Generalized scheme for microbiological examination of foods.

A slide designed with a chamber, known as the Howard mold slide, is used, as its name suggests, to enumerate mold filaments in food products such as fruits, juices, and vegetables. When the mold counts obtained by this procedure exceed certain limits, it indicates raw material of poor quality or unsatisfactory sanitary processing.

Protozoa can be identified and enumerated by direct microscopic examination. Since the protozoa may be present in small numbers, it is frequently necessary to use a procedure which will concentrate these organisms in the food sample prior to microscopic examination.

Culture Techniques

The numerous techniques for cultivating microorganisms described in earlier chapters of this book find application, sometimes with modifications, for the examination of foods. For example, plate culture techniques are available for the enumeration of the "total" microbial population or some particular group of microorganisms, as illustrated in Fig. 28-2. The word *total*, of course, needs qualification; the microorganisms enumerated by a cultural technique are only that portion of the total population which will grow into colonies under the conditions provided, namely, the composition of the medium and the physical conditions of incubation. For example, the standard procedure for counting microorganisms in milk is designed to enumerate bacteria by the standard plate count (SPC). The conditions for the procedure are very specifically articulated in a volume entitled *Standard Methods for the Examination of Dairy Products*. It is mandatory that the procedure be carried out precisely as specified in the publication. Other culture procedures are available for particular physiological or biochemical types of microorganisms.

The cultivation of viruses from food specimens requires the use of tissue-culture techniques as described in Chap. 21. Prior concentration of the food specimen suspected to be contaminated with viruses may be necessary. Additional provisions are necessary to inhibit bacterial growth in the tissue culture.

PRESERVATION OF FOODS

Today we associate food preservation with the refrigerator, the deep freeze, and the canning process, all developments of the nineteenth and twentieth centuries. However, humans have grappled with the problem of food preservation for many centuries. The ancient Egyptians and Romans were aware of the preservative effects of salting, drying, and smoking. It has been suggested that the first salt preservation was accomplished by burying the food along the shore, where seawater effected the cure. The American Indians placed strips of fresh bison and venison at the top of a teepee or over a campfire, where preservation was accomplished through drying and smoking. Dried salt cod was a common food for colonial Americans. Perishable foods were stored in caves and springs, where the low temperature prolonged the preservation.

Modern methods of food preservation employ elaborate refinements of the primitive processes plus additional new techniques. The various practices used for food preservation may be summarized as follows:

1 Aseptic handling
2 High temperatures
   (a) Boiling

(b) Steam under pressure
(c) Pasteurization
(d) Sterilization (of milk)
(e) Aseptic processing
3 Low temperatures
(a) Refrigeration
(b) Freezing
4 Dehydration
5 Osmotic pressure
(a) In concentrated sugar
(b) With brine
6 Chemicals
(a) Organic acids
(b) Substances developing during processing (smoking)
(c) Substances contributed by microbial fermentation (acids)
7 Radiation
(a) Ultraviolet
(b) Ionizing radiations

All methods of food preservation are based upon one or more of the following principles: (1) prevention or removal of contamination, (2) inhibition of microbial growth and metabolism (microbistatic action), and (3) killing of microorganisms (microbicidal action).

Aseptic Handling

Food items undergo considerable handling prior to being processed by some specific method of preservation such as canning, freezing, or dehydration. Each step in the preparation of a food for its final treatment is a potential source of contamination. For example, the shell of an egg provides a protective covering which normally excludes microorganisms. However, when the eggs are cracked open in the process of preparing dehydrated egg powder it is likely that the interior of the egg may become contaminated. The extent of the contamination will depend upon the cleanliness of the eggs and the level of aseptic precautions observed in the process.

One can recognize more vividly the importance of asepic technique in the processing of more perishable foods like oysters and crabmeat, each of which requires considerable handling by people.

High Temperatures

High temperature is one of the safest and most reliable methods of food preservation. Heat is widely used to destroy organisms in food products in cans, jars, or other types of containers that restrict the entrance of microorganisms after processing.

Steam under pressure, such as in a pressure cooker, is the most effective method of high-temperature food preservation since it can kill all vegetative cells and spores. Food preservation by heat requires knowledge of the heat resistance of microorganisms, particularly spores. In addition, one must consider the rate at which heat penetrates through foods of different consistencies as well as the size of the containers in which they are packed. Killing microorganisms by heat involves a time-temperature relationship, as discussed in Chap. 22, and considerable experimentation has been performed to determine the thermal death times of bacteria likely to cause spoilage. From such information it is

possible to establish satisfactory heat-processing conditions. Much research has been done on this subject, and this accounts for the highly successful results achieved in food preservation by canning. Special laboratory equipment has been designed to determine with precision the heat resistance of various bacterial species, particularly the sporeformers.

Canning

Canning has been the basic method of food preservation for approximately 175 years. In 1810 Nicholas Appert, a Frenchman, published *L'Art de Conserver*, which described his successful researches in food preservation; and in the same year Peter Durand was granted an English patent describing the use of tin containers for food preservation.

The temperatures used for canning foods ranges from 100°C for high-acid foods to 121°C for low-acid foods. The canning process does not guarantee a sterile product. For example, spores of some bacterial species may survive these temperatures.

The most important organism to be eliminated in canned foods is the sporeforming anaerobe *Cl. botulinum*, which is capable of producing a very potent lethal toxin.

Pasteurization of Milk

The "Milk Ordinance and Code" of the U.S. Public Health Service comments on the word *pasteurization* as follows:

> The terms *pasteurization*, *pasteurized*, and similar terms shall mean the process of heating every particle of milk or milk product to at least 145°F., and holding it continuously at or above this temperature for at least 30 minutes, or to at least 161°F., and holding it continuously at or above this temperature for at least 15 seconds, in equipment which is properly operated and approved by the health authority . . . .

The original time-temperature relationships for pasteurization were worked out with *Mycobacterium tuberculosis* since this was regarded as the most heat-resistant pathogen likely to occur in milk (see Fig. 28-3). This organism is

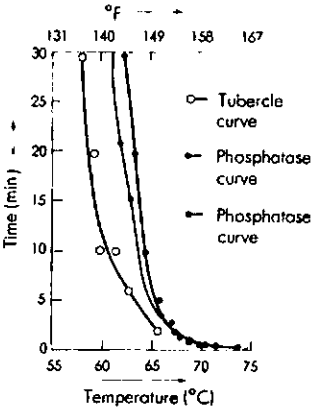

Figure 28-3. Time-temperature curve for the killing of *Mycobacterium tuberculosis* compared with the time and temperature required for the inactivation of the enzyme phosphatase. The two phosphatase curves are plotted from different experimental data. (*Courtesy of McGraw-Hill Encyclopedia of Science and Technology, p. 502, vol. 8. Copyright 1971. McGraw-Hill Book Company.*)

destroyed when exposed to a temperature of 140°F for 10 min. The pasteurization temperature was set at 143°F for 30 min. Later it was discovered that *Coxiella burnetii*, the causative agent of Q fever which can be transmitted by milk, can survive in milk heated to 143°F for 30 min. This observation resulted in the establishment of the present time and temperature for pasteurization.

Pasteurization Processes. Methods of pasteurization of milk used commercially include a low-temperature holding (LTH) method and a high-temperature short-time (HTST) method. The holding method, or vat pasteurization, exposes milk to 145°F (62.8°C) for 30 min in appropriately designed equipment. The HTST process employs equipment capable of exposing milk to a temperature of 161°F (71.7°C) for 15 s (seconds). In either method of pasteurization it is essential that the equipment be designed and operated so that every particle of milk is heated to the required temperature and held for the specified time. Precautions must be taken to prevent recontamination after pasteurization. The finished product should be stored at a low temperature to retard growth of microorganisms which survived pasteurization.

In addition to milk numerous other food products and some fermented beverages like beers and wines are commercially pasteurized.

The Phosphatase Test. Phosphatase is an enzyme, present in raw milk and in many tissues, which is destroyed by adequate pasteurization (see Fig. 28-3). Thus one can determine whether milk has been properly pasteurized by testing for the absence of this enzyme. The principle of the test is illustrated by the following reaction. Milk, which in its raw condition contains the enzyme phosphatase, is added to a substrate upon which the enzyme will react:

$$\underset{\text{Substrate}}{\text{Disodiumphenyl phosphate}} + \underset{\substack{\text{Enzyme from}\\ \text{raw milk}}}{\text{phosphatase}} \rightarrow \underset{\text{Products}}{\text{phenol} + \text{sodium phosphate}}$$

The amount of phenol liberated can be conveniently estimated by the addition of a reagent which turns blue in the presence of phenol. Color standards are used to interpret the results of this test. This is a very simple testing procedure, yet it provides valuable information about the heat treatment milk has received.

Sterilization

Commercial milk-sterilization techniques have been developed which expose milk to ultrahigh temperatures for very short periods of time, for example, 300°F (148.9°C) for 1 to 2 s. In addition, the sterilization process includes steps that eliminate any traces of cooked flavor. The final product is comparable in flavor and nutritional quality to pasteurized milk. The sterile milk product has several attractive features: it does not require refrigeration and it has an indefinite shelf life.

Aseptic Processing

A relatively new commercial development in the food industry is known as aseptic processing. The food item is commercially sterilized and packaged into previously sterilized containers under aseptic conditions.

This process has the advantage that it uses containers other than cans. This provides significant economic and user advantages.

Low Temperatures

Temperatures approaching 0°C and lower retard the growth and metabolic activities of microorganisms. Modern refrigeration and freezing equipment has made it possible to transport and store perishable foods for long periods of time. Refrigerated trucks and railway cars, ships' storage vaults, and the home refrigerator and freezer have improved the quality of the human diet and increased the variety of foods available. Frozen-food production in the United States almost doubled from 11 billion pounds in 1965 to 20 billion in 1975 and is expected to more than double to 48 billion pounds by 1985. Much of this increase will be in prepared frozen foods, whose quantity tripled over the last 10 years and is expected to approach 50 percent of all frozen foods by 1985. The growth and importance of this segment of the food industry places greater emphasis on the study of microorganisms at low temperatures, e.g., their survival, growth, and metabolic activity.

Before freezing, the fresh produce is steamed (blanched) to inactivate enzymes that would alter the product even at low temperatures. Quick-freeze methods, using temperatures of −32°C or lower, are considered most satisfactory; smaller crystals of ice are formed, and cell structures in the food are not disrupted. It should be emphasized that freezing foods, no matter how low the temperature, cannot be relied upon to kill all microorganisms. The number and types of viable and nonviable microorganisms present in frozen foods reflect the degree of contamination of the raw product, the sanitation in the processing plant, and the speed and care with which the product was processed. The microbial count of most frozen foods decreases during storage; but many organisms, including pathogens, e.g., species of *Salmonella*, survive for long periods of time at −9 and −17°C. The temperature ranges at which food-poisoning bacteria and psychrophilic microorganisms are capable of growing are shown in Fig. 28-4.

The increased use of precooked ready-to-serve foods and the prevalence of automatic vending machines for dispensing perishable foods have made it necessary to obtain more data on microbial growth and survival at low temperatures. Figure 28-5 illustrates the growth of salmonellas and staphylococci in prepared foods at various temperatures and times of incubation. Note that the type of

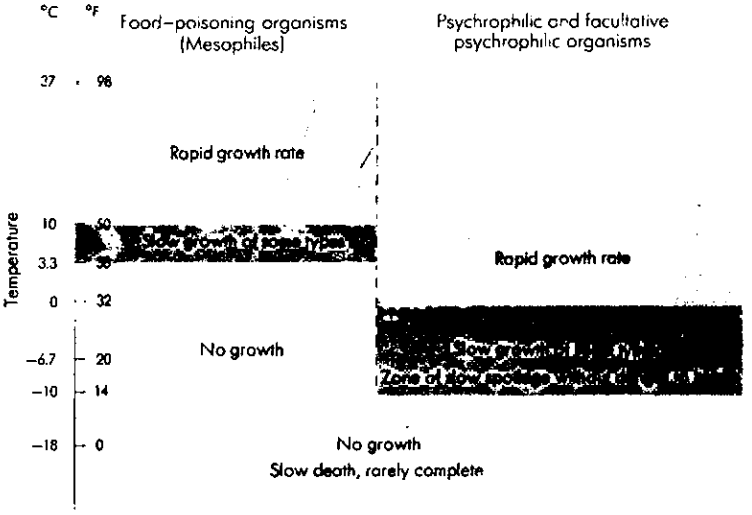

Figure 28-4. Food-poisoning organisms grow in a somewhat higher temperature range than psychrophilic microorganisms. (*Courtesy of R. P Elliot and H. D. Michener, Review of the Microbiology of Frozen Foods, in "Conference on Frozen Food Quality," ARS-74-21, USDA, 1960.*)

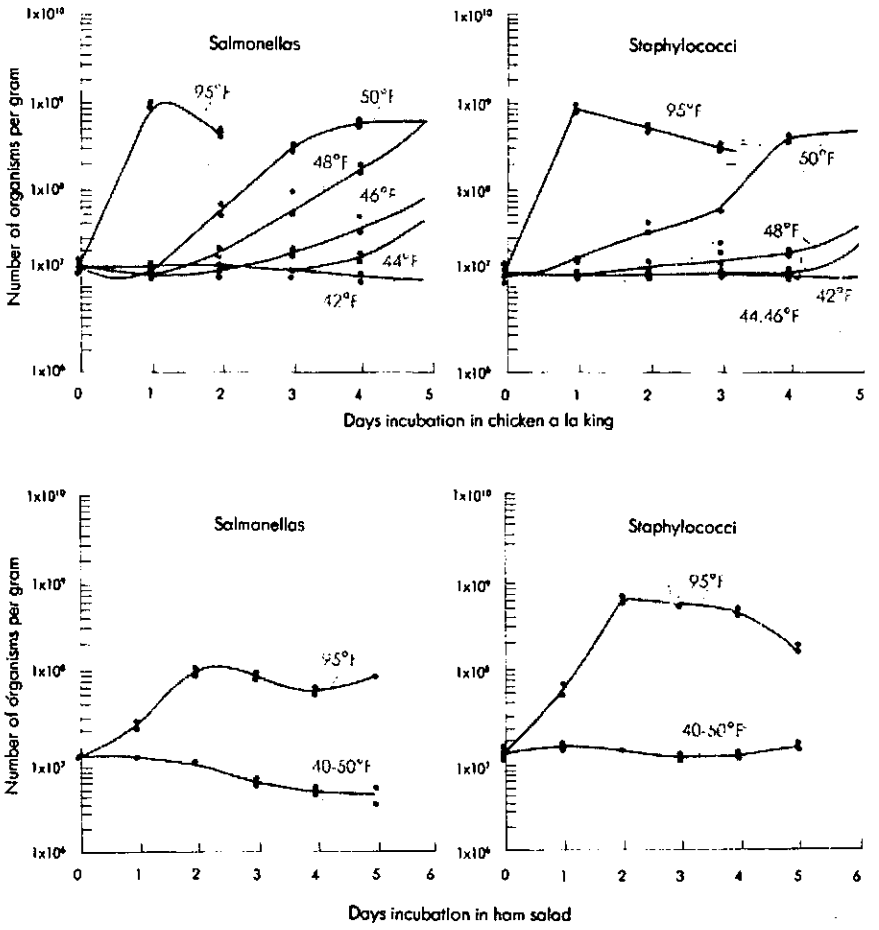

Figure 28-5. Salmonellas and staphylococci multiply rapidly in chicken a la king and ham salad incubated at room temperature. Curves also show growth at other temperatures. (*Courtesy of R. Angelotti, M. J. Foter, and K. H. Lewis, "Time-Temperature Effects on Salmonellae and Staphylococci in Foods," Am J Public Health,* **51:76–88** 1961.)

food product has considerable influence on the rate of bacterial growth at the lower temperatures.

Dehydration

Dried foods have been used for centuries, and they are more common throughout the world than frozen foods. The removal of water by drying in the sun and air or with applied heat causes **dehydration.** The preservative effect of dehydration is due mainly to microbistasis; the microorganisms are not necessarily killed. Growth of all microorganisms can be prevented by reducing the moisture content of their environment below a critical level. The critical level is determined by the characteristics of the particular organism and the capacity of the food item to bind water so that it is not available as free moisture. It will be recalled that lyophilized cultures of microorganisms survive for years.

Osmotic Pressure

Water is withdrawn from microorganisms placed in solutions containing large amounts of dissolved substances such as sugar or salt. The cells are plasmolyzed,

and metabolism is arrested. Thus the antimicrobial condition imposed by increased osmotic pressure is similar in principle to inhibition by dehydration. Although yeasts and molds are relatively resistant to osmotic changes, processes of food preservation based on this principle are, nevertheless, very useful. Jellies and jams are rarely affected by bacterial action because of high sugar content. However, it is not uncommon to find mold growth on the surface of jelly which has been exposed to air. Condensed milk is preserved in part by the increased concentration of lactose and supplemental sucrose. Similar results are obtained by curing meats and other foods in brines. High osmotic pressure may inhibit microbial growth, but it cannot be relied upon to kill all organisms.

Chemicals

Addition of chemicals to foods for the purpose of preservation is subject to the provisions of the United States Food, Drug, and Cosmetic Act as revised in 1972. According to this act, a food is adulterated if any poisonous or deleterious substance has been added which may render it injurious to health. Only a few chemicals are legally acceptable for food preservation. Among the most effective are benzoic, sorbic, acetic, lactic, and propionic acids, all of which are organic acids. Sorbic and propionic acids are used to inhibit mold growth in bread. Nitrates and nitrites used in curing meats, primarily for the preservation of color, are inhibitory to some anaerobic bacteria. This practice has been the subject of considerable controversy because of the potential of nitrates and nitrites as mutagenic agents and the subsequent relationship to carcinogenesis.

Foods prepared by fermentation processes, e.g., sauerkraut, pickles, and silage for animals, are preserved mainly by acetic, lactic, and propionic acids produced during the microbial fermentation. Smoking generates cresols and other antibacterial compounds which penetrate the meat.

Radiation

Ultraviolet light of sufficient intensity and time of exposure is microbicidal to exposed microorganisms. Because ultraviolet light has very limited penetration power, microorganisms that are embedded or covered are unlikely to be affected. Thus, ultraviolet irradiation is limited to control of microorganisms on surfaces or thin, clear layers of liquid. Examples of applications in the food industry include meat-processing plants, control of surface growth on bakery products, sanitation of equipment, and treatment of water used for the depuration (cleansing) of shellfish.

Ionizing radiations are lethal to microorganisms. The fact that they are microbicidal at room temperature and have the ability to penetrate are characteristics that make them attractive candidates for control of microorganisms in foods. Gamma rays and electron beams (beta and cathode rays) have been experimented with extensively for use in the food industry.

Canned and packaged foods can be sterilized by an appropriate radiation dosage. This "cold sterilization" produces a rise in temperature of the product of only a few degrees. Radiation pasteurization is a term describing the killing of over 98—but not 100—percent of the organisms by intermediate doses of ionizing radiation.

