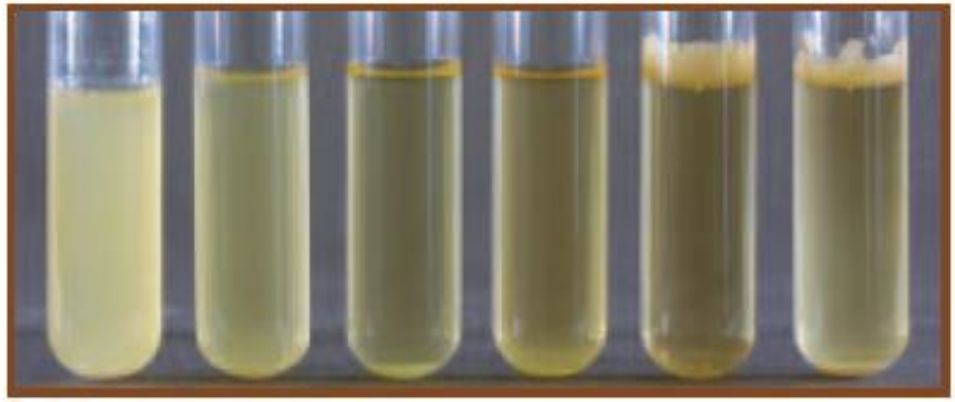
Growth Patterns in Broth

Purpose

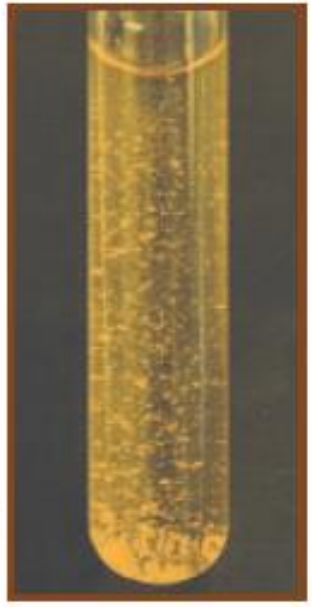
Bacterial genera—and frequently different species within a genus—demonstrate characteristic growth patterns in broth that provide useful information when attempting to identify an organism.

Principle

Microorganisms cultivated in broth display a variety of growth characteristics. Some organisms float on top of the medium and produce a type of surface membrane called a **pellicle**; others sink to the bottom as **sediment**. Some bacteria produce **uniform fine turbidity** while others appear to clump in what is called **flocculent** growth. Refer to Figures 3-35 and 3-36. Figure 3-37 shows an example of a pigmented species (*Rhodospirillum rubrum*) in broth.



3-35 Growth Patterns in Broth From left to right in pairs (by type of organism): *Enterobacter aerogenes* and *Citrobacter diversus*—motile members of *Enterobacteriaceae* (uniform fine turbidity), *Enterococcus faecalis* and *Staphylococcus aureus*—nonmotile Gram-positive cocci (sediment), *Mycobacterium phlei* and *Mycobacterium smegmatis* (relatives of *Mycobacterium tuberculosis*)—nonmotile with a waxy cell wall (pellicle).



3-36 Flocculence in Broth This is a *Streptococcus* species from a throat culture demonstrating flocculence in Todd-Hewitt Broth.

3-37 Pigment in Broth *Rhodospirillum rubrum* has a red color due to carotenoid pigments. It grows as a photoheterotroph in the presence of light and the absence of oxygen.