The ionizing radiation resistance of microorganisms does not correspond to their thermal resistance. *Clostridium botulinum* appears to be the most radioresistant organism of importance to the food technologist. Figure 28-6 illus-

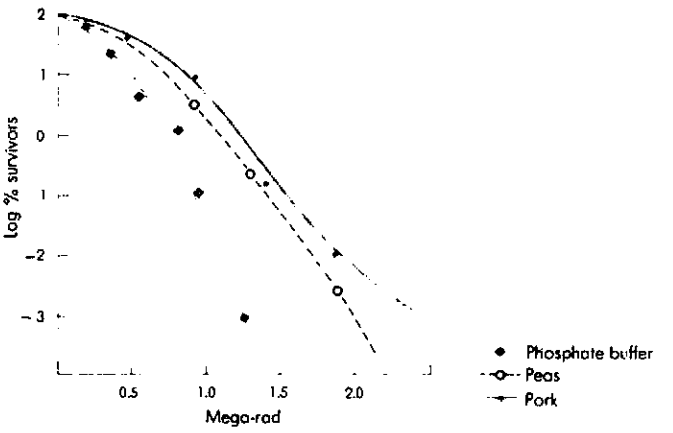

Figure 28-6. Gamma radiation kills spores of *Clostridium botulinum* in frozen foods. Curves show the effect on spores in pork, peas, and phosphate buffer. (*Courtesy of C. B. Denny, C. W. Bohrer, W. E. Perkins, and C. T. Townsend, "Destruction of Clostridium botulinum by Ionizing Radiation," Food Res,* **24**:44–50, 1959.)

trates the lethal effect of gamma radiation on spores of *C. botulinum*. Note that the survival of spores is influenced by the material in which they are suspended and that time is not a factor. In the case of radiation, unlike temperature, the radiation death dose rather than radiation death time is determined.

Ionizing radiation sterilization provides the possibility of an entirely new approach to food preservation; it could bring about a radical change in industrial methods of food processing. However, despite the extensive research and documentation on the effectiveness of ionization radiation for the preservation of foods, this method of preservation has not been approved in the United States. This is due in part to economic factors as well as to some lingering uncertainties about the effect of the radiation on the food material. In addition, the United States already has well-developed systems for food preservation. This is not the case for all countries. The World Health Organization approved (1976) radiation of poultry at a specified level, as has Canada for controlling salmonellas. In July 1983, the U.S. Food and Drug Administration approved the use of ionizing radiation for sterilization of specific spices and vegetable seasonings.

FERMENTED FOODS

Thus far we have stressed the undesirable characteristics of microorganisms in food. However, there are many useful applications of microorganisms in the food industry. A variety of important products in our diet are produced with the aid of microbial activity.

Fermented Dairy Products

In the dairy industry, fermented milks are produced by inoculating pasteurized milk with a known culture of microorganisms, sometimes referred to as a starter culture, which can be relied on to produce the desired fermentation, thus assuring a uniformly good product. (See Fig. 28-7, which shows *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, organisms used as starter cultures in the preparation of yogurt.)

Several hundred varieties of cheese are manufactured, and with few exceptions, most of them can be made from the same batch of milk. Microorganisms—

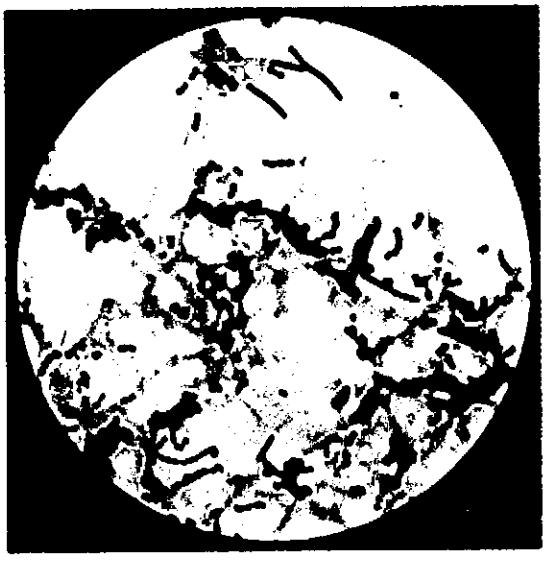

Figure 28-7. Photomicrograph of yogurt, illustrating microbial flora, *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (X800). (*Courtesy of K. J. Demeter, Bakteriologische Untersuchungsmethoden der Milchwirtschaft, Eugen Ulmer, Stuttgart, 1967.*)

bacteria or molds—convert the curd of the milk into the desired cheese. For the manufacture of some cheeses, such as blue cheese or Roquefort (blue cheese made in Roquefort, France), it is necessary to inoculate the curd with the mieroorganism which brings about the changes (in this case, *Penicillium roqueforti*). Some of the steps in the process of making Roquefort cheese are shown in Fig. 28-8.

Other Fermented Foods

Important food items produced in whole or in part by microbial fermentations include pickles, sauerkraut, olives, and certain types of sausage. Lactic acid bacteria are chiefly responsible for the desirable type of fermentation required for the production of each of these products. The microorganisms that produce the changes may be the natural flora on the material to be fermented or may be something added as a starter culture. Most commercial sour, sweet, mustard, and mixed pickles are made from fermented salt-stock pickles. The other major type of pickled cucumber is the fermented dill pickle. An illustration of a commercial fermentation process for the production of dill pickles is shown in Fig. 28-9.

The list of food products produced by microbial fermentation is very long. A few examples are shown in Tables 28-5 and 28-6.

MICROORGANISMS AS FOOD—SINGLE-CELL PROTEIN

Bacteria, yeasts, and algae, produced in massive quantities, are attractive sources of food for animals as well as humans. These microorganisms can be cultivated on industrial wastes or by-products as nutrients and yield a large cell crop that is rich in protein (single-cell protein). Bacterial cells grown on hydrocarbon wastes from the petroleum industry are a source of protein in France, Japan, Taiwan, and India. Yeast-cell crops harvested from the vats used to produce

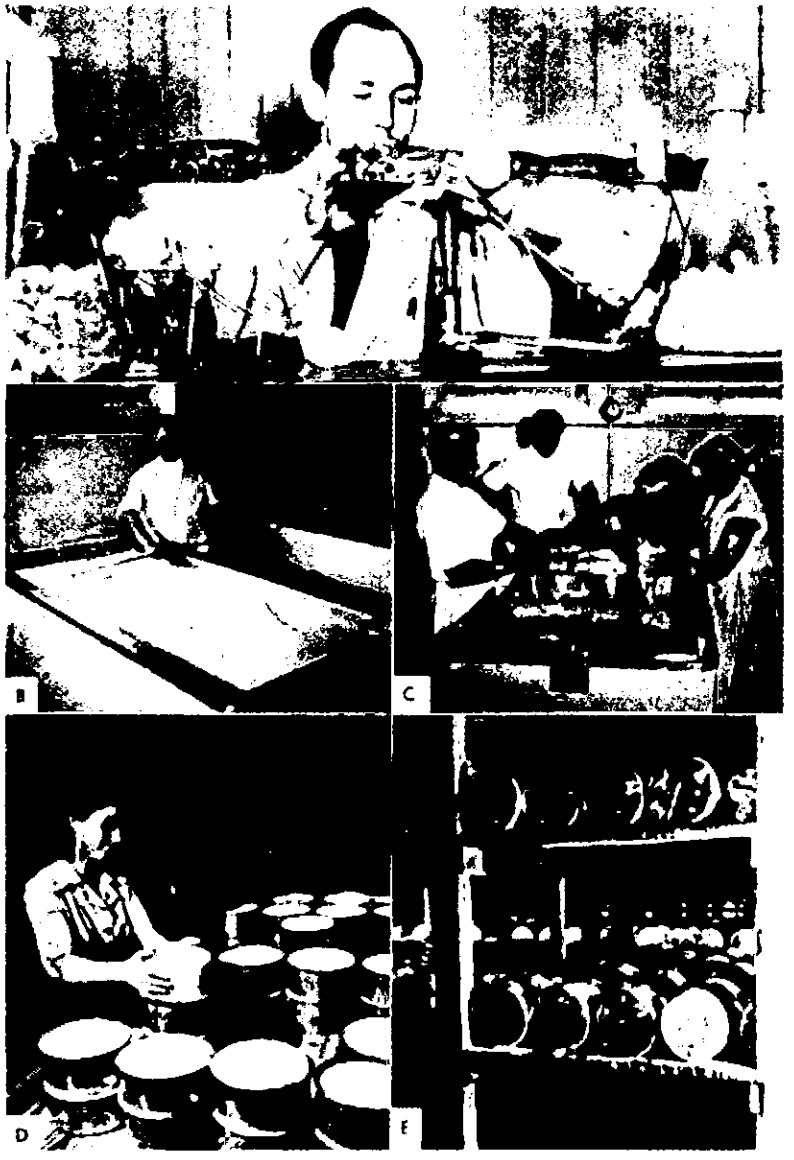

Figure 28-8. Roquefort and blue cheese. (A) Cubes of sterile whole wheat bread are inoculated with *Penicillium roqueforti*. After extensive growth of the mold on the bread cubes, the cubes are removed, dried, and powdered and used as inoculum for making cheese. (*Courtesy of the Borden Company.*) (B) The addition of a lactic culture and rennet curdles the milk. The curd is cut when it becomes firm. (C) The curd particles are removed and placed in metal hoops. The addition of the spores of *P. roqueforti* may take place in either of these steps. (D) The hoops are placed on a draining board to facilitate whey drainage and matting of the curd, after which the curd is removed, salted periodically, and (E) eventually placed in an area of high humidity (95 to 98 percent) and low temperature (9 to 12°C), where the ripening process occurs over a period of several months. The hoops of cheese shown ripening here are wrapped in foil. [(*B to E*) *Courtesy of Roquefort Association, Inc.*]

alcoholic beverages have been used as a food supplement for generations. The attractiveness of single-cell protein as a food substitute or supplement is apparent from the following characteristics of the process.

1 Microorganisms grow very rapidly and produce a high yield. It has been calculated that one can obtain a gain of 1 lb of protein in 1 day's growth from a 1000-lb steer; 1000 lb of yeast would produce several tons of protein in one day! Algae grown in ponds can produce 20 tons (dry weight) of protein per acre per year.

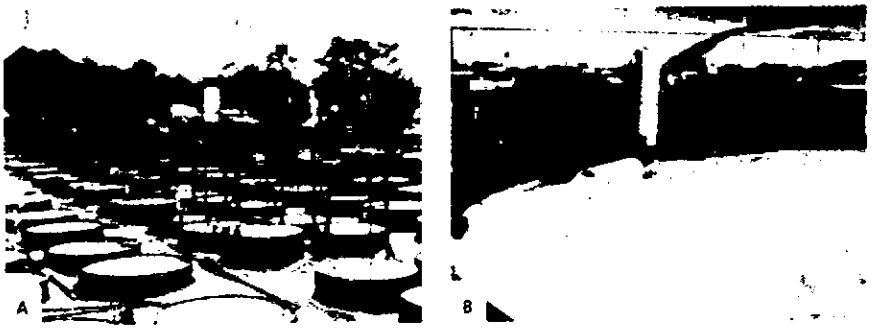

Figure 28-9. (A) Typical tank yard for fermentation and storage of brined cucumbers. The wooden tanks shown are 600- to 1000-bushel capacity. Some pickle companies now use fiberglass tanks. Note the tall, white tank in the background, which is for storage of liquid nitrogen. Nitrogen gas is piped to each brine tank for use in purging of dissolved CO_2 from fermenting brines to prevent bloater damage in the cucumbers. (*Courtesy of H. P. Fleming, USDA.*) (B) Surface of a cucumber brine tank being nitrogen-purged with a sidearm purger. Nitrogen gas purges dissolved CO_2 from the brine and also serves to circulate the brine. The white frothing on the surface is caused by the purging action. (*Courtesy of H. P. Fleming, USDA.*)

Table 28-5. Some Characteristics of Fermented Milks

Fermented Product	Principal Microorganisms Responsible for Fermentation	General Remarks
Cultured buttermilk	A mixture of lactic streptococci (*Streptococcus lactis* or *S. cremoris*) with aroma-producing bacteria (*Leuconostoc citrovorum* or *L. dextranicum*)	The function of the lactic acid streptococci is to produce lactic acid that gives the sour taste and to curdle the milk; the function of the leuconostocs is to produce volatile and neutral products that impart a characteristic desirable odor; the starter culture must contain vigorously growing bacteria; incubation is performed at 21°C.
Cultured sour cream	Same as used for cultured buttermilk, i.e., streptococci and leuconostocs	Not strictly a fermented milk but manufacture resembles that of cultured buttermilk; cream is inoculated and incubated until the desired acidity develops; flavor and aroma compounds are also contributed by the starter culture
Bulgarian milk	*Lactobacillus bulgaricus*	Incubation of inoculated milk at 37°C, but otherwise similar to cultured buttermilk; product differs from commercial buttermilk in having higher acidity and lacking aroma
Acidophilus milk	*L. acidophilus*	Milk for propagation of *L. acidophilus* and the bulk milk to be fermented is sterilized, since this organism is eaily overgrown by contaminating bacteria; incubation is at 37°C; acidity allowed to develop to 0.6 to 0.7%

Table 28-5. (*continued*)

Fermented Product	Principal Microorganisms Responsible for Fermentation	General Remarks
Yogurt	*Streptococcus thermophilus* *L. bulgaricus*	Made from milk in which solids are concentrated by evaporation of some water and addition of skim milk solids; product has consistency resembling custard; now common in Europe and North America; similar products with different names are produced elsewhere (see Fig. 28-7)
Kefir	*S. lactis* *L. bulgaricus* Lactose-fermenting yeasts	A mixed lactic acid and alcoholic fermentation; bacteria produce acid (0.6 to 1.0% lactic acid), and yeasts produce alcohol (0.5 to 1.0% ethanol); the organisms conglomerate to form small granules called kefir grains; the granules are used as the starter culture; in the Balkans, the fermentation is carried out in leather bags made of goatskin; the fermentation process may be continuous by adding fresh milk as the fermented product is removed; Kefir is made from cow, goat, or sheep milk
Kumiss	Similar to those found in kefir grains	A mixed acid-alcoholic fermentation product made from mares' milk in some parts of Russia

Table 28-6. Some Examples of Fermented Food Products

Fermented Food	Starting Product	Microorganisms Involved
Sauerkraut	Shredded cabbage	Early stage: *Enterobacter cloacae* *Erwinia herbicola* Intermediate stage: *Leuconostoc mesenteroides* Final stage: *Lactobacillus plantarum*
Pickles	Cucumbers	Early fermentation: *L. mesenteroides* *Streptococcus faecalis* *Pediococcus cerevisiae* Later fermentation: *Lactobacillus brevis* *L. plantarum*
Green olives	Olives	Early stage: *L. mesenteroides* Intermediate stage: *L. plantarum* *L. brevis* Final stage: *L. plantarum*
Sausage	Beef and pork	*Pediococcus cerevisiae* *Micrococcus* spp.

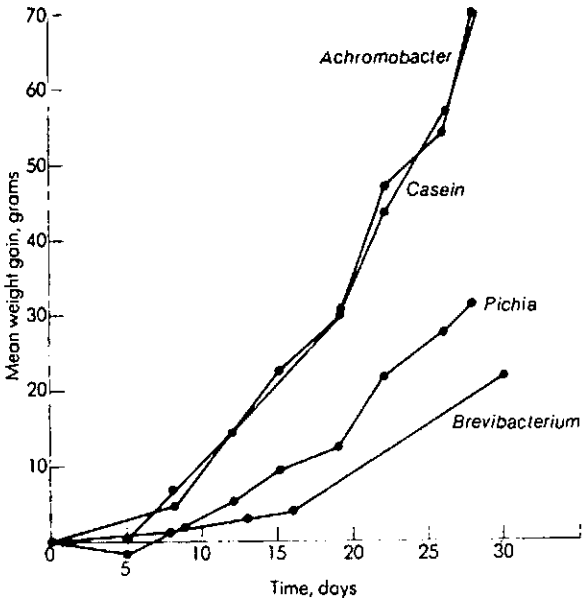

Figure 28-10. The nutritional quality of microbial proteins. This experiment shows the mean weight gain (per group) of rats fed various proteins: casein, bacterial proteins (*Achromobacter, Brevibacterium*), or yeast protein (*Pichia*). Note that the growth response to casein and *Achromobacter* protein was very similar. (*Courtesy of V. F. Coty and R. I. Leavitt, Dev Ind Microbiol, 12, 1971.*)

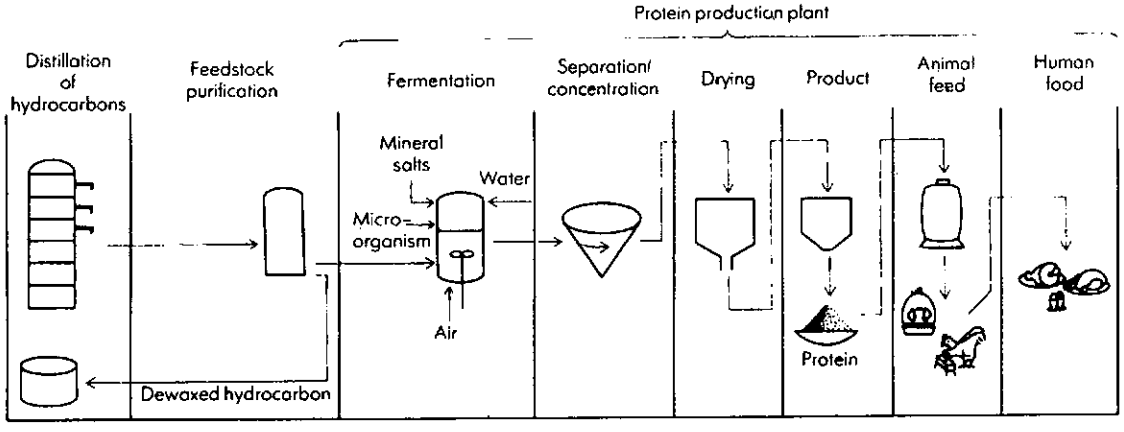

Figure 28-11. The process used by British Petroleum to produce single-cell protein from hydrocarbons. *n*-Alkanes are distilled for use in the fermenter. Minerals are added. Following fermentation, the cells are separated and dried for use as animal feed. (*Courtesy of British Petroleum Co. Ltd.*)

This yield is 10 to 15 times higher than soybeans and 25 to 50 times higher than corn.

2 The protein content of the microbial cells is very high. Dried cells of *Pseudomonas* spp. grown on petroleum products have 69 percent protein; yeast cells have a protein content in a 40 to 50 percent range; for algae, the range is from 20 to 40 percent.

3 The proteins of selected microorganisms contain all the essential amino acids. An example of the nutritional quality of microbial proteins is shown in Fig. 28-10.
4 Some microorganisms, particularly yeasts, have a high vitamin content.
5 The medium (nutrients) for growth of microorganisms may contain industrial wastes or by-products, e.g., liquid paraffins (hydrocarbons) from oil refineries, spent sulfite liquors from the pulp and paper industry, beet molasses, and wood hydrolysates.

A fermentation system using yeast cells for single-cell protein production is shown in Fig. 28-11. The growth medium consists of hydrocarbons (*n*-alkanes) supplemented with mineral salts. The cell crop is harvested by centrifugation, dried, and used as animal feed.

Despite the very attractive features of single-cell protein as a nutrient for humans there are problems which deter its adoption on a global basis. For example, individual tastes and customs make microorganisms unattractive as a food substance to many persons. More specifically, the high nucleic acid content of microbial cells can produce intestinal disturbances. There is also the need to ascertain if the amino acid composition and content of the microbial protein meet the dietary requirements of the consumer.