MacConkey Agar

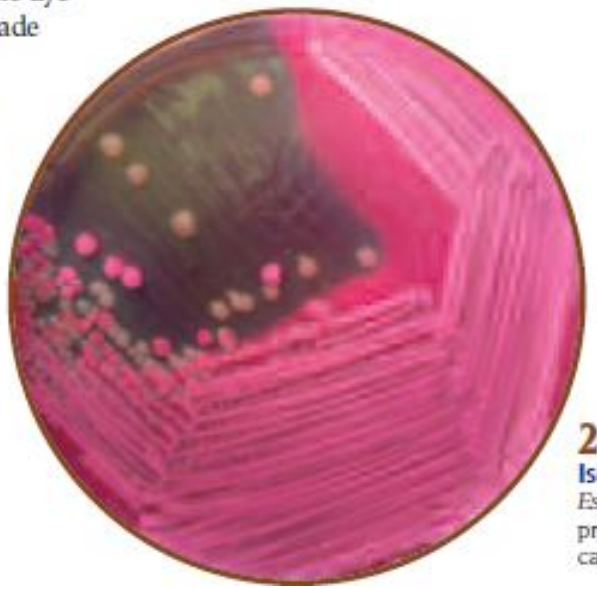
Purpose

MacConkey Agar is used to isolate and differentiate members of the *Enterobacteriaceae* based on the ability to ferment lactose. Variations on the standard medium include MacConkey Agar w/o CV (without crystal violet) to allow detection of Gram-positive cocci or MacConkey Agar CS to control swarming bacteria (such as *Proteus*) that interfere with other results.

Principle

MacConkey Agar is a selective and differential medium containing lactose, bile salts, neutral red, and crystal violet. Bile salts and crystal violet inhibit growth of Gram-positive bacteria. Neutral red dye is a pH indicator that is colorless above a pH of 6.8 and red at a pH below 6.8. Acid accumulating from lactose fermentation turns the dye red. Lactose fermenters turn a shade of red on MacConkey agar whereas lactose nonfermenters remain their normal color or the color of the medium (Figures 2-19 and 2-20). Formulations without crystal violet allow growth of *Enterococcus* and some species of *Staphylococcus*, which ferment the lactose and appear pink on the medium.

2-19 MacConkey Agar MacConkey Agar inoculated with (clockwise from top) *Escherichia coli, Enterobacter aerogenes, Shigella sonnei,* and *Proteus mirabilis. E. coli* and *E. aerogenes* produce pink color from acid-producing lactose fermentation. *S. sonnei* and *P. mirabilis*, both lactose nonfermenters, remain their normal color. Note the precipitated bile salts around the *E. coli*, also shown in Figure 2-20.



2-20 MacConkey Agar Streaked for Isolation MacConkey Agar inoculated with *Escherichia coli* and *Shigella flexneri*. Note the precipitated bile salts around the *E. coli* caused by acid from lactose fermentation.

Mannitol Salt Agar

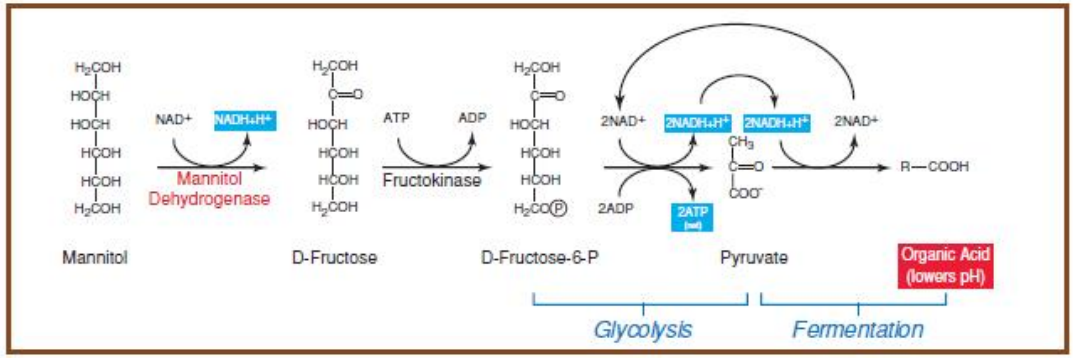
Purpose

Mannitol Salt Agar (MSA) is used for isolation and differentiation of pathogenic staphylococci, principally *Staphylococcus aureus*.