QUESTIONS

1 List and describe the principles upon which methods of food preservation are based.
2 Compare the antimicrobial action of the following methods of food preservation: canning, refrigeration, dehydration, and increased osmotic pressure.
3 What is the lowest temperature range at which food-poisoning bacteria will grow?
4 What physiological types of bacteria are most likely to be present when canned food spoils?
5 Compare the types of microorganism that might be involved in the spoilage of refrigerated foods with those incriminated in the spoilage of canned foods.
6 List several types of microbial food spoilage, and name the organisms responsible in each instance.
7 Why is milk an excellent bacteriological culture medium?
8 Is milk sterile as it is drawn from the cow? Explain.
9 List the major sources of bacterial contamination of milk.
10 Describe the various types of biochemical changes brought about in milk by microorganisms. Identify the predominant types of bacteria responsible for each of these changes.
11 Of what particular significance are psychrophilic, thermoduric, and thermophilic bacteria in milk and milk products?
12 Is pasteurized milk sterile milk? Explain.
13 Compare the heat resistance of *Mycobacterium tuberculosis* and *Coxiella burnetii*. What bearing does this have on requirements for adequate pasteurization time and temperature?
14 What information does the phosphatase test reveal about milk?
15 What are the attractive features of food preservation through use of radiation?
16 Outline a procedure suitable for enumeration, isolation, and identification

of the following groups of microorganisms from a sample of food: thermophilic sporeformers, coliforms, and viruses.

17 Compare the microscopic and cultural techniques for microbiological analysis of foods. What are the advantages and limitations of each of these procedures?

18 Name several foods that are prepared by microbial fermentations. Describe the role of microorganisms in each example.

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Chapter 29 Industrial Microbiology

OUTLINE

Microorganisms were exploited for useful purposes long before anything was known about their existence or their characteristics. As early as 6000 B.C., the Babylonians and Sumerians used yeast to make alcohol. History reveals many other applications of microbial processes that resulted in the production of desirable materials, particularly foods and beverages. However, as we have mentioned earlier, it was not until the studies of Louis Pasteur in the second half of the nineteenth century that the role of microorganisms in these processes was understood.

From the standpoint of industrial microbiology, microorganisms can be considered chemical factories in miniature. They have the capacity to convert a raw material (nutrient or substrate) into end products. If the end products have value for human use, then it becomes attractive to exploit the microbiological process, that is, to produce the end products on a commercial scale.

Industrial microbiology has experienced two dramatic explosions during the last few decades. In the 1940s the discovery of antibiotics, led by penicillin, initiated a major new industry built upon the products of microorganisms. More recently, as a result of the great advances in our knowledge of microbial genetics, it is possible to manipulate microorganisms genetically to produce new products. The process is called **recombinant DNA technology**. This development, namely, the "engineering" of microorganisms to produce needed valuable chemical substances, is likely to revolutionize the field of industrial microbiology.

MICROORGANISMS AND INDUSTRY

Microorganisms, under natural conditions, produce an extremely large number, as well as a very large variety, of chemical substances. Some of these substances are very useful for the treatment of diseases and disorders of people and other animals and hence are attractive to the pharmaceutical industry; others are valuable as raw materials for the chemical industry for precursors for other products, solvents, and for other uses. We have already discussed the role of microorganisms in the production of many foods. Other applications of large-scale microbial activity can be found in mining, where microorganisms are used to leach metals from low-grade ores; in dealing with environmental pollution where, for example, microorganisms are used to degrade obnoxious pollutants; and in agriculture for enhancement of plant growth, control of insect pests, and other purposes.

The overall reaction characterizing the industrial application of microorganisms can be summarized as follows:

Substrate (raw material) + microorganisms → new products

Prerequisites to Practical Industrial Microbiological Processes

If a microorganism converts cheap raw materials into a useful product, it may be feasible to perform this reaction on a large industrial scale. Some of the prerequisites to an economically practicable industrial microbiological process are the following, in terms of the organism, the medium, and the product:

1 ***The organism.*** The organism to be used must be able to produce appreciable amounts of the product. It should have relatively stable characteristics and the ability to grow rapidly and vigorously, and it should be nonpathogenic.
2 ***The medium.*** The medium, including the substrate from which the organism produces the new product, must be cheap and readily available in large quantities. In several instances it has been found practicable to utilize nutrient-containing wastes from the dairy industry (whey), the paper industry (waste liquors resulting from the cooking of wood, waste sulfite liquors), and other commercial operations.
3 ***The product.*** Industrial fermentations are performed in large tanks; capacities of 50,000 gal are not unusual. The product formed by the metabolism of the microorganism is present in a heterogenous mixture that includes a tremendous crop of microbial cells and unused constituents of the medium, as well as products other than those being sought. Thus, an efficient and economical mass-scale method of recovery and purification of the desired end product must be developed.

Major Classes of Products and Processes

The major commercial products of microorganisms can be classified as follows: (1) the microbial cells; (2) large molecules like enzymes that are synthesized by the microorganisms; (3) primary metabolic products, i.e., compounds essential for cell growth; (4) secondary metabolic products, i.e., compounds not required for cell growth. Substances in groups 3 and 4 are generally much smaller in molecular size than those in group 2.

One may also group industries on the basis of the type of microbial products they market, as listed below:

1 ***Pharmaceutical chemicals.*** Most prominent in this category are the antibiotics and steroid drugs, but other substances, such as insulin and interferon, are now being produced by genetically engineered bacteria. Many other new products are likely through genetic biotechnology.
2 ***Commercially valuable chemicals.*** Solvents, enzymes, and intermediate compounds for the synthesis of other substances are representative of the kinds of substances produced commercially by microorganisms. Specific examples are provided later in this chapter.
3 ***Food supplements.*** Mass production of yeasts, bacteria, and algae from media containing inorganic nitrogen salts and other readily available and cheap nutrients provides a good source of protein and other organic nutrients useful as food supplements. Large-scale microbial production of amino acids is an attractive industrial process being employed in many parts of the world.
4 ***Alcoholic beverages.*** Brewing, wine making, and production of other alcoholic beverages constitute some of the oldest and largest microbiological industries.
5 ***Vaccines (immunizing antigens).*** Some microorganisms are grown in very large quantities for use as vaccines. The whole cell or some part or product of the cell is used for the preparation of vaccines.
6 ***Deterioration of materials by microorganisms.*** All kinds of material such as leather, textiles, wood, metals, and even optical equipment are subject to deterioration by contamination with and growth of microorganisms. The magnitude of potential destruction with resulting financial losses demands that methods for prevention of this destruction be developed. Industry is responsive to this need and produces many chemicals and treatment processes for this purpose.
7 ***Analytical microbiology.*** Microbiological techniques have been developed for assaying a variety of products like antibiotics, amino acids, and vitamins. Other microbiological procedures are available for evaluating wood and paint preservatives and for testing the efficacy of sterilization procedures.

Microorganisms Used in Industrial Processes

Industrial microbiological processes have been developed using specific strains of algae, fungi (yeasts and molds), bacteria, protozoa, and viruses. Microbial species which have potential for industrial application are continually being sought. The attractiveness of a microorganism may reside in its ability to produce a new product, e.g. an antibiotic. Or the industrial application might involve the use of a microorganism in a process such as cleaning up oil spills; the microorganism degrades the oil to nonobjectionable compounds.

Once a species has been found to have industrial application, a research program is undertaken to increase the capacity of the microorganism to produce the desirable change, that is, to give a higher yield of the end product or a greater rate of change in the substrate being decomposed. The customary approach to achieve these ends has been through improvements in culture media and cultural conditions, selection of new strains, and development of mutants.

However, research in molecular biology and more specifically research in bacterial genetics, as described in Chap. 12, has provided the knowledge and the technology to deliberately change the genetic makeup of a microorganism. This process, known as genetic recombination, has dramatically altered industrial microbiology.

BIOENGINEERING OF MICROORGANISMS FOR INDUSTRIAL PURPOSES

What is commonly referred to today as bioengineering of microorganisms is, in fact, an application of recombinant DNA technology—the in vitro incorporation of segments of genetic material from one cell into another cell. This technology was made possible from the knowledge accumulated over the last few decades in biological research at the molecular level which elucidated the structure and synthesis of DNA. The fundamental aspects of this subject were presented in Chaps. 11 and 12.

Genetic Engineering of Microorganisms

The essential steps in the technology of producing a genetically engineered bacterium are shown in Fig. 29-1A. They can be summarized as follows:

1 ***Source of donor genetic material.*** DNA containing the genetic code for the property to be transferred into a bacterium is isolated from cells, or it may be synthesized. The DNA is *tailored* to form the gene which contains the genetic information to code for a desired characteristic such as production of human insulin.

2 ***Production of hybrid DNA molecule.*** The donor genetic material (DNA segment) is incorporated into the DNA molecule of a bacteriophage or a bacterial plasmid. This is accomplished by the use of two enzymes: restriction endonucleases ligases. Restriction endonucleases cut double-stranded DNA molecules at particular nucleotide sequences and thus produce a well-defined DNA fragment for a given enzyme and a given DNA. In this process both the donor DNA and the agent (vehicle) into which the fragment of the donor DNA is to be incorporated are treated with the same restriction endonuclease. As shown in Fig. 29-1B, the endonuclease Eco R1 cuts the plasmid DNA and the donor DNA in a manner such that the ends of each are identical and self-complementary. The fragments can be connected by the addition of an enzyme called DNA ligase.

   Hybrid DNA can also be produced by other more elaborate experimental techniques.

3 ***Incorporation of hybrid DNA into host cell.*** Transformation in genetic engineering is the process by which plasmid hybrid DNA molecules are introduced into a competent host bacterial cell. Transfection involves the introduction of phage hybrid DNA into the host cell. The most common technique for transformation depends on treating the recipient bacteria with calcium chloride to make the membrane permeable to the DNA. The recipient bacteria are capable of receiving recombinant DNA molecules on the basis of only one molecule per bacterium.

When bacteria are transformed or transfected, a mixture of bacteria of various genotypes is usually produced. But each bacterial cell is capable of binary fission, yielding a colony of identical cells possessing equivalent genetic, and therefore physiological, traits. Once a colony with the proper phenotype is identified, the bacteria in it can be grown in limitless quantity to amplify the gene.

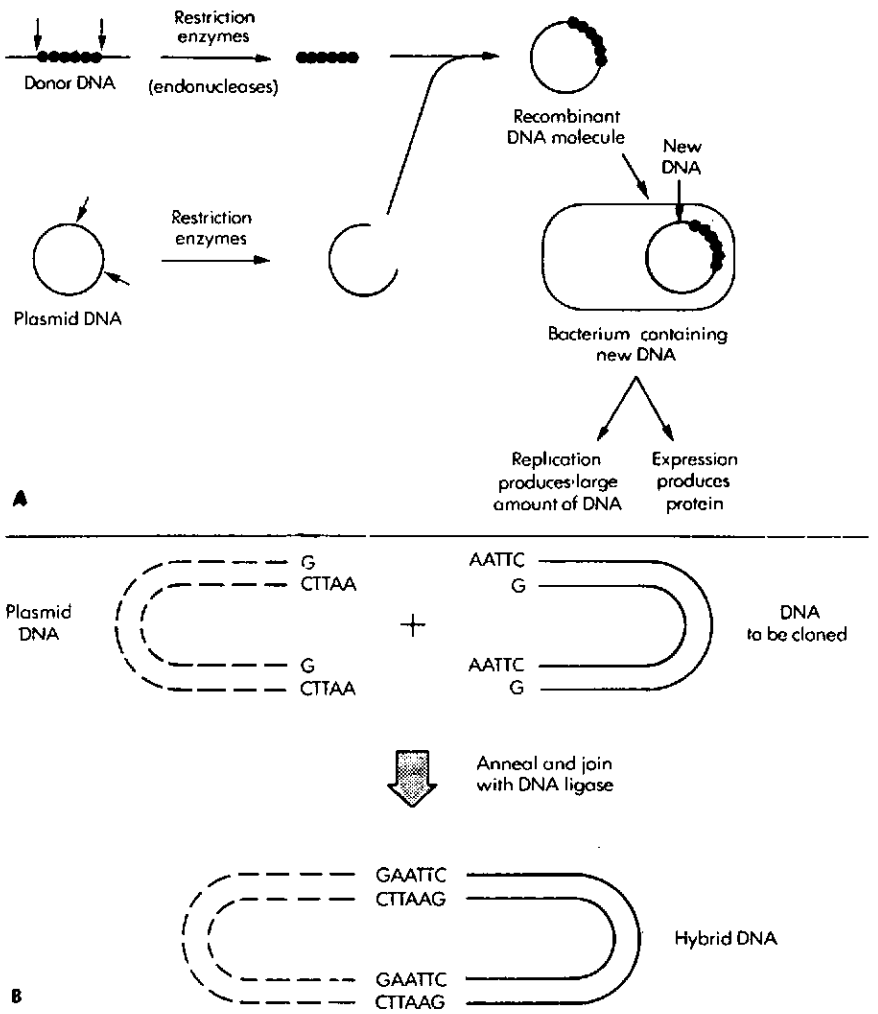


Figure 29-1. (A) The major steps in producing a "genetically engineered" bacterium. (B) Fragments of donor DNA and plasmid DNA excised by endonucleases and formation of hybrid DNA by joining these fragments using DNA ligase. *(Erwin F. Lessel, illustrator.)*

Thus it is seen that the difficult problem of the chemical purification of a gene has been surmounted by the screening of bacterial colonies. **Cloning**, the isolation and proliferation of individual, genetically unique cells, thus provides one type of a high-resolution separation method for DNA molecules which would be almost impossible to fractionate by any other means. The progeny of the selected bacterium constitutes a **clone** and the gene is said to have been **cloned**. Since there is no difficulty in physically separating the plasmid DNA from the rest of the bacterial DNA, it is possible to obtain the DNA of the cloned gene in pure state and in unlimited amounts. Furthermore, one plasmid inserted into an *Escherichia coli* bacterium may generate a hundred or more copies of itself within the cell. Cloned genes have been obtained in great numbers from a wide variety of species, ranging from bacteria through brewing-yeasts, fruit flies, sea urchins, toads, and mice.

The Potential and Problems of Genetic Engineering

The genetic alteration of plants, animals, and microorganisms has been an important practice in many of our major industries, such as agriculture, the beverage industry, and more recently the pharmaceutical industry (antibiotic production). These genetic alterations have been achieved through mutation and selection. As a result of new genetic technologies our capabilities to manipulate the inherited characteristics of all species of life has been enormously increased. This technology provides almost limitless possibilities for the benefit of society and at the same time poses serious problems.

Benefits from Genetic Engineering

Genetic technologies, present and future, can contribute to the improvement of our health, our environment, our supply of food, and many other aspects of our

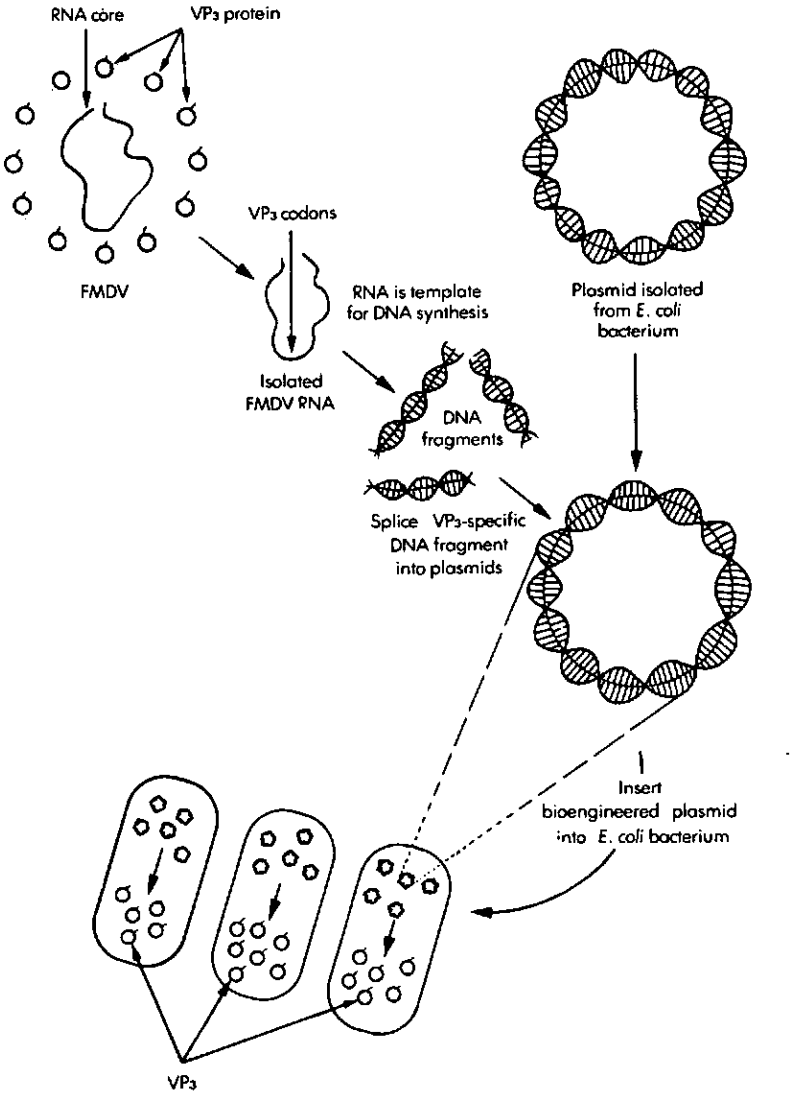


Figure 29-2. Recombinant DNA strategy for making foot-and-mouth disease vaccine. VP_3 is the protein from the shell of the foot-and-mouth disease virus (FMDV), which can act as a vaccine for immunizing livestock against foot-and-mouth disease. The idea is to make this VP_3 protein without making any virus or infectious RNA. *(Erwin F Lessel, illustrator.)*

lives. The pharmaceutical industry has already produced several products for human therapy, such as human insulin, interferon, urokinase (for the treatment of blood clots), and somatostatin (a brain hormone), and new techniques for vaccine development have emerged (see Fig. 29-2). A major research effort is underway to produce genetically engineered microorganisms that can fix nitrogen in cereal crops and thus greatly improve soil fertility.

Microorganisms have been genetically engineered to decompose oil in oil spills, and other commercial applications are likely in pollution-control industries. mining, and oil recovery.

Potential Problems of Genetic Engineering

The practical application of molecular genetic technology allows the movement of genes across species lines, such as from animals and fruit flies to bacteria! This results in the creation of new, redesigned organisms. This has raised questions of risks that might be involved.

There is concern that production of recombinant DNA molecules that are functional in vivo could prove biologically hazardous. If they are carried in a microbe like *E. coli*, which is a commensal bacterium in the human gut and can exchange genetic information with other types of bacteria, they might possibly become widely disseminated among human, bacterial, plant, or animal populations, with unpredictable results.

Of special concern is construction of new autonomously replicating bacterial plasmids that could, if not very carefully controlled, introduce genetic determinants for antibiotic resistance or bacterial toxin formation into bacterial strains that do not presently carry such determinants. Experiments to link all, or segments of, DNA from oncogenic or other animal viruses to autonomously replicating DNA elements, such as bacterial plasmids or other viral DNAs, also pose threats.

Because of the concerns associated with genetic engineering, The National Institutes of Health have established guidelines for research involving recombinant DNA molecules. Under these guidelines, The National Institutes of Health serve an overseeing role by sponsoring risk-assessment programs, certifying new host-vector systems, serving as an information clearing house, and coordinating federal and local activities.