Principle

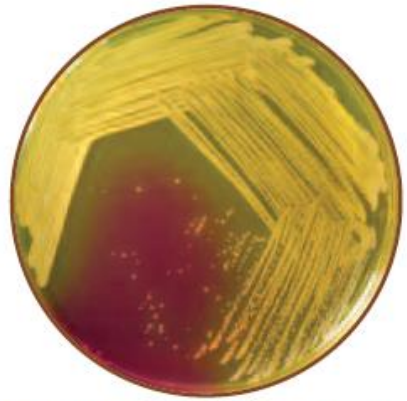
Mannitol Salt Agar contains the carbohydrate mannitol, 7.5% sodium chloride (NaCl), and the pH indicator phenol red. Phenol red is yellow below pH 6.8, red at pH 7.4 to 8.4, and pink above 8.4. The sodium chloride makes this medium selective for staphylococci since most other bacteria cannot survive in this level of salinity. The pathogenic species of *Staphylococcus* ferment mannitol (Figure 2-21) and produce acid, which turns the pH indicator yellow. Nonpathogenic staphylococcal species grow, but produce no color change. Refer to pages 71–73 and Figure A-5 in the Appendix for more information on fermentation.

The development of yellow halos around the bacterial growth is presumptive evidence that the organism is a pathogenic *Staphylococcus* (usually *S. aureus*). Good growth that produces no color change is presumptive evidence for nonpathogenic *Staphylococcus* (Figures 2-22 and 2-23). With few exceptions, organisms that grow poorly on the medium are not staphylococci.



2-21 Mannitol Fermentation with Acid End-Products The organic acids produced lower the pH and turn the medium yellow.

2-22 Mannitol Salt Agar MSA inoculated with *Staphylococcus aureus* (top) and *S. epidermidis* (bottom). (*Note:* Some strains of *S. epidermidis* are inhibited by this medium). The yellow halo around *S. aureus* is due to mannitol fermentation with acid end products.



2-23 Mannitol Salt Agar Streaked for Isolation MSA inoculated with *Staphylococcus aureus* and *Staphylococcus epidermidis*. The growth shown in this photo is typical of the two species on this medium; the colonies of *S. epidermidis* are small and red whereas those of *S. aureus* are slightly larger and yellow.

Sulfur Reduction (SIM Medium)

Purpose

The Sulfur Reduction Test is used to differentiate members of *Enterobacteriaceae*, especially the sulfur-reducing *Salmonella*, *Francisella*, and *Proteus* from the non-reducing *Morganella morganii* and *Providencia rettgeri*.

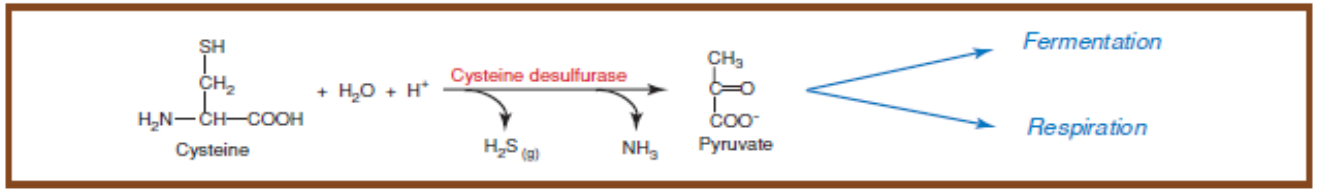
Principle

The Sulfur Reduction Test, as it appears in this manual, is performed using SIM medium. SIM medium also tests for indole production (page 74) and motility (page 82). It is a semi-solid medium that is formulated with casein and animal tissue as sources of amino acids, an iron-containing compound, and sulfur in the form of sodium thiosulfate.

Sulfur reduction to H_2S is an anaerobic activity and can be accomplished by bacteria in two different ways, depending on the enzymes present.

1. The enzyme **cysteine desulfurase** catalyzes the putrefaction of the amino acid cysteine to pyruvate (Figure 7-87).
2. The enzyme **thiosulfate reductase** catalyzes the reduction of sulfur (in the form of sulfate) at the end of the anaerobic respiratory electron transport chain (Figure 7-88).

Both systems produce hydrogen sulfide gas (H_2S). When either reaction occurs in SIM medium, the H_2S produced combines with iron (ferrous ammonium sulfate in the medium) to form ferric sulfide (FeS), a black precipitate (Figure 7-90). Any blackening of the medium is an indication of sulfur reduction and a positive test. Absence of blackening in the medium indicates no sulfur reduction and a negative reaction (Figure 7-90).



7-87 Putrefaction of Cysteine Putrefaction involving cysteine desulfurase produces H_2S. The reaction is a mechanism for getting energy out of the amino acid cysteine.

7-88 Reduction of Thiosulfate Anaerobic respiration with thiosulfate as the final electron acceptor also produces H_2S.

7-89 Indicator Reaction Hydrogen sulfide, a colorless gas, can be detected when it reacts with ferrous ammonium sulfate in the medium to produce the black precipitate ferric sulfide.

7-90 Sulfur Reduction in SIM Medium On the left is *Escherichia coli* (H_2S-negative); on the right is *Proteus mirabilis* (H_2S-positive).

Indole Test (SIM Medium)

Purpose

The Indole Test identifies bacteria capable of producing indole using the enzyme **tryptophanase**. The Indole Test is one component of the IMViC battery of tests (*I*ndole, *M*ethyl Red, *V*oges-Proskauer, and *C*itrate) used to differentiate the *Enterobacteriaceae*.

Principle

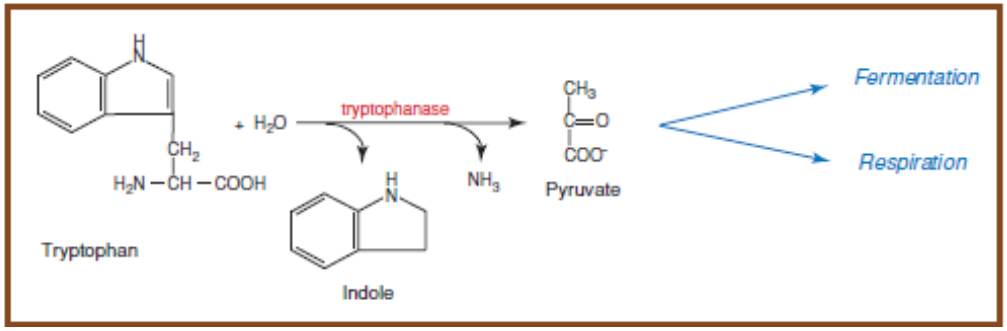
The Indole Test, as it appears in this manual, is performed using SIM medium. SIM medium also tests for motility and sulfur reduction (SIM is an acronym for Sugar-Indole-Motility). It is a semi-solid medium that is formulated with casein and animal tissue as sources of amino acids, an iron-containing compound, and sulfur in the form of sodium thiosulfate.

Indole production in the medium is made possible by the presence of tryptophan (contained in casein and animal protein). Bacteria possessing the enzyme tryptophanase can hydrolyze tryptophan to pyruvate, ammonia (by deamination), and indole (Figure 7-46).