INDUSTRIAL USES OF BACTERIA

Some products of bacterial origin together with their uses are shown in Table 29-1. The production processes for lactic acid, vinegar, amino acids (lysine and glutamic acid), and insulin will be described as examples of the many others in operation today.

Lactic Acid Production

Several carbohydrate substances such as corn and potato starch, molasses, and whey can be used for the production of lactic acid. Starch must first be hydrolyzed to glucose by acid or enzymatic treatment. The choice of carbohydrate material depends upon its availability, treatment required prior to fermentation, and cost. We shall describe the production of lactic acid from whey.

Large quantities of whey constitute a waste product in the manufacture of certain dairy products such as cheese. From the standpoint of pollution problems created by the disposal of untreated whey, as well as for economic reasons,

Table 29-1. Some Industrial Products (Other than Antibiotics) Produced by Bacteria

Product	Microorganism	Uses
Acetone-butanol	*Clostridium acetobutylicum* and others	Solvents; chemical manufacturing
2,3-Butanediol	*Bacillus polymyxa* *Enterobacter aerogenes*	Solvent; humectant; chemical intermediate
Dihydroxyacetone	*Gluconobacter suboxydans*	Fine chemical
2-Ketogluconic acid	*Pseudomonas* spp.	Intermediate for D-araboascorbic acid
5-Ketogluconic acid	*G. suboxydans*	Intermediate for tartaric acid
Lactic acid	*Lactobacillus delbrueckii* *L. bulgaricus*	Food products; textile and laundry; chemical manufacturing; deliming hides
Bacterial amylase	*Bacillus subtilis*	Modified starches; sizing paper; desizing textiles
Bacterial protease	*B. subtilis*	Bating hides; desizing fibers; spot remover; tenderizing meat
Dextran	*Leuconostoc mesenteroides*	Stabilizer in food products; blood-plasma substitute
Sorbose	*G. suboxydans*	Manufacture of ascorbic acid
Cobalamin (vitamin B_{12})	*Streptomyces olivaceus* *Propionibacterium freudenreichii*	Treatment of pernicious anemia; food and feed supplementation
Glutamic acid	*Brevibacterium* spp.	Food additive
Lysine	*Micrococcus glutamicus*	Animal-feed additive
Streptokinase-streptodornase	*Streptococcus equisimilis*	Medical use (dissolving blood clots)
Bioinsecticides	*Bacillus thuringiensis* *Bacillus popilliae*	Control of insects
Insulin, interferon, somatostatin (human growth hormone)	Recombinant DNA Varieties of *E. coli*	Human therapy
Microbial protein (SCP)	Methane-oxidizing bacteria	Food supplement

it is desirable to use it to make some useful product. Whey represents a satisfactory medium for the growth of certain bacteria, since it contains carbohydrate (lactose), nitrogenous substances including vitamins, and salts. The first requirement for the development of a method of producing lactic acid is an organism capable of growing in whey and fermenting most if not all the lactose to lactic acid. Lactobacilli are suitable for this purpose, particularly *Lactobacillus bulgaricus*. This organism grows rapidly and is homofermentative and thus is capable of converting the lactose to the single end product—lactic acid. Stock cultures of the organism used are maintained in a skim-milk medium. To prepare a sufficient amount of inoculum for addition to the main fermentation tank, the culture is successively transferred and incubated in increasing amounts of sterile skim milk, pasteurized skim milk, and finally whey. Milk is used in "building up" the inoculum, since it is a superior medium. Inoculum from the whey-incubation tank is added to the fermentation tank in an amount equivalent to 5

to 10 percent of the volume to be fermented. An incubation temperature of 43°C is used and has the desirable effect of inhibiting growth of many extraneous microorganisms. During the fermentation, a slurry of lime, $Ca(OH)_2$, is added intermittently to neutralize the acid, and calcium lactate is formed; otherwise the accumulation of acid would retard fermentation. Upon completion of fermentation (approximately 2 days) the material in the tank is boiled to coagulate the protein, which is then filtered and processed for use as an animal-feed supplement. The filtrate containing the calcium lactate is then concentrated by removal of water under a vacuum, followed by additional treatments to purify the compound. The process is shown schematically in Fig. 29-3.

The biochemical reactions performed by the microorganism in producing the lactic acid can be summarized:

$$\underset{\text{Lactose}}{C_{12}H_{22}O_{11}} + H_2O \xrightarrow{\text{lactase}} \underset{\substack{\text{Glucose}\\ +\ \text{galactose}}}{2C_6H_{12}O_6} \xrightarrow[\text{of enzymes}]{\text{system}} \underset{\text{Lactic acid}}{2CH_3CHOHCOOH}$$

Derivatives of lactic acid are used in the treatment of calcium deficiency (calcium lactate) and of anemia (iron lactate), as a solvent in lacquers (n-butyl lactate), and as a plasticizer and humectant (sodium lactate).

Vinegar Production

The word *vinegar* is derived from the French term *vinaigre*, meaning "sour wine." It is prepared by allowing a "wine" to go sour under controlled conditions.

The production of vinegar involves two types of biochemical changes: (1) an alcoholic fermentation of a carbohydrate and (2) oxidation of the alcohol to acetic acid. There are several kinds of vinegars, and the differences among them are primarily associated with the kind of material used in the alcoholic fermentation, e.g., fruit juices, sugar-containing syrups, and hydrolyzed starchy materials. The definition and standards for one type as given by the U.S. Food and Drug Administration are as follows:

Vinegar, cider vinegar, apple vinegar. The product made by the alcoholic and subsequent acetous fermentations of the juice of apples. It contains, in 100 cubic centimeters (20°C), not less than 4 grams of acetic acid.

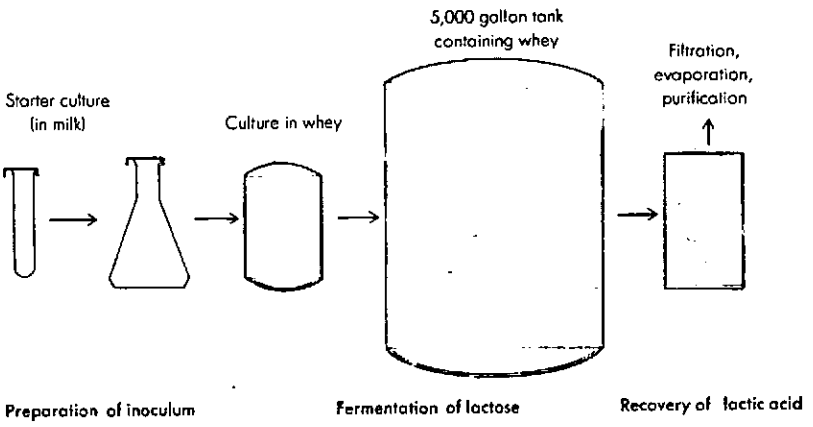


Figure 29-3. Lactic acid production from whey by *Lactobacillus bulgaricus*.

A yeast fermentation is used for production of the alcohol. The alcohol concentration is adjusted to between 10 and 13 percent and then exposed to the action of acetic acid bacteria. Many types of equipment have been designed for industrial production of vinegar. All depend upon providing a suitable environment for the bacterial oxidation of alcohol to acetic acid. The essential features of one of the industrial processes for vinegar production, the Frings method, is shown in Fig. 29-4 and may be summarized as follows. A mix is prepared which consists of an adjusted solution of alcohol acidified with acetic acid and special nutrients for the growth of acetic acid bacteria. Acetic acid bacteria, species of the genus *Acetobacter*, are inoculated onto the beechwood shavings. The mix is applied in a trough at the top of the chamber and allowed to trickle down over the shavings. As the alcohol solution passes over the shavings, the acetobacters oxidize some of the alcohol to acetic acid. The mix is collected at the bottom of the unit and may be recirculated over the shavings, resulting in more oxidation of alcohol until vinegar of the desired strength is produced.

Since this is an aerobic process, oxygen is required as shown in the following reaction accounting for the formation of acetic acid:

$$\underset{\text{Ethyl alcohol}}{2CH_3CH_2OH} + 2O_2 \rightarrow \underset{\text{Acetic acid}}{2CH_3COOH} + 2H_2O$$

An abundant supply of air must be available throughout the chamber. It is also necessary to keep the temperature between 15 and 34°C, the optimum for growth and metabolism of the acetobacters. The Frings vinegar generator is equipped with various accessories which permit control of these factors. Deviation in temperature below or above this range not only has an adverse effect on the acetobacters but permits growth of other microorganisms with different metabolic characteristics.

Amino Acid Production

Many microorganisms can synthesize amino acids from inorganic nitrogen compounds. The rate and amount of synthesis of some amino acids may exceed the cells' need for protein synthesis, whereupon the amino acids are excreted into the medium. Some microorganisms are capable of producing amounts of certain

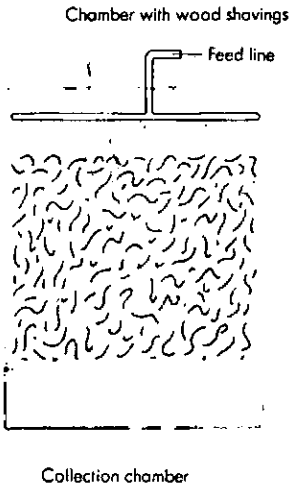
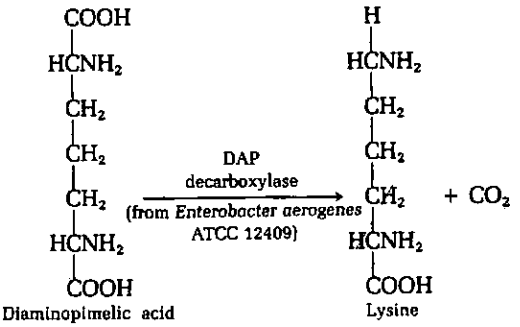


Figure 29-4. Frings vinegar generator. A dilute solution of alcohol percolates through wood shavings that are covered with a growth of acetobacters. The bacteria oxidize the alcohol to acetic acid.

amino acids (lysine, glutamic acid, and tryptophan) sufficient to justify their commercial production. Among the advantages of the microbial fermentation processes is that the biologically active forms of the amino acids (L optical isomers) are produced.

L-Lysine Production

One of the commercial methods for production of lysine consists of a two-stage process using two species of bacteria: (1) the formation of diaminopimelic acid (DAP) by *E. coli* and (2) the decarboxylation of the diaminopimelic acid by an enzyme (DAP decarboxylase) obtained from *Enterobacter aerogenes*:



E. coli is grown in a medium consisting of glycerol, corn-steep liquor, and $(NH_4)_2HPO_4$ under controlled conditions of aeration, temperature, and pH for optimum production of DAP. After approximately 3 days' incubation, DAP decarboxylase is added to convert the DAP to lysine, as shown in the reaction above.

Lysine is an essential amino acid for the nutrition of humans and is of particular interest since cereal proteins are often deficient in this amino acid. It is used as a supplement for bread and other foodstuffs.

L-Glutamic Acid Production

Many species of microorganisms, especially bacteria and fungi, are capable of producing large amounts of glutamic acid. Species of *Micrococcus*, *Arthrobacter*, and *Brevibacterium* are used for its industrial production. The medium generally consists of a carbohydrate, peptone, inorganic salts, and biotin; the concentration of biotin has a significant influence on the yield of glutamic acid. α-Ketoglutaric acid produced via the tricarboxylic acid cycle (Krebs cycle) is the precursor of glutamic acid.

The conversion of α-ketoglutaric acid to glutamic acid is accomplished by glutamic dehydrogenase.

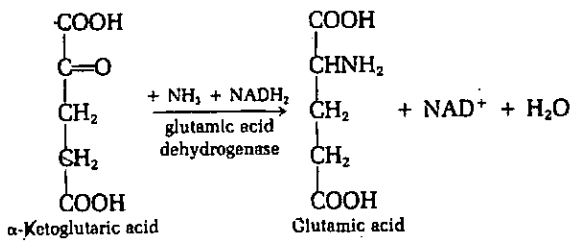


Figure 29-5. (A) Transmission electron micrograph of *E. coli* (X35,000) containing insulin A chain chimeric protein. Arrows indicate concentrations of this chimeric protein in the cells. (B) The first crystals ever obtained of human insulin made by recombinant DNA technology. *(Courtesy of Eli Lilly Co.)*

Glutamic acid is in demand as a condiment and flavor-enhancing agent in the form of monosodium glutamate. Millions of pounds are produced annually.

Insulin is one of the important pharmaceutical products produced commercially by a genetically engineered bacterium. Prior to this development, commercial insulin for the therapy of diabetes was isolated from animal pancreatic tissue.

Earlier research on purified insulin isolated from pancreatic tissue led to the

Table 29-2. Some Commercial Products of Yeast

Product	Microorganism	Uses
Bakers' yeast, beer, wine, ale, bread	*Saccharomyces cerevisiae*	Baking industry; brewing industry
Soy sauce	*Saccharomyces rouxii*	Food condiment
Sour French bread	*Candida milleri*	Baking
Commercial alcohol (ethanol)	*S. cerevisiae* *Kluyveromyces fragilis*	Fuel; solvent
Riboflavin	*Eremothecium ashbyi*	Vitamin supplement
Microbial protein	*Candida utilis*	Animal food supplement (single-cell protein) from paper-pulp waste
	Saccharomycopsis lipolytica	Microbial protein from petroleum products

establishment of the amino acid sequence of this protein hormone molecule. From this information it was possible to establish the DNA code for the synthesis of insulin. This was followed by the isolation of the gene from human tissue which controls insulin production. By using recombinant DNA technology, the human insulin gene was introduced into a bacterium (*E. coli*). This genetically engineered bacterium is grown in large quantities, as is characteristic of industrial microbiological processes, to produce human insulin. Following maximum production of insulin in the commercial culture, the insulin is extracted, purified, and evaluated for biological response (see Fig. 29-5A and B).

Human insulin produced by genetically altered bacteria was made available to diabetics in September of 1982.

Two of the major advantages of insulin production by microorganisms is that the resulting insulin is chemically identical to human insulin and it can be made available in unlimited quantities.

INDUSTRIAL USES OF YEASTS

The best known and one of the most important uses of yeasts is in the production of ethyl alcohol from carbohydrate materials. This fermentation process is used by brewers of malt beverages, distillers, bakers, wine makers, chemical manufacturers, homemakers, and many others. A list of some of the commercial products of yeasts is shown in Table 29-2.

Alcohol Fermentations

Next to water, alcohol is the most common solvent and raw material used in the laboratory and chemical industry. The microbiological aspects of the process of ethyl alcohol production can be summarized as follows.

The Substrate

Ethyl alcohol can be produced from any fermentable carbohydrate by yeasts. When starches, such as corn, and other complex carbohydrates are used as the raw material, it is first necessary to hydrolyze them to simple fermentable sugars. The hydrolysis can be accomplished with enzymes from barley malt or molds or by heat treatment of acidified material. Corn, molasses, sugar beets, potatoes, and grapes are some of the common raw materials employed throughout the world.

The Organism

Selected strains of *Saccharomyces cerevisiae* are commonly employed for the fermentation. It is imperative that the culture be one that grows vigorously and has a high tolerance for alcohol as well as a capacity for producing a large yield of alcohol. Much attention has been directed toward the selection and development of strains of yeasts which excel in these particular characteristics.

The Reaction

The biochemical change accomplished by the yeast is as follows:

$$\underset{\substack{\text{Glucose}\\\text{(fermentable}\\\text{carbohydrate)}}}{C_6H_{12}O_6} \xrightarrow[\text{enzymes}]{\text{yeast}} \underset{\text{Ethyl alcohol}}{2C_2H_5OH} + \underset{\text{Carbon dioxide}}{2CO_2}$$

Bakers' Yeast

The use of yeast as a leavening agent in baking dates back to the very early histories of the Jews, Egyptians, Greeks, and Romans. In those days leavened bread was made by mixing some leftover dough from the previous batch of bread with fresh dough. Another practice, since the Middle Ages, has been to use excess yeasts from brewing and winemaking operations. The variable quality of such products made this practice unsatisfactory. In modern baking practice, pure cultures of selected strains of *S. cerevisiae* are mixed with the bread dough to bring about desired changes in texture and flavor. Desirable characteristics of *S. cerevisiae* strains selected for commercial production of bakers' yeast include the ability to ferment the sugar in the dough vigorously and to grow rapidly; these as well as other characteristics for which the strain was selected should be relatively stable. The carbon dioxide produced during the fermentation is responsible for the leavening, or rising, of the dough. The quality of the product depends on the proper selection of yeasts and the incubation conditions as well as on the choice of raw materials.

In the manufacture of bakers' yeast the "stock" strain is inoculated into a medium which frequently contains molasses and corn-steep liquor. The medium is adjusted to an acid pH (pH 4 to 5), which helps retard bacterial growth. The inoculated medium is aerated during the incubation period. At the end of incubation the yeast cells are harvested by centrifugation and washed by suspending the cells in water and then centrifuging the cells out. The cells are finally recovered on a filter press, small amounts of vegetable oil are added as a plasticizer, and then this mass of cells is molded into blocks. Some steps in this process are illustrated in Fig. 29-6.

Food Yeasts

Mass cultivation of yeasts, as well as of algae and bacteria, offers a possible source of food supplement or substitute for human and animal consumption. This subject is presented in Chap. 28, where the production of single-cell protein (yeast) from petroleum constituents is discussed. It appears, at the present time, that the major technical problems associated with producing a new type of protein for animal foods have been solved. Thus, massive production of microbial cells may provide the way of bridging the "protein gap" in a protein-hungry world.

INDUSTRIAL USES OF MOLDS

Many substances are produced commercially by molds. Perhaps the most significant is the antibiotic penicillin. Molds are used for the fermentation of rice

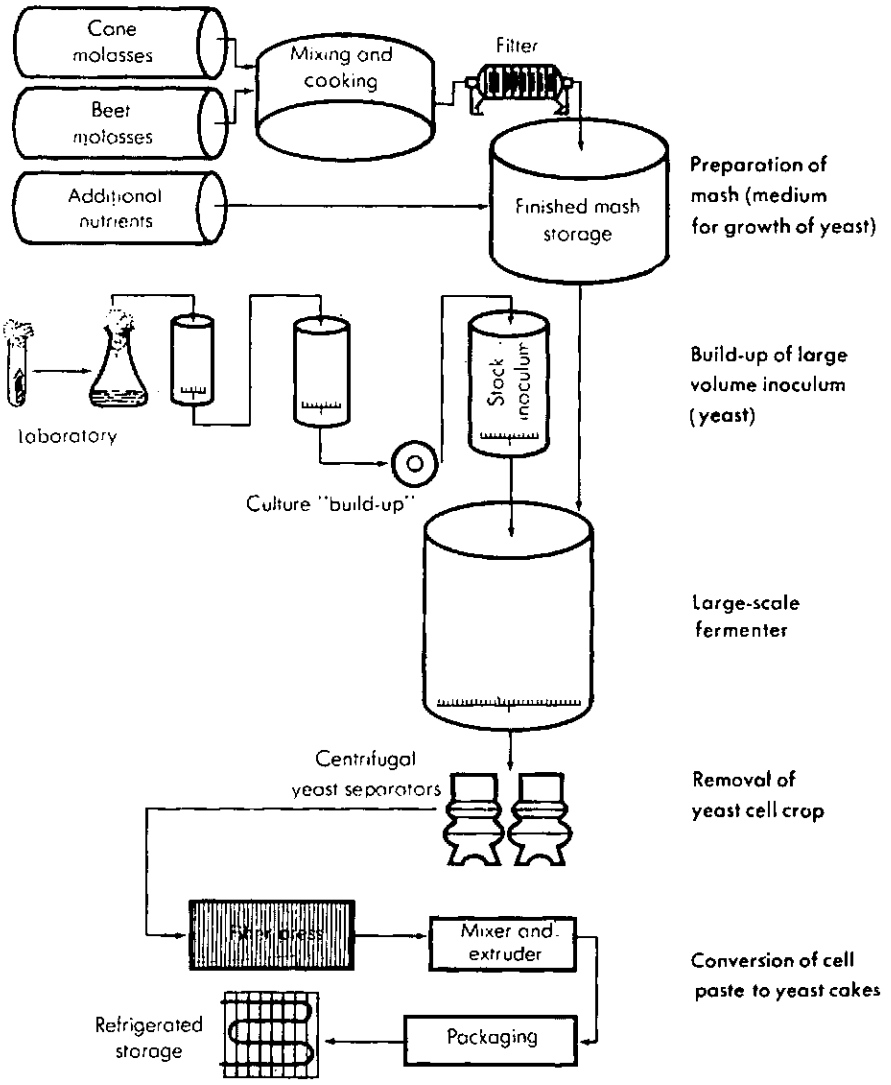


Figure 29-6. Steps in the commercial production of bakers' yeast.