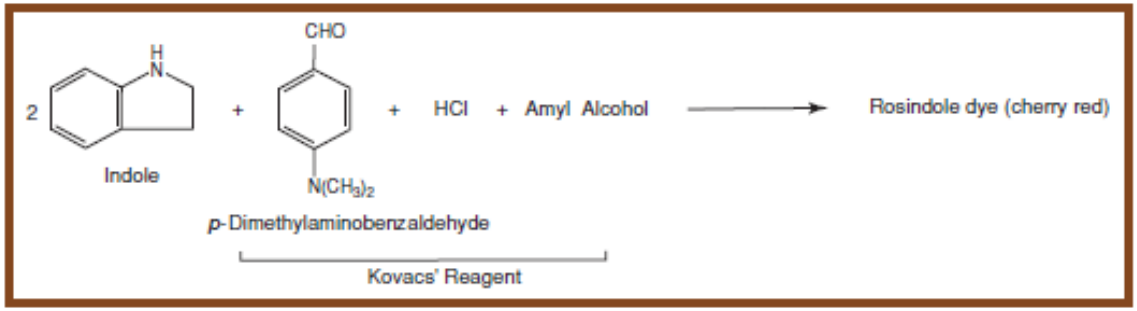
The hydrolysis of tryptophan in SIM medium can be detected by the addition of Kovacs' reagent after a period of incubation. Kovacs' reagent contains dimethylaminobenzaldehyde (DMABA) and HCl dissolved in amyl alcohol. When a few drops of Kovacs' reagent are added to the tube, it forms a liquid layer over the solid medium. DMABA then reacts with any indole present and produces a quinoidal compound that turns the reagent layer red (Figures 7-47 and 7-48). The formation of red color in the reagent layer indicates a positive reaction and the presence of tryptophanase. No red color is indole-negative.

An instantaneous indole test is available and done by placing bacterial growth on a paper slide impregnated with 5% DMABA (Figure 7-49). A positive result is formation of pink on the paper slide.

7-46 Tryptophan Catabolism in Indole-Positive Organisms
Tryptophanase hydrolyzes the amino acid tryptophan to indole, ammonia, and pyruvate. Subsequently, pyruvate can be used in the Krebs Cycle.



7-47 Indole Reaction with Kovacs' Reagent
Kovacs' Reagent is added to the medium following incubation. If the organism is indole-positive, a red color is produced by this reaction.



7-48 Indole Test Results
This is SIM medium inoculated with *Morganella morganii* (+) on the right and *Enterobacter aerogenes* (−) on the left.

Cultivation of Anaerobes—Thioglycollate Broth

Purpose

Fluid Thioglycollate Medium is a simple, inexpensive system for cultivating small numbers of anaerobic or microaerophilic bacteria. It is a liquid medium formulated to promote growth of a wide variety of fastidious anaerobic and microaerophilic microorganisms.

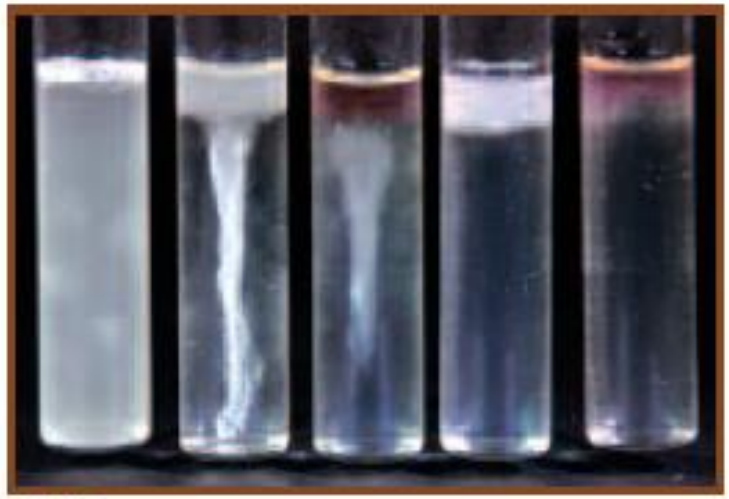
Principle

Fluid Thioglycollate Medium is prepared as a basic medium or with a variety of supplements, depending on the specific needs of organisms being cultivated. As such, it is appropriate for a broad variety of aerobic and anaerobic, fastidious and nonfastidious organisms. It is particularly well adapted for cultivation of strict anaerobes and microaerophiles.