Table 29-3. Some Industrial Products (Other than Antibiotics) Derived from Molds

Product	Microorganism	Uses
Citric acid	*Aspergillus niger* or *Aspergillus wentii*	Food products, medicinal citrates; in blood for transfusion
Fumaric acid	*Rhizopus nigricans*	Manufacture of alkyd resins, wetting agents
Gluconic acid	*A. niger*	Pharmaceutical products, textiles, leather, photography
Itaconic acid	*Aspergillus terreus*	Manufacture of alkyd resins, wetting agents
Pectinases, proteases	*A. wentii* or *Aspergillus aureus*	Clarifying agents in fruit juice industries
11-γ-Hydroxyprogesterone	*Rhizopus arrhizus, R. nigricans*, others	Intermediate for 17-γ-hydroxycorticosterone
Gibberellic acid	*Fusarium moniliforme*	Setting of fruit, seed production
Lactic acid	*Rhizopus oryzae*	Foods and pharmaceuticals

to produce a variety of oriental foods and food additives. They also produce several enzymes—proteases, amylases, and pectinases—that are manufactured for use in industry. A list of some commercially important products, other than penicillin, is shown in Table 29-3.

Penicillin Production

The commercial production of penicillin and other antibiotics represents one of the most dramatic case histories in the development of industrial microbiology. The antibiotic industry did not exist in 1941, but 10 years later net sales of these products had reached $344 million per year. Data reported in 1983 by the U.S. International Trade Commission revealed that 32.518 million pounds of bulk antibiotics were manufactured in 1982.

Penicillin was the first antibiotic to be produced industrially. Much of what was learned in transforming Fleming's laboratory observations into an economically feasible large-scale operation paved the way for successful production of other chemotherapeutic antibiotics as they were discovered.

The mold isolated by Fleming (*Penicillium notatum*), and as grown in his laboratory, yielded only a few units of penicillin per milliliter, an exceedingly small amount when one considers that a patient may require treatment with millions of units. The remarkable chemotherapeutic effectiveness of penicillin was demonstrated by Florey and Chain during 1939 and 1941. Because of the pressures of war, the British scientists brought the mold to the United States in hope of developing production of the antibiotic on a large scale. An extensive research program having one of the highest wartime priorities was initiated. In a relatively short time the yield of penicillin was increased about a thousand times. The developments contributing to this enormous increase in yield were as follows:

1 Improvements in composition of the medium.
2 Isolation of a better penicillin-producing mold species, *Penicillium chrysogenum*.
3 Development of the submerged-culture technique: cultivation of the mold in large volumes of liquid medium through which sterile air is forced.
4 The production of mutant strains of P. chrysogenum which were capable of producing large amounts of penicillin. A series of mutants, produced by x-ray and ultraviolet radiation, resulted in strains with a remarkable capacity for synthesis of penicillin.
5 The addition of chemicals to the medium which served as precursors for synthesis of penicillin.
6 Refinements in methods of recovering penicillin from the fermentation mixture.

The major steps in the commercial production of penicillin are:

1 Preparation of inoculum
2 Preparation and sterilization of medium
3 Inoculation of the medium in the fermenter
4 Forced aeration with sterile air during incubation
5 Removal of the mold mycelium after fermentation
6 Extraction and purification of the penicillin

This process is shown schematically in Fig. 29-7; a commercial production facility is shown in Fig. 29-8. The changes which occur during the fermentation process (growth, synthesis of penicillin, etc.) are shown in Fig. 29-9.

The production of most other antibiotics follows a similar plan: The major differences relate to the organism, composition of medium, and method of extraction. Some manufacturers employ the same fermentation equipment for the production of several different antibiotics.

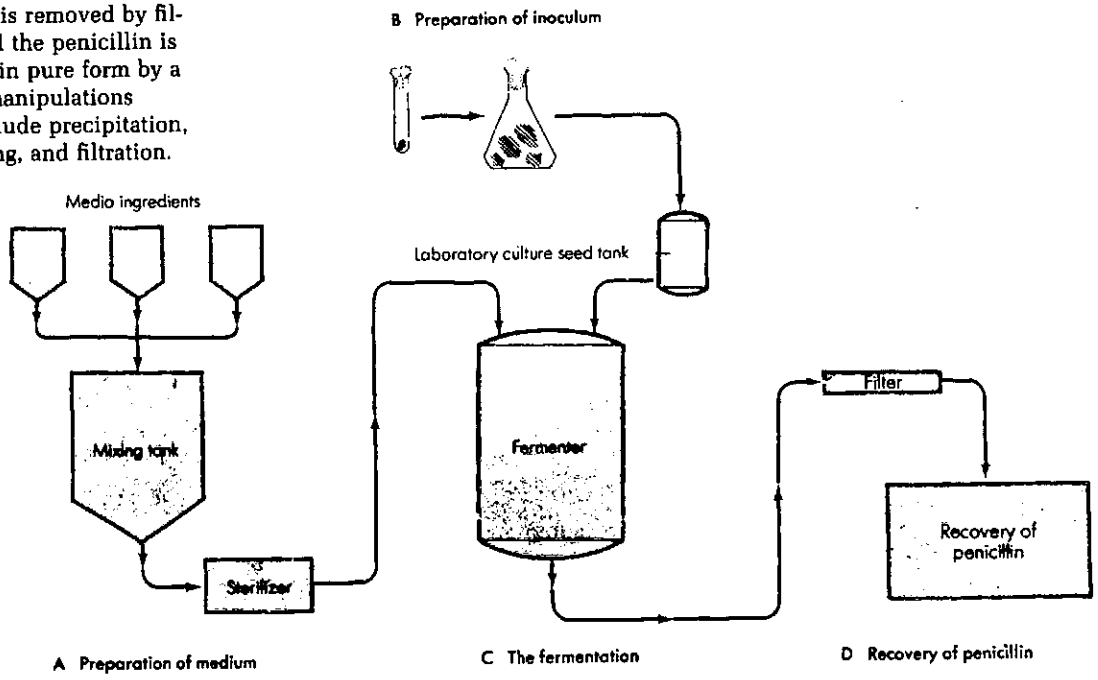


Figure 29-7. Manufacture of penicillin shown schematically. (A) A medium of corn-steep liquor, lactose, salts, and other ingredients is mixed, sterilized, cooled, and pumped into the fermenter. (B) The mold *Penicillum chrysogenum* is transferred from slant cultures to bran, and spore suspensions from bran are transferred to a sterile vessel with medium, which in turn is used to inoculate the seed tank. (C) The fermenter is inoculated from the seed tank; sterile air is forced through the fermenter during incubation. (D) After the maximum yield of penicillin is produced, the mold mycelium is removed by filtration and the penicillin is recovered in pure form by a series of manipulations which include precipitation, redissolving, and filtration.

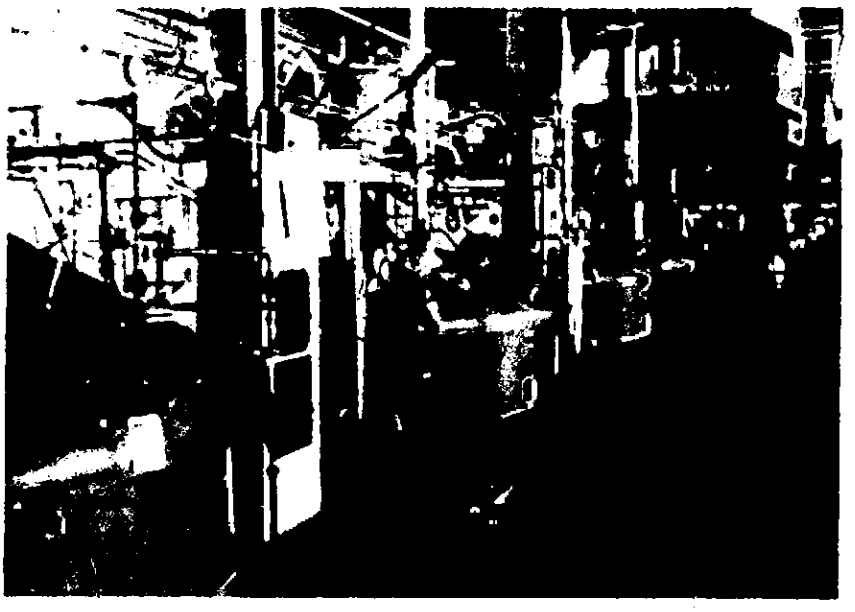


Figure 29-8. Tops of large fermentation tanks of the type used to produce antibiotics. *(Courtesy of Merck and Co., Inc.)*

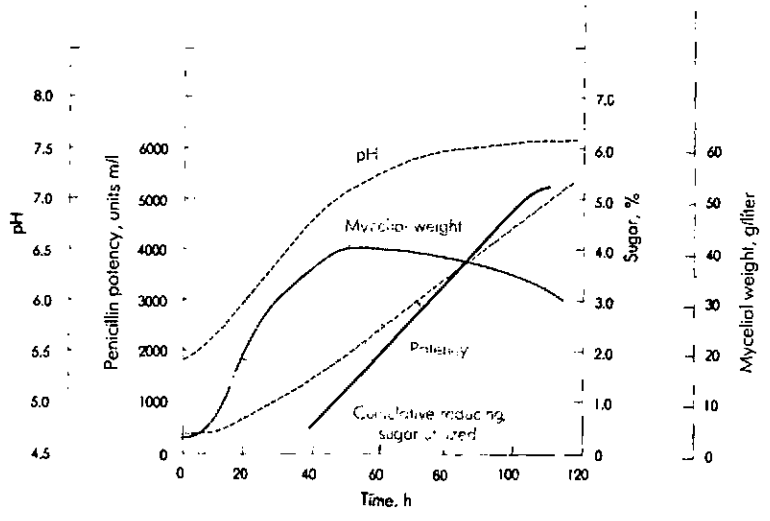


Figure 29-9. Biochemical changes that occur in the fermenter during production of penicillin by *Penicillium chrysogenum*. *(Courtesy of R. Donovick, Appl Microbiol,* **8**:117, 1960.)

Citric Acid

Citric acid is an important chemical used in medicines, flavoring extracts, food and candies, the manufacture of ink, dyeing, and engraving. Several different species of molds have the ability to convert sugar to citric acid, but *Aspergillus niger* is most widely used for its commercial production. The development of this industry in the United States illustrates the value of applying new ideas in an old industry. Until 1923, most of the citric acid used in America was imported from Italy, and all of it was obtained from citrus fruits. At that time, the pro-

duction of citric acid by mold fermentation was undertaken, and the industry has grown until today the annual production exceeds 20 million pounds. For several years after production by this method became practical, the United States not only did not import citric acid but exported large quantities. Since other countries now employ a similar method of production, exports have decreased.

Many sugars may serve as the substrate for the production of citric acid; however, molasses is generally used. The carbohydrate is incorporated into a medium containing an inorganic nitrogen compound as well as inorganic salts. The sterile medium is dispensed into shallow pans and inoculated with the mold spores. This is an aerobic process; consequently a large surface area provides an adequate supply of oxygen. An alternative to this method of production is the submerged-culture technique, in which the inoculated medium is contained in large tanks through which a supply of sterile air is forced. The strain of mold employed, the composition of the medium, the degree of aeration, and the temperature of incubation all have an effect on the yield of citric acid.

Enzyme Production

Many molds synthesize and excrete large quantities of enzymes into the surrounding medium. It is industrially feasible to concentrate and purify enzymes from cultures of molds such as *Aspergillus*, *Penicillium*, *Mucor*, and *Rhizopus*. Mold enzymes, e.g., amylases, invertase, proteases, and pectinase, are useful in the processing or refining of a variety of materials. Amylases hydrolyze starch to dextrin and sugars and are used in preparing sizes and adhesives, desizing textiles, clarifying fruit juices, manufacturing pharmaceuticals, and for other purposes. Invertase hydrolyzes sucrose to form glucose and fructose (invert sugar). It is widely used in candy making and production of noncrystallizable syrups from sucrose, which is partially hydrolyzed by this enzyme. The term *protease* refers to a mixture of proteolytic enzymes. Proteases are used for bating (treatment of hides to provide a finer texture and grain) in leather processing, manufacture of liquid glue, degumming of silks, and clarification of beer protein haze, and as an adjunct to soap for cleaning in laundries. For centuries—long before the role of enzymes in the bating of hides was understood—this treatment was accomplished by soaking the hides in suspensions of dog or fowl manure. Today, standard enzyme solutions have replaced the concoctions of dung. Pectinase is used in the clarification of fruit juices and to hydrolyze pectins in the retting of flax for the manufacture of linen.

Immobilized Enzyme Technology

The commercial uses of microbial enzymes have greatly expanded following the development of immobilized enzyme technology. The refinements and advances in this technology result from collaboration between the fields of enzymology, engineering, and microbiology. In principle, the enzyme is bound (immobilized) on a material through which the substance to be changed by the enzyme is passed. The process is analogous to passing a solution through a filter pad, the enzyme being present (immobilized) in the filter pad. A variety of substances including paper, wood chips, ceramic and glass beads, and ion-exchange resins have been used to immobilize enzymes. Among the advantages of this technology are (1) reuse of the enzyme and (2) more convenient recovery and purification of the end product of the enzyme reaction since it does not contain the enzyme.

Table 29-4. Net Distribution of Selected Biologics (United States)

Product Description	Net Doses Distributed, January–June, 1983
Influenza vaccine	45,630
Trivalent	
Bivalent	
Diphtheria toxoid and tetanus toxoid (pediatric)	447,213
Diphtheria and tetanus toxoids with pertussis vaccine	8,897,380
Tetanus and diphtheria toxoid (adult)	4,733,327
Tetanus toxoid	3,818,283
Poliomyelitis vaccine, inactivated	17,320
Poliomyelitis vaccine, live, oral, trivalent	9,362,070
Measles virus vaccine, live, attenuated*	2,993,000
Rubella virus vaccine, live*	3,015,030
Mumps virus vaccine, live*	2,605,329
Smallpox vaccine	2,246,507
Immune serum globulin, human (reported in cc)	1,010,602
Tetanus immune globulin, human (reported in cc)	353,380

* All products containing this antigen.

SOURCE: From Rept. No. 86, January–June 1983. Centers for Disease Control, Biologics Surveillance, U.S. Dept. of Health and Human Services, Public Health Service.

HYBRIDOMAS AND MONOCLONAL ANTIBODIES

Genetic research with microorganisms, particularly at the molecular level, has provided techniques which have been applied to studies with mammalian cells. One of the important outcomes of this research has been the fusion of myeloma cells (cancer cells) with antibody-producing white blood cells (B lymphocytes). The resulting hybrid cell is called a **hybridoma** (see Chap. 33). The hybridoma cells can be grown in vitro. Furthermore, as explained in Chap. 33, hybridoma cells can be selected and grown to produce a single, specific antibody. Such an antibody is called a **monoclonal antibody.**

Monoclonal antibodies are now produced on a commercial scale. They have great potential for therapeutic use in combating malignant cells, in immunosuppression in organ transplantation, and for passive immunization in a variety of infectious diseases. They also serve as powerful analytical reagents for diagnosis of cancer and infectious diseases and for determination of hormone levels. Many investigations of cellular biology involving proteins, antigenic structure, and other phenomena employ monoclonal antibodies as analytical reagents primarily because of their high level of specificity and sensitivity.

BIOLOGICS FOR IMMUNIZATION

Control of infectious diseases through immunization requires the manufacture, on a commercial scale, of a variety of microbiological antigens. The wide practice of disease control through active immunization is discussed in Part 8. Development of effective immunizing antigens together with the stringent test requirements to ensure their safe use constitute major programs in many of the major pharmaceutical houses. The total doses of a selected number of biological products distributed in the United States during January–June 1983 are shown in Table 29-4.

PETROLEUM MICROBIOLOGY

Microorganisms are associated with petroleum in its formation, its recovery by drilling, its decomposition, and its utilization. Only in recent decades has a significant amount of attention been directed to research in this field. Studies in petroleum microbiology require interdisciplinary participation. The microbiologist needs to work closely with chemists, engineers, physicists, and perhaps representatives from other fields of study. Some aspects of microbial involvement in this area are summarized as follows.

Petroleum Formation

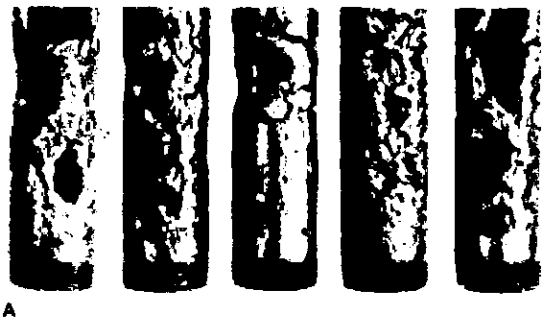
Much of the sedimentary material of the marine environment consists of dead microbial cells. Furthermore, biochemical changes in the sedimentary deposit are accomplished by a variety of microorganisms. It is speculated that these changes are associated with the formation of petroleum.

Petroleum Exploration

Soil in the region of a petroleum reservoir may contain vapors of hydrocarbon compounds such as methane or ethane. These may be detected by exposing microbial cultures in a test system which contains all nutrients for growth with the exception of a carbon source. Alternatively, the isolation of a large number of hydrocarbon-oxidizing microorganisms from soil may suggest that their presence is due to continued release of hydrocarbons from a petroleum deposit.

Petroleum Recovery

When an oil well is drilled, the initial recovery is made possible by the pressure within the rock formation. Later, as the original pressure decreases and the oil flow lessens, additional wells are drilled and water or steam is injected to force oil to the surface. Microbial activity has been suggested as a potential means of enhancing the yield of trapped oil. For example, bacteria injected into the oil might produce acid to dissolve rock formations, thereby releasing oil. Through other metabolic activities, microorganisms may decrease the viscosity of the oil.