Key components of the medium are yeast extract, pancreatic digest of casein, dextrose, sodium thioglycollate, L-cystine, and resazurin. Yeast extract and pancreatic digest of casein provide nutrients; sodium thioglycollate and L-cystine reduce oxygen to water; and resazurin (pink when oxidized, colorless when reduced) acts as an indicator. A small amount of agar is included to slow oxygen diffusion.

Oxygen is removed from the medium during autoclaving but begins to diffuse back in as the tubes cool to room temperature. This produces a gradient of concentrations from fully aerobic at the top to anaerobic at the bottom. Thus, fresh media will appear clear to straw colored with a pink region at the top where the dye has become oxidized (Figure 3-42). Figure 3-43 demonstrates some basic bacterial growth patterns in the medium as influenced by the oxygen gradient.

3-42 Aerobic zone in Thioglycollate Medium Note the pink region in the top (oxidized) portion of the broth. The bottom (reduced) portion of the medium remains colorless.



3-43 Growth Patterns in Thioglycollate Medium Growth patterns of a variety of organisms are shown in these Fluid Thioglycollate Broths. Pictured from left to right are: aerotolerant anaerobe, facultative anaerobe, strict anaerobe, strict aerobe, and microaerophile. Compare these tubes with the uninoculated broth in Figure 3-42.

Catalase Test

Purpose

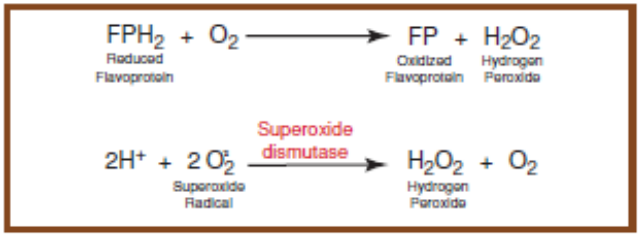
The Catalase Test is used to identify organisms that produce the enzyme **catalase**. It is most commonly used to differentiate members of the catalase-positive *Micrococcaceae* from the catalase-negative *Streptococcaceae*. Variations on this test may also be used in identification of *Mycobacterium* species.

Principle

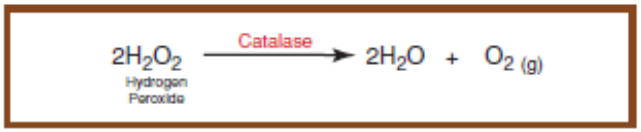
The electron transport chains of aerobic and facultatively anaerobic bacteria are composed of molecules capable of accepting and donating electrons as conditions dictate. As such, these molecules alternate between the oxidized and reduced form, passing electrons down the chain to the final electron acceptor (FEA). Energy lost by electrons in this sequential transfer is used to perform oxidative phosphorylation (*i.e.*, produce ATP from ADP).

One carrier molecule in the ETC called **flavoprotein** can bypass the next carrier in the chain and transfer electrons directly to oxygen (Figure 7-18). This alternate pathway produces two very potent cellular toxins—hydrogen peroxide (H_2O_2) and superoxide radical (O_2^-).

Aerobic and facultatively anaerobic bacteria produce enzymes capable of detoxifying these compounds. **Superoxide dismutase** catalyzes conversion of superoxide radicals (the more lethal of the two compounds) to hydrogen peroxide (Figure 7-18). Catalase converts hydrogen peroxide into water and gaseous oxygen (Figure 7-19).



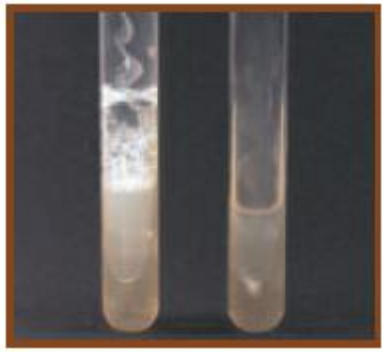
7-18 Microbial Production of H_2O_2 Hydrogen peroxide may be formed through the transfer of electrons from reduced flavoprotein to oxygen or from the action of superoxide dismutase.



7-19 Catalase Mediated Conversion of H_2O_2 Catalase is an enzyme of aerobes, microaerophiles, and facultative anaerobes that converts hydrogen peroxide to water and oxygen gas.

Bacteria that produce catalase can be detected easily using typical store-grade hydrogen peroxide. When hydrogen peroxide is added to a catalase-positive culture, oxygen gas bubbles form immediately (Figures 7-20 and 7-21). If no bubbles appear, the organism is catalase-negative. This test can be performed on a microscope slide or by adding hydrogen peroxide directly to the bacterial growth.

7-20 Catalase Slide Test Shown is the catalase slide test in which visible bubble production indicates a positive result. *Staphylococcus aureus* (+) is on the left, *Enterococcus faecium* (–) is on the right. It is a good idea to cover the slide with a Petri dish lid immediately after addition of peroxide to contain aerosols produced in positive reactions.



7-21 Catalase Tube Test The catalase test may also be performed on an agar slant. *Staphylococcus aureus* (+) is on the left, *Enterococcus faecium* (–) is on the right.

Oxidation–Fermentation Test

Purpose

The Oxidation-Fermentation (O–F) Test is used to differentiate bacteria based on their ability to oxidize or ferment specific sugars. It allows presumptive separation of the fermentative *Enterobacteriaceae* from the oxidative *Pseudomonas* and *Bordetella*, and the nonreactive *Alcaligenes* and *Moraxella*.

Principle

The O–F Test is designed to differentiate bacteria on the basis of fermentative or oxidative metabolism of carbohydrates. In oxidation pathways a carbohydrate is directly oxidized to pyruvate and further converted to CO_2 and energy by way of the Krebs cycle and the electron transport chain, where an inorganic molecule such as oxygen is required to act as the final electron acceptor. Fermentation also converts carbohydrates to pyruvate, but uses it to produce one or more acids (as well as other compounds). Consequently, fermenters identified by this test acidify O-F medium to a greater extent than do oxidizers.

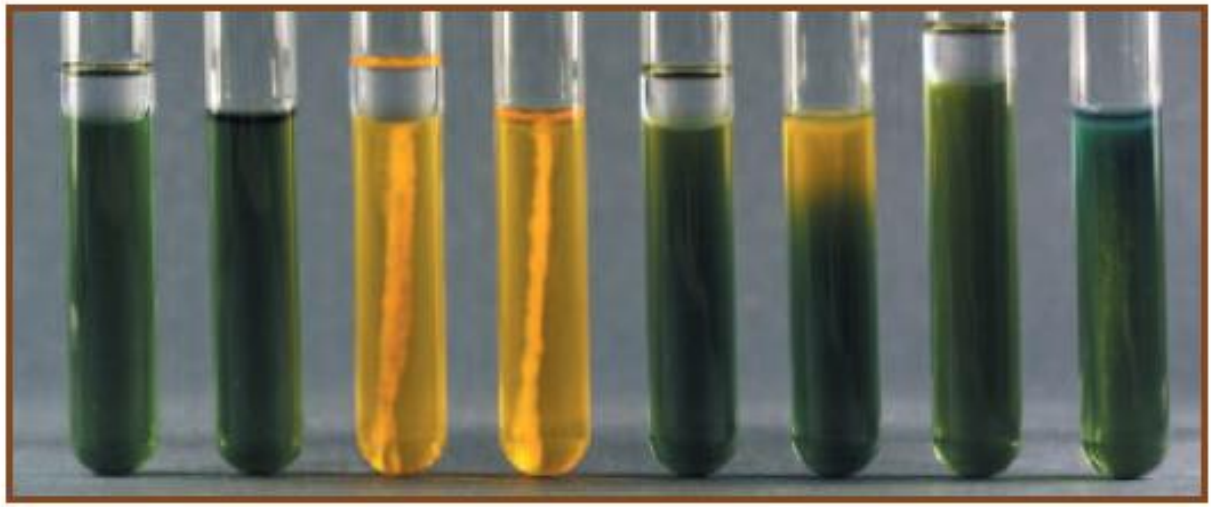
Hugh and Leifson's O–F medium includes a high sugar-to-peptone ratio to reduce the possibility that alkaline products from peptone utilization will neutralize weak acids produced by oxidation of the carbohydrate. Bromthymol blue dye, which is yellow at pH 6.0 and green at pH 7.1, is added as a pH indicator. A low agar concentration makes it a semi-solid medium that allows determination of motility.