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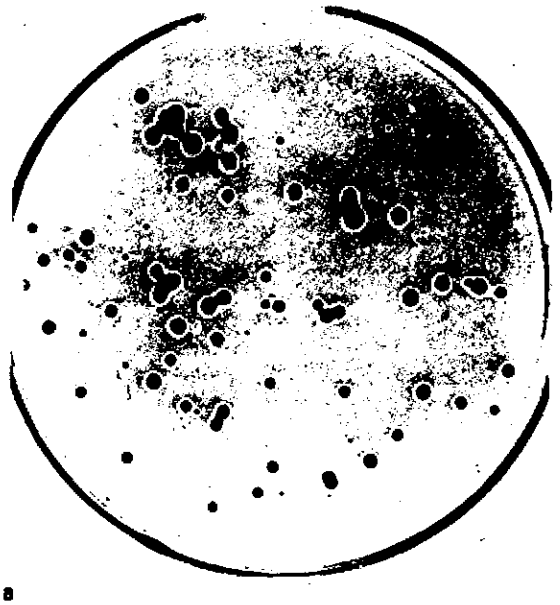

B

Figure 29-10. (A) Corroded cast iron pipes from a tidal marsh. Corrosion is due primarily to activities of sulfate-reducing bacteria. (B) *Desulfovibrio* sp. growing on an iron salts-agar medium. The colonies appear black because of iron sulfide formation. *Desulfovibrio* spp. occur widely in fresh, polluted, marine, and brackish waters. *(Courtesy of W. P. Iverson and the National Bureau of Standards, U.S. Department of Commerce.)*

Corrosion of iron pipe by *Desulfovibrio* spp. is a major problem in the oil industry (see Fig. 29-10). Contamination of drilling fluids by various bacterial species is likewise a serious and costly problem.

Oil Spills

The international traffic of oil in supertankers, with the occasional accidents that result in huge oil spills, has created a major threat to the environment. How do we clean up the oil? One approach is to inoculate the spill area with a microorganism that has the ability to degrade petroleum oil. This concept has been enhanced by genetically engineering a species of *Pseudomonas putida* so that it has the capacity to metabolize the four major hydrocarbons of petroleum: camphor, octane, xylene, and naphthalene. A bacterium with this metabolic capability made legal history by being the first genetically engineered microorganism ever patented.

MICROBIOLOGY AND MINING

Microorganisms play a role in the recovery of minerals from ores. Their importance as agents in the process of extracting metals from ores is likely to increase for the following reasons:

1 The richer mineral deposits are being depleted. Lower-quality ores are being processed, and they require development of techniques which yield more nearly complete extraction of metals.
2 The traditional method of processing ores, namely smelting, is a major cause of air pollution and is under attack from environmental groups.

Microorganisms are capable of improving both these situations. For example, some autotrophic, aerobic bacteria (*Thiobacillus thiooxidans* and *Thiobacillus ferrooxidans*) when grown in the presence of copper ores produce acid and effect oxidation of the ore with subsequent precipitation (removal) of the metal. This process is known as **leaching**. This technique improves the recovery of metal from an ore and is nonpolluting to the atmosphere.

An example of a low-grade ore undergoing bacterial leaching is shown in Fig. 29-11.

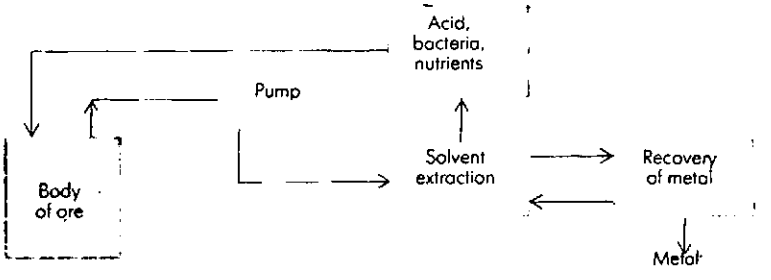

Figure 29-11. Leaching of low-grade ores using bacteria. *Thiobacillus ferrooxidans* plays an important role in the extraction (leaching) of metal from low-grade ores. This scheme shows an arrangement whereby the bacteria, nutrients, and acid are pumped into the ore bed. Continued growth of *Thiobacillus ferrooxidans* produces more acid, which solubilizes the metal content, promoting its extraction. The metal is then recovered from this acid solution.

DETERIORATION OF MATERIALS

The term *materials*, in the sense in which it is used here, refers to all products other than foodstuffs—paper, textiles, wood, rubber, and metals. It has been estimated that deterioration of such materials from all causes represents a loss running into several billions of dollars annually. Microorganisms are responsible for a significant amount of this destruction. Virtually nothing is immune or totally resistant to attack by microorganisms. Metals used in marine environments or the walls of a fuel storage tank on dry land are susceptible to microbiological corrosion. The glass components of optical equipment have been etched by microbial growth on their surfaces. We shall discuss a few examples of the role of microorganisms in deterioration.

Paper

The manufacture of paper involves two major operations. The first consists of the physical or chemical treatment of cellulosic material (e.g., wood, cotton, and linen rags) for the purpose of separating and purifying the cellulose fibers. The second consists of the fabrication of the resulting fibrous pulp, after further refinement, for redeposition of the fibers in the form of a sheet. Microbial deterioration in the form of paper-pulp slime may be encountered in the pulp, and other defects may appear on the finished product.

The development of slime depends largely upon the nature of the pulping operations. Slimes appear in the paper sheet in the form of undesirable slime spots. Bacteria, yeasts, molds, algae, and protozoa have been isolated from pulp slimes. Bacteria, particularly capsulated bacilli, are the most important single group of slime formers.

Finished paper is also subject to microbiological attack. Cellulose, the principal constituent of paper, is susceptible to degradation by a great many species of fungi and some bacteria. Other components of paper, such as glue or casein, may also serve as substrates for microbes. Under conditions permitting growth of microorganisms, the paper may be stained or discolored by the products of microbial metabolism. Growth of cellulolytic microorganisms will produce weakening of fibers, perforations, and even complete destruction of the paper.

Textiles and Cordage

Textiles made from natural fibers—cotton, wool, linen, and silk—are susceptible to deterioration by microorganisms. The same is true of cordage. Estimates on the annual losses due to microbiological attack on fabrics and ropes in the United States extend into millions of dollars. Enormous losses of cellulosic fabrics were experienced during World War II in tropical climates. Molds are the principal microorganisms responsible for this damage; many cellulolytic species inhabit the soil and are readily available as contaminants. *Myrothecium verrucaria* is notorious for its ability to degrade cellulose, and laboratory studies on this subject generally make use of this organism. Mold growth is favored by high humidity, moderate temperature, and diminished light. When this combination of conditions prevails, deterioration is greatly enhanced. For example, a lightweight canvas, when exposed to fungi under ideal conditions for mold growth, can be altered to the extent that it has no measurable strength after a few weeks.

Painted Surfaces

Painted surfaces are not always resistant to microbial disfiguration. Unless the paint film contains an effective fungicidal ingredient, it may under certain

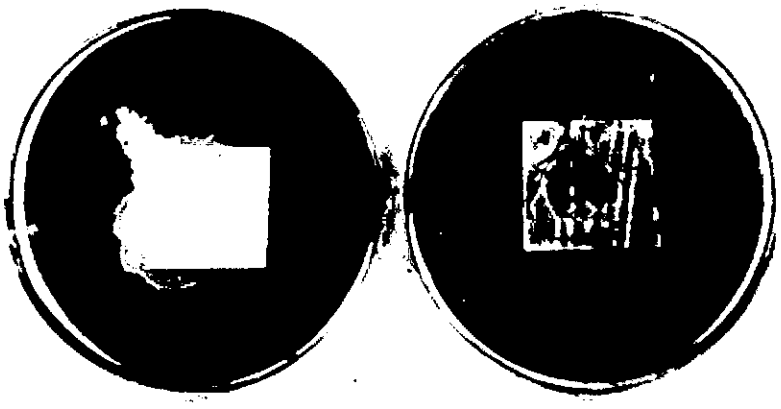

Figure 29-12. Agar-plate test demonstrates the effectiveness of an antifungal agent incorporated into paint. In the plate on the left is paint containing antifungal agent; in the plate on the right is untreated paint overgrown with test fungus. *(Courtesy of Nuodex Products Company, Inc.)*

environmental conditions exhibit evidence of mold spotting, or discoloration. This deterioration is due to products of microbial metabolism of organic constituents of the paint. Many species of molds have been isolated from mildewed or "moldy" painted surfaces. Included among these are species of *Aspergillus, Penicillium, Cladosporium, Pullularia,* and *Alternaria. Pullularia* spp. appear to be the most common cause of mildewed paint. The effectiveness of incorporating an antifungal agent in paint is shown in Fig. 29-12.

Prevention of Microbial Deterioration

To minimize microbiological deterioration of materials, more efficient methods of preservation are continually being developed. Prevention of deterioration is accomplished through application of the principles for controlling microbial populations discussed in Part 6: incorporation of microbicidal agents into the material, packaging that protects material from contamination, and storage under conditions that inhibit microbial growth (e.g., dehumidification).

ANALYTICAL MICROBIOLOGY

Many techniques have been developed whereby a specific microorganism is used to assay quantitatively substances such as vitamins, amino acids, and antibiotics. Microbiological methods are routinely employed to determine the potency of all antibiotic preparations at various stages of development, from their crude forms to the finished product (see Fig. 29-13). This type of assay involves measurement of inhibition of growth caused by the antibiotic. Within established limits of antibiotic concentration there is proportionality between the degree of inhibition and the amount of drug.

Another type of microbiological assay is based on measurement of increase in growth or metabolic activity. The principle of this technique is that a *single* nutrient, e.g., a vitamin or amino acid, may be the limiting factor for growth or metabolic activity of a specific organism in an otherwise complete assay medium. Within limits, the magnitude of the growth or metabolic response is proportional to the amount of the specific nutrient added to the assay medium. The following will serve as an example of this type of assay.

Lactobacillus arabinosus requires the vitamin niacin for growth. When this

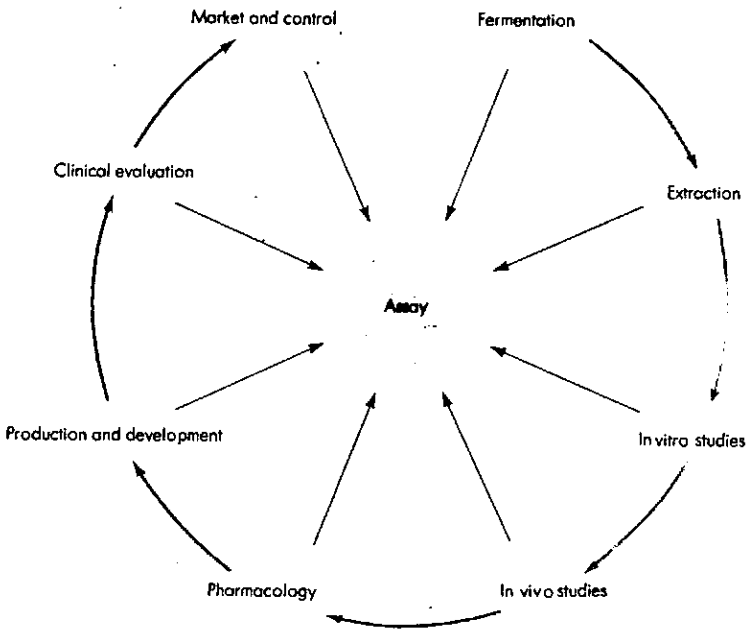

Figure 29-13. Development of an antibiotic involves a number of quantitative assays. (*Prog Ind Microbiol* **1**, 1959.)

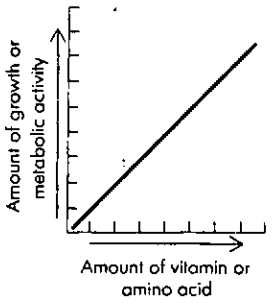

Figure 29-14. Principle of microbiologic assays as used for the measurement of vitamins and amino acids. Within limits of concentration of the substance being assayed, the amount of growth of the organism is proportionate to the amount of substance present.

organism is inoculated into a medium containing all the necessary nutrients (e.g., amino acids and other nitrogen compounds and vitamins other than niacin, glucose, and salts), growth will not occur. If niacin is added to this medium, the organisms will grow and the total growth obtained, within limits, will increase as the niacin is increased. It is therefore possible to prepare a standard curve relating growth to the amount of the vitamin, as shown in Fig. 29-14. If a substance of unknown niacin content is added to the medium and the test is carried out in the usual manner, the amount of growth measured can be referred to the standard curve, and from this the amount of niacin in the unknown sample can be extrapolated.

The measurement of the response of the test organism in these assays varies with the particular tests. It may be growth in terms of turbidity readings, dry weight, or cellular nitrogen. Other assay procedures rely upon measurement of metabolic activity such as production of acid or gas. Many procedures have been developed using bacteria (particularly lactobacilli), yeasts, fungi, algae, and protozoa. Some examples are shown in Table 29-5.

Microbiological techniques are extensively employed for the assay of vitamins and amino acids in pharmaceutical preparations and foods. Theoretically it is possible to assay any chemical to which the organism displays a measurable response. In practice, a wide range of substances, from simple mineral elements to complex organic compounds, are assayed.

Microbiological assays are highly specific and unusually sensitive. For example, as little as 0.1 nanogram (0.0000000001 g) of biotin per milliliter can be detected by using *Lactobacillus casei*.

Table 29-5. Microorganisms Used in Assay Techniques

Microorganism	Substance Assayed
Streptococcus faecalis (bacterium)	Several amino acids
Tetrahymena geleii (protozoan)	Folic acid
Saccharomyces carlsbergensis (yeast)	Pantothenic acid
Neurospora crassa (mold)	Biotin
Ochromonas malhamensis (alga)	Vitamin B_{12}

FUTURE PROSPECTS

The basic knowledge of molecular biology and genetics accumulated during the past few decades is rapidly being translated into practical objectives and is revolutionizing industrial microbiology. More than 200 companies built upon the new biotechnology have come into being during the last several years, and a large sector of the industry is feverishly pursuing numerous leads suggested by studies in the genetic engineering of microorganisms and other cells. Most state governments, aware of what the new technology promises, have placed a high priority on establishing centers where universities and industry can collaborate.

The impact of the new biotechnology, or applied genetics, has already been significant in many areas and, in time, will affect all industries that are involved with biological systems or their products. Indeed, modern technology is likely to present attractive new options for industries that presently do not use biological systems.

The impact of advanced biotechnology and applied genetics will affect society throughout the world. Agriculture and food production, waste management and environmental quality, raw materials for the chemical industry, new pharmaceutical products, and disease control are some of the areas wherein new accomplishments will occur.

QUESTIONS

1 What is the basis for using microorganisms in a manufacturing industry?
2 What are some of the prerequisites for using microorganisms to manufacture a product?
3 What is meant by a "genetically engineered" bacterium? How is this accomplished?
4 What is a hybridoma? What is the practical significance of hybridomas?
5 Explain how it is possible to produce human insulin from bacteria. What are the advantages of this method of production?
6 Identify the microorganisms and describe the general biochemical processes involved in the production of lactic acid, vinegar, and glutamic acid.
7 What is meant by the term *food yeast*? *Bakers' yeast*?
8 Outline the procedure for industrial production of penicillin.
9 What developments contributed to the increase in yield of penicillin over that originally obtainable?
10 The technique of immobilized enzymes may increase the use of enzymes in industry for product modification. Why is this likely?
11 What are the desirable features of the use of enzyme preparations for refinement of a product?

12 What is the significance of paper-pulp slime in the manufacture of paper? What is its source?
13 Why is the deterioration of textiles and fabrics of particular concern in tropical climates?
14 What general measures are applicable for the overall control of microbial deterioration?
15 List and describe the various ways in which microorganisms are involved with the petroleum industry.
16 What is the principle upon which microbiological assay techniques are based? What types of microbial response may be measured by these procedures?
17 How has industrial microbiology been affected by the new biotechnology (applied genetics)?
18 What are the prospects for the future of biotechnology?

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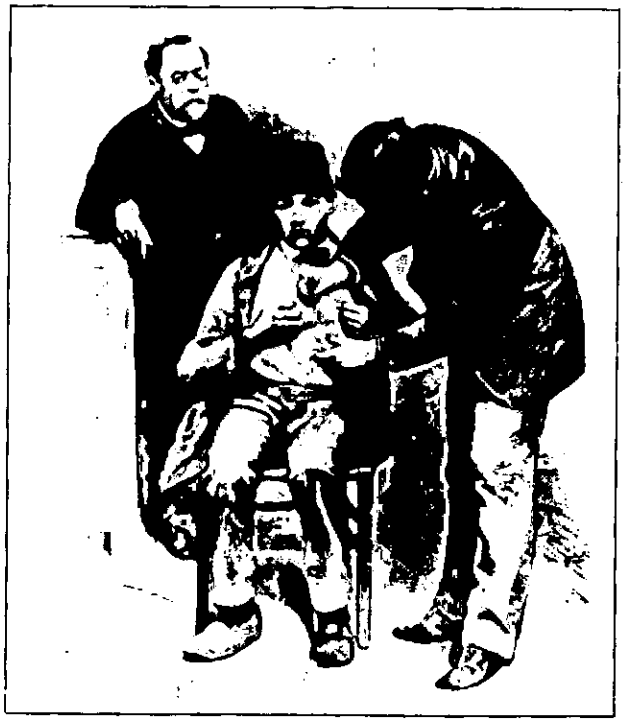
PART EIGHT
MICROORGANISMS AND DISEASE



An old "new" agent of human infection

The latter half of the nineteenth century was the "golden age" of microbiology, when the infectious agents of many human diseases were being discovered. However, new causative agents of human disease are still being discovered today. In most instances it is not that a new microorganism suddenly appears on the scene; rather it is that our own knowledge of the occurrence and activities of an already existing organism is new. Such a situation is exemplified by *Campylobacter*, a genus of tiny, Gram-negative, vibrio-like bacteria.

Veterinarians had been aware since 1913 that campylobacters were important pathogens of domestic animals, causing abortion in cattle and sheep. However, before 1972, few clinical microbiologists were aware that some campylobacters were important and widespread human pathogens. In 1957, pioneering studies by Elizabeth King at the Communicable Disease Center in Atlanta had indicated that tiny, vibrio-like bacteria could cause human blood infections. King recognized that the human isolates resembled, but were not identical to, the vibrio-like organisms that veterinary microbiologists frequently isolated, and thus she called them "related vibrios." King suspected that the organisms might be able to cause gastrointestinal infections. However, this could not be confirmed until 1972, when P. DeKeyser and his colleagues in Belgium were the first to isolate "related vibrios" from diarrheic stools. They used a selective method based on (1) the removal of larger bacteria from diarrheic stools by filtration, and (2) the use of antibiotic-containing media that suppressed the growth of other intestinal bacteria.

We know today that the species *Campylobacter jejuni* is a major cause of diarrhea in humans, especially in children and young adults. In fact, it causes as many cases of diarrhea in humans as the more familiar *Salmonella* and *Shigella bacteria*, and it has a worldwide distribution. Why then did it take so long to recognize its clinical significance?

The answer lies not only in the lack of suitable selective methods, but also in the general cultivation methods used by hospitals and public health laboratories. Cultures from diarrheic stools were incubated aerobically on media designed to grow salmonellae and shigellae. However, *C. jejuni* grows neither aerobically nor anaerobically. Veterinary microbiologists had long known that the campylobacters they isolated from animals were *microaerophiles*; i.e., although the organisms required oxygen they could not tolerate the level of oxygen that is present in air (21 percent). The same is true of campylobacters isolated from humans; they grow only when the oxygen level is low—usually about 6 percent. Oxygen is both a blessing and a curse for campylobacters: they need it as a terminal electron acceptor for respiration, but they are poisoned by too much oxygen.

Today it is routine in clinical laboratories to culture diarrheic stool specimens for *C. jejuni*. We now know that successful isolation of this important pathogen depends on the use of suitable selective media and also on incubation of the cultures in jars which have a gaseous atmosphere containing a low level of oxygen.

Preceding page. Pasteur supervises inoculation of rabies vaccine by an assistant into a patient bitten by a rabid animal. *(Courtesy of National Library of Medicine.)*

Chapter 30 Microbial Flora of the Healthy Human Host

OUTLINE

We are constantly in contact with a myriad of microorganisms in the environment. However, we are in even more intimate contact with an enormous number of microorganisms that inhabit our bodies. It is estimated that the adult human body is composed of approximately 10^{13} eucaryotic cells; what may be less apparent is that the human body also serves as the natural habitat for 10 times that number of microbial cells! These microorganisms, most of which are bacteria comprise the **normal flora**, also termed the **normal microbiota**. They inhabit mainly the skin and the inner surfaces of the body such as the mucous membranes of the oral cavity, upper respiratory tract, intestinal tract, and genitourinary tract. Most are highly adapted to survival and growth in these areas despite physical and chemical conditions that discourage many other kinds of microorganisms.

It is useful to know the normal flora of the healthy human body for the following reasons:

1 The term *normal flora* implies that these microbes are harmless, and for the most part they do not cause disease and are even beneficial. Most are **commensals**: they benefit from the association with the host, but the host is not affected. Others have a **mutualistic association** with the host: they benefit the host in some fashion while thriving in the host's body. It is of interest to learn what these beneficial effects are and how they may be lost due to changes in the normal flora caused by the use of antibiotics or other means.
2 Some normal flora organisms can be *opportunistic pathogens*; i.e., they may cause infections if tissue injury occurs at specific sites or if the resistance of the body to infection is decreased. This is especially important because in recent years there has been a rising incidence of infections from these microorganisms.

ORIGIN OF THE NORMAL FLORA

Before birth a healthy human fetus is free of bacteria. Under natural circumstances, the fetus first acquires microorganisms while passing down the birth

canal. It acquires them by surface contact, swallowing, or inhaling. These microbes are soon joined by other microbes from many sources in the newborn's immediate surroundings. Microorganisms which find suitable environments, either on the outer or inner body surfaces, quickly multiply and establish themselves. The initial flora may change considerably in composition during the first few days or weeks after birth until a stable flora becomes established and forms the normal flora. Each part of the human body, with its special environmental conditions, has its own particular mixture of microorganisms. For example, the oral cavity acquires a different natural microbial population than the intestines. In a short time, depending on factors such as the frequency of washing, diet, hygienic practices, and living conditions, the child will have the same kind of normal flora as an adult person in the same environment.

Even though an individual has a "normal" flora, it often happens that during his or her life there are fluctuations in the composition of this flora due to general health conditions, diet, hormonal activity, age, and many other factors.

NORMAL FLORA AND THE HUMAN HOST

What effect does the establishment of a normal flora (colonization) have on the body? Three approaches have been used to answer this question:

1 ***Use of germfree animals.*** If the colonization of experimental animals by microorganisms can be prevented, one can compare the properties of such germfree animals with those of normal animals. The results can be helpful in understanding the functions of the normal flora of human beings.
2 ***Use of antimicrobial agents.*** If the balance that occurs between the normal flora and the human host is altered by the use of such agents, various effects may occur that are useful for indicating the role of the normal flora for the human body.
3 ***Knowledge of certain characteristics of normal flora organisms.*** The nature of these characteristics suggests that normal flora organisms may help to discourage the growth of microorganisms that are not part of the indigenous flora.

Germfree and Gnotobiotic Life

Pasteur did not believe that animals could live in the absence of microorganisms, and in 1897, following his suggestion, an abortive attempt was made to rear germfree chickens. Between 1899 and 1908, Schottelius, a German, was successful in raising chickens that were bacteria-free. However, because his birds did not develop normally and died in about a month, he concluded that intestinal bacteria are essential in the nutrition of vertebrates. In 1912, Cohendy at the Pasteur Institute raised 17 germfree chickens for 40 days and concluded that vertebrate life is possible in the absence of microorganisms. We now know that when an adequate diet is provided, germfree chickens live long, healthy lives and reproduce as well as normal birds.

Rearing Germfree Animals

In 1928 James A. Reyniers at the University of Notre Dame started work on germfree chickens. He and his associates developed equipment and techniques for rearing chickens, rats, mice, and other animals in the absence of detectable living microorganisms for several generations. They emphasized the anatomical and physiological description of these animals and made comparisons with conventional nongermfree animals of the same species. As a result of these studies, germfree animals no longer belong to the realm of biological curiosities but have become practical models for solving problems of importance in biology

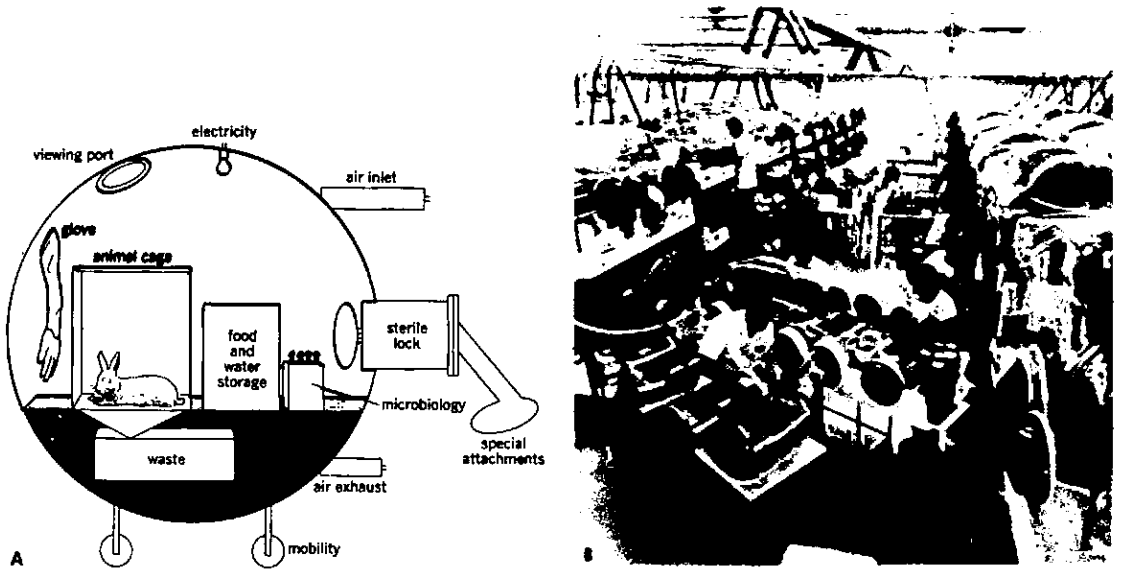


Figure 30-1. Germfree equipment. (A) Schematic diagram of a germfree isolator unit. The interior can be sterilized prior to an experiment and maintained in that condition. *(From McGraw-Hill Encyclopedia of Science and Technology, 3d ed., vol. 6, McGraw-Hill, New York, 1971.)* (B) Germfree colonies of mice reared in plastic isolator units. *(Courtesy of Charles River Breeding Laboratories, Inc.)*

and medicine. Germfree laboratory units are shown in Fig. 30-1. Animals that are either germfree or that live in association with one or more known organisms are said to be **gnotobiotic.**

The first germfree animals reared by Reyniers were chickens obtained by sterilizing the shells of 20-day-old embryonated eggs with an effective germicide and placing them in sterile containers such as glass churn jars or steel tanklike cages into which filtered (sterile) air is passed and from which waste gases are removed. Sterile food and water are placed in the cages prior to introducing the ready-to-hatch chicks. Periodic bacteriological examination of the exhaust air, feathers, excreta, and body orifices are made to confirm the absence of microorganisms in the cages or on the birds. Germfree rats, mice, guinea pigs, and other mammals can be obtained by cesarean section of gravid mothers under sterile operative conditions in a special chamber that allows the young animals to be introduced directly into a rearing cage. These babies must be hand-fed hourly for 2 or 3 weeks with specially devised nipples attached to medicine droppers with a formula containing, as nearly as can be determined, all of the components of the natural mothers' milk. Once established, a colony of germfree animals can be maintained by natural reproduction under germfree conditions.

Germfree Animals versus Normal Animals

Compared with normal animals, germfree animals exhibit an underdeveloped immune system, making them unusually susceptible to infection if subsequently exposed to pathogenic bacteria. They also require higher levels of B vitamins than do normal animals, and they require vitamin K, which normal animals do not require. These findings indicate that the normal flora may make a significant contribution to the vitamin requirements of the host.

Other Uses for Germfree Animals

Gnotobiotic techniques have been used to assess the effect of *particular species* of microorganisms on a host. Here, the germfree animal is reared in the presence of one or more known microbial species to determine the effect of those species

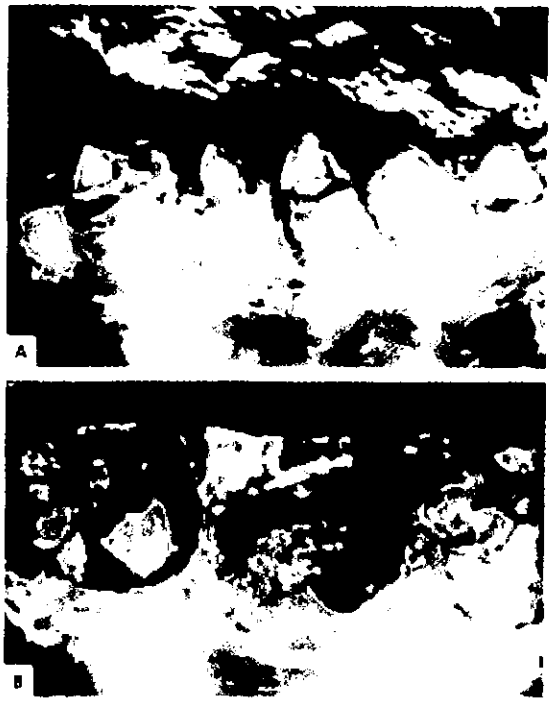


Figure 30-2. Gnotobiotic techniques have been widely used for studies of the role of bacteria in dental caries. (A) Normal noncarious teeth from a noninfected hamster. (B) Extensive plaque formation and caries following inoculation of a hamster with cariogenic streptococci of the *Streptococcus mutans–Streptococcus sanguis* group from a carious lesion in another infected hamster. *(Courtesy of Morrison Rogosa and the National Institute of Dental Research.)*

on growth and development of the animal or on various physiological processes. Similarly, one can inoculate a germfree animal with a known species of microorganism, or a mixture of known species, to determine their ability to produce disease or cause pathological or immunologic changes in the animal. For example, gnotobiotic techniques have helped to elucidate the role of bacteria in causation of dental caries (Fig. 30-2).

Effect of Antimicrobial Agents

Suppression of the normal flora by use of antibiotics or other antimicrobial agents indicates that the normal flora may serve as a defense against colonization by potential pathogens. For instance, treatment of the skin of humans with antibacterial agents such as hexachlorophene results in suppression of the normal Gram-positive flora and promotes colonization and clinical infection by Gram-negative bacilli and other organisms that cannot normally establish themselves on skin. In another example, hospital patients receiving antibiotics may undergo suppression of the normal flora of the large bowel, leading to pseudomembranous colitis, which is a severe disease caused by excessive growth of toxin-producing strains of *Clostridium difficile*. In humans not receiving antibiotic therapy, such strains are ordinarily held in check by the normal flora and do not grow to high numbers. Normal flora organisms can prevent the establishment of pathogens by various means such as successful competition for available nutrients or formation of inhibitory metabolic products and antibiotics.

Characteristics of Normal Flora Organisms

Many species of normal flora organisms have the ability to *adhere* to the surface of host epithelial cells. Thus they have a selective advantage over other micro-

organisms in colonizing the host. Adherence occurs as the result of a molecular interaction between the microbial cell surface and a chemical receptor on the body cell. Proteins or polysaccharides on the surface of the microbial cells, as well as the fibrillar structures known as pili which extend out from the microbial cell, have been implicated in adherence; the particular means of adherence varies with the species. Microorganisms may often adhere specifically to one body site. For example, *Streptococcus salivarius* adheres mainly to the surface of the tongue, whereas *Streptococcus mutans* selectively binds to the smooth surface of the teeth.

Adherence to Host Cells

A phenomenon that bears on microbial adherence is desquamation, the detachment of host epithelial cells from body surfaces and replacement of the lost cells by new cells. In some body sites, e.g., the intestinal tract, the rate of desquamation may be very high. One result of desquamation is the elimination of microorganisms that are not part of the normal flora and that are only feebly attached to the epithelial cells. Normal flora microorganisms, however, have the ability to reattach firmly to the fresh epithelial layer and thus persist at these body sites.

Production of Antimicrobial Substances

In some instances normal flora microorganisms have been shown to be capable of producing metabolic products or other agents that can inhibit other microbes. For example, in the large bowel certain anaerobic bacteria produce various organic acids, e.g., acetic, lactic, or butyric acid, as metabolic waste products which can inhibit the growth of other bacteria. Some strains of skin staphylococci have been shown to produce antibiotics that inhibit a wide variety of other bacteria. In still another example, many strains of *Escherichia coli* in the intestines produce colicins (see Chap. 12) which may help to protect the intestinal tract from closely related pathogenic bacteria.

DISTRIBUTION AND OCCURRENCE OF THE NORMAL FLORA

Bacteria make up most of the normal flora of the human body, and, therefore, this chapter deals mainly with the distribution and numbers of various bacterial genera and species. Although various fungi (mainly yeasts) and protozoa may also inhabit the body, their numbers are usually very low compared to the bacterial flora. As for viruses, it is not clear whether any can be considered as true normal flora even though some may be harbored for long periods in the absence of disease symptoms. For instance, certain human intestinal viruses were discovered only by noting their cytopathogenic effects in tissue cultures; thus they were termed "orphan" viruses (enteric cytopathogenic human orphans, or echoviruses). Similarly, coxsackieviruses, which occur only in human hosts, were initially discovered only because of their pathogenicity for suckling mice; they apparently were not associated with human disease. Many echoviruses and coxsackieviruses have since been found to cause a number of human diseases such as nonspecific febrile illnesses, acute respiratory disease, exanthematous disease, aseptic meningitis, enteritis, and paralytic and encephalitic disease. However, it must be recognized that not all of these viruses have been shown to cause disease in humans. In another example, chronic adenovirus infections are known to occur without disease: the presence of certain adenoviruses in the lymphoid tissue of normal individuals may become evident only after the tissue cells have been cultured in vitro in the laboratory. Consequently,

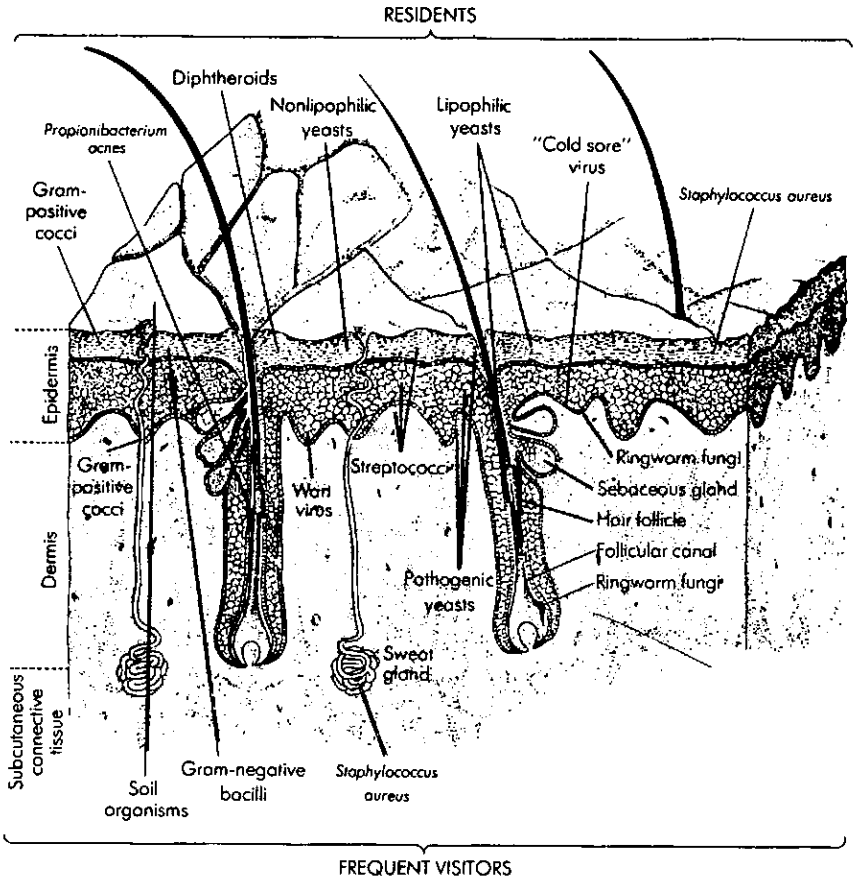


Figure 30-3. The major microbial symbionts found on or in the skin of humans. *(Courtesy of B. C. Block and J. Ducas, Man, Microbes, and Matter, McGraw-Hill, New York, 1975.)*

there do seem to be viruses that cause chronic asymptomatic infections, but whether they are so consistently present as to justify considering them as normal flora is not yet known.

The following sections describe the distribution and occurrence of the bacteria, fungi, and protozoa that comprise the normal flora of different regions of the human body, beginning with the external surfaces.

Skin

The skin is composed of the **epidermis** at the outer surface and of the connective tissue layer, **dermis**, underneath it (Fig. 30-3). The outermost layer of the epidermis consists of a layer of dead, anucleated, horny cells and is constantly in contact with bacteria from the surrounding environment. It is normally impermeable to bacteria; however, cuts, abrasions, or burns can allow bacteria to penetrate. The skin has a wide variation in structure and function in various sites of the body. These differences serve as selective ecological features, determining the types and numbers of microorganisms that occur on each skin site.

The skin surface is hostile to survival and growth of many kinds of bacteria.

For instance, the pathogen *Streptococcus pyogenes* does not survive for more than a few hours when applied to the skin, whereas it may survive for weeks in room dust. Several factors are responsible for discouraging skin colonization:

1 ***Dryness.*** The relatively dry surface of the skin is inhibitory to microbial growth. When allowed to dry, many bacteria remain in a dormant condition; some species die in a matter of hours. Some regions of the skin are more moist than others, e.g., the **axillary region**, **toe webs** (skin between the toes), and the **perineum** (skin at the lower end of the trunk between the thighs). These regions have higher numbers of normal flora organisms [about 10^5 colony-forming units (cfu)/cm^2] than do the drier areas of skin (about 10^2 to 10^4 cfu/cm^2).
2 ***Low pH.*** Skin has a normal pH between 3 and 5 (higher in moist regions), which is due in part to lactic or other organic acids produced by normal skin microorganisms such as staphylococci. This low pH can discourage the growth of many kinds of microorganisms.
3 ***Inhibitory substances.*** Several bactericidal or bacteriostatic compounds occur on the skin. For example, **sweat glands** (Fig. 30-3) secrete lysozyme, an enzyme that destroys bacterial cell walls. **Sebaceous glands** (Fig. 30-3) secrete complex lipids, which may be partially degraded by some bacteria such as *Propionibacterium acnes*; the resulting long-chain unsaturated fatty acids, e.g., oleic acid, are highly toxic to other bacteria.

Despite these formidable antimicrobial factors, some bacteria not only survive on the skin but even grow, forming the normal flora. The secretions of the sweat glands and sebaceous glands provide water, amino acids, urea, salts, and fatty acids, which can serve as nutrients for these microorganisms. Most of these bacteria are species of *Staphylococcus* (mainly *Staphylococcus epidermidis*) and aerobic corynebacteria, or diphtheroids. In the deep sebaceous glands are found lipophilic anaerobic bacteria such as *P. acnes*. The latter organism is a normal skin inhabitant and is usually harmless; however, it has been associated with the skin disease known as acne vulgaris. The numbers of these propionibacteria are little affected by washing because of their deep location. The location of various skin bacteria in or on the skin is shown in Fig. 30-3.

Propionibacterium acnes and Acne Vulgaris

Acne vulgaris is a disease of the sebaceous glands of the skin. In the first stage of the disease **comedones** (singular, *comedo*) are formed, i.e., distensions of the glands caused by an accumulation of sebum (fluid secreted by the gland), hair, and bacteria. Comedones may progress no further, or they may become closed (no longer able to eliminate their contents to the skin surface); such comedones can develop into disfiguring inflammatory lesions (papules, pustules, and nodules). *P. acnes* is the predominant organism in comedones; however, since it is also abundant in normal sebaceous glands, it is not yet clear that the organism is actually the causative agent of acne vulgaris. The ability of antibiotics to achieve clinical improvement in acne and at the same time reduce the number of *P. acnes* suggests that the organism may play an important role.

Eye

Lining the eyelids and covering the eyeball is a delicate membrane called the **conjunctiva**. This membrane is continually being washed by a flow of **fluid** (tears), which tends to remove microorganisms. Moreover, lysozyme is secreted in tears. Consequently, the conjunctival flora is sparse. The main or-

ganisms found are *S. epidermidis, Staphylococcus aureus, Corynebacterium* species, *Streptococcus pneumoniae, Neisseria* species, *Moraxella* species, and *Haemophilus parainfluenzae*; other organisms may be isolated occasionally.

Respiratory Tract

Upper Respiratory Tract

Although more moist than skin, the mucous membranes of the upper respiratory tract (that portion of the respiratory tract above the larynx) nevertheless represent an environment which is difficult for many kinds of bacteria to colonize. As inspired air containing microorganisms passes along the tortuous nasal passages (sometimes referred to as the **nasal baffle**) and the **nasopharynx** (the region of the respiratory tract above the soft palate; Fig. 30-4), it is likely that the microorganisms will impinge on and stick to the thin moist layer of highly viscous **mucus** that overlies the surfaces. Because of the rhythmic beating of **cilia** (hairlike appendages) on the surface of the epithelial cells lining the nasopharynx, the mucus layer continuously flows downward toward the **oropharynx** (Fig. 30-4); thus, trapped bacteria are eventually swallowed and are destroyed by the acid of the stomach. In addition to this mechanical removal of bacteria, a bactericidal effect is exerted by lysozyme present in nasal mucus. Despite these factors, the nose and nasopharynx are inhabited by numerous normal flora microorganisms. Much of their success in colonization is due to their ability to adhere to the epithelial cell layer of the mucous membranes, thereby avoiding being carried away by the flow of mucus. The bacteria most frequently and most consistently found in the nose are *S. epidermidis* and *S. aureus*; in the nasopharynx avirulent strains of *S. pneumoniae* and other α-hemolytic streptococci predominate, but species of the genera *Staphylococcus, Corynebacterium, Neisseria, Branhamella, Haemophilus*, and *Micrococcus* are also common.

Lower Respiratory Tract

The mucous membrane surfaces of the **trachea** (windpipe) and its branches (**bronchi**) do not have a normal flora, as a result of the mechanical removal of organisms by an upward, cilia-driven flow of mucus. Bacteria that manage to traverse the air passages all the way to the **alveoli** (air sacs) of the lungs are

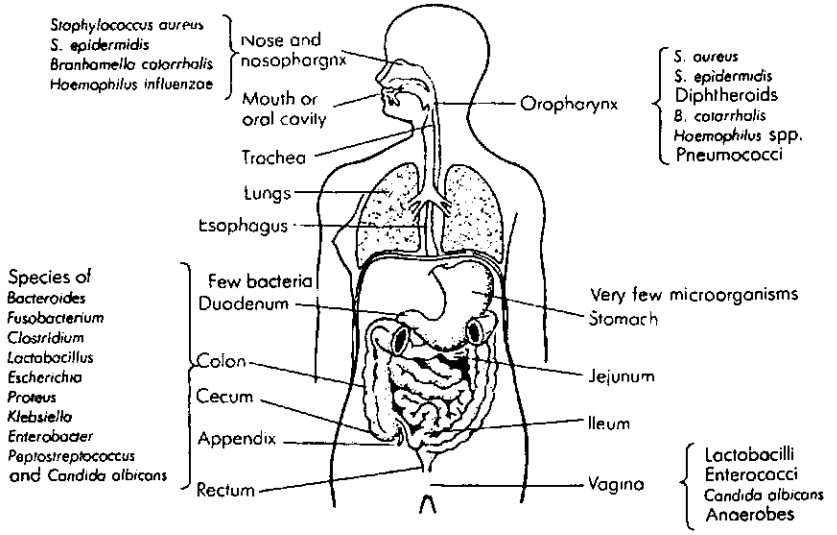


Figure 30-4. Distribution of normal flora of the human body.

usually engulfed and destroyed by phagocytic body cells known as *alveolar macrophages*.

Mouth

The abundant moisture and constant presence of dissolved food as well as small food particles would seem to make the mouth an ideal environment for bacterial growth. However, the continuous flow of saliva through the mouth exerts a mechanical flushing action that removes many microorganisms, causing them to be swallowed and destroyed by the acid of the stomach. Desquamation of epithelial cells is a second mechanical factor that removes microorganisms from the oral cavity. Consequently, it is not surprising that many of the microbes that constitute the normal flora of the mouth resist such mechanical removal by being able to adhere firmly to various surfaces of the oral cavity.

Acquisition of the Oral Flora

At birth the oral cavity is essentially a sterile, warm, and moist incubator containing a variety of nutritional substances. The saliva is composed of water, amino acids, proteins, lipids, carbohydrates, and inorganic compounds. It is thus a rich and complex medium that can be used as a source of nutrients by microbes at various sites in the mouth. (Saliva itself generally contains transient microbes from other sites of the oral cavity, particularly from the upper surface of the tongue, and generally has a microbial population of about 10^8 bacteria per milliliter.)

The normal flora of a newborn is established within a few days after birth. The predominant bacterial species belong to the genera *Streptococcus*, *Neisseria*, *Veillonella*, *Actinomyces*, and *Lactobacillus*; yeasts are also present. The number and kinds of microbial species found are related to the infant's diet and to association with the mother, attendants, and objects such as towels and feeding bottles. The only species consistently recovered from the oral cavity, even as early as the second day after birth, is *S. salivarius*. This species has an affinity for epithelial tissues and appears in large numbers on the dorsal surface of the tongue.

Normal Flora of the Teeth

Until eruption of the teeth, most microorganisms in the mouth are aerobes and facultative anaerobes. As the first teeth appear, obligate anaerobes, such as species of the genera *Bacteroides* and *Fusobacterium*, become more evident because the tissue surrounding the teeth provides an anaerobic environment.

The teeth themselves can become areas for microbial adherence. *Streptococcus mutans* is associated with the tooth surface and appears to be the major etiological (causative) agent of dental caries, or tooth decay. *S. mutans* produces a highly branched, extracellular glucan (polymer of glucose) that acts like a cement which binds the bacterial cells together as well as causing them to adhere to the tooth surface. This glucan is formed only in the presence of the disaccharide sucrose (glucose-1-fructose, the type of sugar found in confections) by means of an enzyme called glycosyl transferase located on the surface of the cocci. The reaction catalyzed by this enzyme is as follows:

$$n\ \text{Sucrose} \xrightarrow[\text{transferase}]{\text{Glycosyl}} \underset{\text{Glucan}}{(\text{Glucose})_n} + n\ \text{Fructose}$$

Figure 30-5. Scanning electron micrograph of a human dental plaque, fixed and freeze-dried in situ on an extracted tooth. Seen are cocci-coated filaments in a "corncob" arrangement. Field width is 38 μm. *(Courtesy of Dr. Sheila J. Jones, University College, London.)*

The fructose liberated from the sucrose by this reaction, as well as sucrose itself and other sugars that may be present, can be fermented by the streptococci, giving rise to lactic acid, which can etch the surface of the teeth. Although *S. mutans* initiates dental caries, other bacteria such as *Lactobacillus* and *Actinomyces* species can contribute to caries as secondary invaders. The aggregation of bacteria and organic matter on the surface of teeth is termed **plaque** (Fig. 30-5). Dental plaque contains a very high number of bacteria, about 10^8 cells per milligram.

Once teeth are present, the normal flora in infants appears to be generally similar to that in adults. Then, for reasons which are not well understood at present, but probably as a result of hormonal changes, anaerobic bacteria, especially oral spirochetes (*Treponema* spp.) and *Bacteroides melaninogenicus*, colonize the oxygen-deficient gingival crevices at puberty.

In addition to bacteria, certain commensalistic protozoa may inhabit the oral cavity. For instance, the flagellated protozoan *Trichomonas tenax* may occur in the gingival margins and in the tartar and cavities of teeth. Its presence is usually associated with poor oral hygiene.

Intestinal Tract

Stomach

Although the stomach constantly receives numerous bacteria from the oral cavity, the fluid contents of the healthy stomach generally contain less than 10 bacteria per milliliter because of the bactericidal effect of the hydrochloric acid in the gastric secretion. The few organisms found are mainly lactobacilli and yeasts such as *Candida* spp. Following the ingestion of food the number of bacteria increases (10^3 to 10^6 organisms per gram of contents), but it soon falls as gastric juice is secreted and the pH of the stomach's fluid drops.

Small Intestine

The upper portion (or **duodenum**; Fig. 30-4) of the small intestine has few bacteria (usually $<10^3$ per milliliter of fluid). Of those present, the majority are Gram-positive cocci and bacilli. In the **jejunum** (second part of the small intestine, between the duodenum and ileum; Fig. 30-4) there are occasionally found species of enterococci, lactobacilli, and diphtheroids. The yeast *Candida albicans* may also be found in this part of the small intestine.

In the distal portion (**ileum**; Fig. 30-4) of the small intestine, the flora begins to resemble that of the large intestine. Anaerobic bacteria and members of the family *Enterobacteriaceae* grow extensively here and appear in large numbers.

Large Intestine

In the human body, the **colon**, or large intestine (Fig. 30-4), has the largest microbial population. It has been estimated that the number of microorganisms in stool specimens is about 10^{11} organisms per gram wet weight. (Fifty or sixty percent, dry weight, of fecal material may consist of bacteria and other microorganisms.) Over 300 different bacterial species have been isolated from human feces. It has also been calculated that an adult excretes in the feces 3×10^{13} bacteria daily.

Various factors tend to remove microorganisms from the large intestine. The continual movement of intestinal contents through the channel of the intestine by peristalsis is one factor. Desquamation of surface epithelial cells to which bacteria are attached is another. Mucus is a third factor; just as this substance is important in the mechanical removal of microorganisms from the respiratory tract, it plays a similar role in the intestine. However, the mucus in the intestine forms a discontinuous, meshlike layer rather than a continuous layer. Movement of intestinal contents causes contact and adherence of microorganisms to the mucus, which subsequently rolls up into small masses that, with their attached microorganisms, are eliminated from the body in the feces.

There are about 300 times as many anaerobic bacteria as facultatively anaerobic bacteria (such as *E. coli*) in the large intestine. The anaerobic Gram-negative bacilli present include species of *Bacteroides* (*B. fragilis, B. melaninogenicus, B. oralis*) and *Fusobacterium*. The Gram-positive bacilli are represented mainly by species of *Bifidobacterium, Eubacterium*, and *Lactobacillus*.

The facultatively anaerobic species found in the intestine belong to the genera *Escherichia, Proteus, Klebsiella*, and *Enterobacter*. Peptostreptococci (anaerobic streptococci) are common. The yeast *C. albicans* is also present.

Some protozoa may also occur as harmless commensals in the intestine, where they grow anaerobically by ingesting the bacteria that are present. For instance, a flagellated protozoan, *Trichomonas hominis* (also known as *Pentatrichomonas hominis*), inhabits the intestinal tract in the area of the cecum. In another example, a number of amoebae belonging to the genera *Entamoeba, Endolimax*, and *Iodamoeba* are commensals of the colon. One species, *Entamoeba histolytica*, can live as a commensal but can also be pathogenic, causing amoebic dysentery; it is capable of penetrating the intestinal mucosa and invading various organs of the body.

It is interesting that the intestinal flora of the young breast-fed infant consists mainly of bifidobacteria, which are Gram-positive; in bottle-fed infants, lactobacilli, which are also Gram-positive, predominate. With eventual substitution of solid food and an adult-type diet, a Gram-negative flora consisting mainly of *Bacteroides* spp. predominates.

Factors Influencing the Normal Flora

The composition of the normal flora of the intestine can be influenced by various factors, such as strong emotional stress, changes in air pressure due to altitude, and starvation. It should be noted that in diarrhea, as a result of rapid movements of the intestinal contents, the intestinal flora undergoes considerable change.

Alteration of the flora also occurs in persons receiving antibiotic treatment; organisms susceptible to the antibiotic being administered may be replaced by antibiotic-resistant strains. Other factors that may possibly affect or regulate the normal flora are diet, the bile acids secreted into the duodenum from the gallbladder, and the presence of antibodies secreted into the intestine; however, the importance of these factors is not yet clear, and present evidence suggests that their influence may be negligible.

Implantation of Lactobacilli

As indicated above, prolonged therapy with certain antibiotics may eliminate many normal intestinal microorganisms, permitting antibiotic-resistant species to thrive. This, in turn, may cause gastrointestinal disturbances such as constipation or diarrhea. The oral administration of *Lactobacillus acidophilus* has been found to alleviate the intestinal disorders in some instances. The principle is that ingestion of large numbers of the lactobacilli may result in replacement of undesirable intestinal organisms by harmless or beneficial organisms, a concept first proposed by the Russian bacteriologist Metchnikoff in the early days of bacteriology. The implantation of the lactobacilli seems to depend on ingestion of a large number of the organisms and on supplying a suitable carbohydrate such as lactose that is not readily absorbed by the body but can be easily used by the organisms. A milk product known as **sweet acidophilus milk** has been devised for therapeutic use. This product is made by adding a concentrated suspension of a human strain of *L. acidophilus* to cold pasteurized milk, with subsequent storage at a temperature below 4°C.

Normal Flora and Colon Cancer

An interesting finding that is related to the normal flora of the large intestine is the discovery in 1977 of the presence of a mutagenic compound in the feces of normal humans. Because compounds that are mutagenic may also be carcinogenic, the relationship of the fecal mutagenic compound to the occurrence of colon cancer in humans is being actively investigated. Diet is highly correlated with the occurrence of colon cancer, and populations at high risk for this disease—such as those in the United States, where nearly 125,000 cases of colon cancer occur per year—have been found to have significantly higher levels of this mutagen in their feces than do populations at low risk. Studies in 1982 have demonstrated the ability of several normal flora organisms (species of the genus *Bacteroides*) to produce this mutagen from a precursor compound present in the feces of persons who excrete the mutagen. Whether this precursor arises from the diet of such human excreters, from some product of host metabolism, or from the microbial flora of the intestine, is not known. It must also be emphasized that, as suggestive as these findings are, at the time of this writing it is not yet certain that the mutagenic substance is carcinogenic or is actually responsible for colon cancer.

Genitourinary Tract

In a healthy person, the **kidney, urinary bladder,** and **ureters** (the tubes that convey the urine from the kidney to the bladder) are free of microorganisms. However, bacteria are commonly found in the lower portion of the **urethra** (canal that conveys the urine to the outside of the body) of both males and females. *S. epidermidis, Streptococcus faecalis,* and corynebacteria are found frequently; neisseriae and members of the family *Enterobacteriaceae* are occa-

sionally present. The upper portion of the urethra, near the bladder, has few microorganisms, apparently because of some antibacterial effect exerted by the urethral mucosa and because of the mechanical removal of microorganisms by the frequent flushing of the urethral epithelium by urine. Urine itself is an excellent growth medium for many bacteria; however, as indicated above, urine in the bladder of a healthy person is sterile. It acquires microorganisms as it passes from the bladder to the outside of the body, the source of these organisms being the surface of the lower portion of the urethra. During voiding, if the periurethral surfaces (tip of penis, labial folds, vulva) are first thoroughly cleansed, the first portion of the voiding discarded, and the subsequent urine collected in a sterile container, the urine sample will normally contain less than 10^3 organisms per milliliter. (A count of 10^5 organisms or higher is indicative of a urinary tract infection.)

The adult female genital tract has a very complex normal flora. The character of this population changes with the variation of the menstrual cycle. The main inhabitants of the adult vagina are the acid-tolerant lactobacilli; these break down glycogen produced by the vaginal epithelium, forming lactic acid in the process. As a result, the pH in the vagina is maintained at about 4.4 to 4.6. Microorganisms capable of multiplying at this low pH are found in the vagina and include enterococci, diphtheroids, the yeast *C. albicans*, and large numbers of anaerobic bacteria (Fig. 30-4). The accumulation of glycogen in the vaginal wall is due to ovarian activity. Thus it is not present before puberty or after menopause, and in its absence the vaginal secretions are mildly alkaline and contain normal skin microorganisms.

QUESTIONS

1 Define the following terms:

Commensal	Glucan
Lysozyme	Plaque
Desquamation	Gnotobiotic
Nasal baffle	Echovirus
Comedo	Glycosyl transferase

2 Give the major reason or reasons for the occurrence of the following species of bacteria in their natural habitat:
   (a) *Propionibacterium acnes* in skin.
   (b) *Lactobacillus* species in the vagina.
   (c) *Bacteroides melaninogenicus* in the gingival crevice of teeth.
   (d) *Streptococcus mutans* on teeth surfaces.
3 How are germfree animals obtained and reared?
4 What benefits might a human host derive from the normal flora?
5 What factors make the following environments difficult to colonize: (a) skin, (b) upper portion of the urethra, (c) trachea, (d) stomach, (e) conjunctiva?
6 Why is it useful to know about the normal flora of the healthy human body?
7 Provide an example to illustrate that the laws of natural selection also govern the ecology of the normal flora of the human body.
8 What role does microbial adherence play in establishment of the normal flora? Give an example.

9 In what part of the body do most of the normal flora microorganisms occur? What would a pathogenic microorganism need to do to establish itself, if only temporarily, in this part of the body?
10 How might colon cancer possibly be related to the normal flora of the body?
11 What role does mucus play in preventing microorganisms of inhaled air from reaching the alveoli of the lungs?

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