The medium is prepared with glucose, lactose, sucrose, maltose, mannitol, or xylose and is not slanted. Two tubes of the specific sugar medium are stab-inoculated several times with the test organism. After inoculation, one tube is sealed with a layer of sterile mineral oil to promote anaerobic growth and fermentation. The other tube is left unsealed to allow aerobic growth and oxidation. (***Note:*** Tubes of O–F medium are heated in boiling water and then cooled prior to inoculation. This removes free oxygen from the medium and ensures an anaerobic environment in all tubes. The tubes covered with oil will remain anaerobic, whereas the uncovered medium quickly will become aerobic as oxygen diffuses back in.)

Organisms able to ferment the carbohydrate or ferment *and* oxidize the carbohydrate will turn the sealed and unsealed media yellow throughout. Organisms that are able to oxidize only will turn the unsealed medium yellow (or partially yellow) and leave the sealed medium green or blue. Slow or weak fermenters will turn both tubes slightly yellow at the top. Organisms that are not able to metabolize the sugar will either produce no color change or turn the medium blue because of alkaline products from amino acid degradation. The results are summarized in Table 7-4 and shown in Figure 7-80.

TABLE 7-4 O–F Medium Results and Interpretations

TABLE OF RESULTS			
Sealed	**Unsealed**	**Interpretation**	**Symbol**
Green or blue	Any amount of yellow	Oxidation	O
Yellow throughout	Yellow throughout	Oxidation and fermentation or fermentation only	O–F or F
Slightly yellow at the top	Slightly yellow at the top	Oxidation and slow fermentation or slow fermentation only	O–F or F
Green or blue	Green or blue	No sugar metabolism; organism is nonsaccharolytic	N



7-80 Oxidation–Fermentation Test These pairs of tubes represent three possible results in the Oxidation–Fermentation (O–F) Test. Each pair contains one tube sealed with an overlay of mineral oil and one unsealed tube. The mineral oil creates an environment unsuitable for oxidation because it prevents diffusion of oxygen from the air into the medium. The result is that an organism capable of fermentation will turn both tubes yellow, whereas an organism capable only of oxidizing glucose will turn only the oxygen-containing portion of the unsealed medium yellow. An organism incapable of utilizing glucose by any means either will not change the color of the medium or will turn it blue-green as a result of alkaline products from protein degradation. Reading from left to right, the first pair of tubes on the left are uninoculated controls for color comparison. The second pair of tubes was inoculated with *Shigella flexneri*, an organism capable of both oxidative and fermentative utilization of glucose (O–F). Unfortunately, this determination cannot be made simply by visual examination, as the results of a fermentative organism (F) look exactly the same as an organism capable of both oxidation and fermentation (O–F). Therefore, when both tubes are yellow, the organism is assumed to be either (F) *or* (O–F). The third pair of tubes was inoculated with *Pseudomonas aeruginosa*, a glucose nonfermenter. This organism is capable only of oxidation. Note the yellowing only of the oxygenated portion of the unsealed tube. The fourth pair of tubes (right) was inoculated with *Alcaligenes faecalis*, an organism incapable of utilizing glucose. The blue color in the oxygenated portion of the unsealed tube suggests that the organism is both nonsaccharolytic (N) and a strict aerobe.

Casein Hydrolysis Test

Purpose

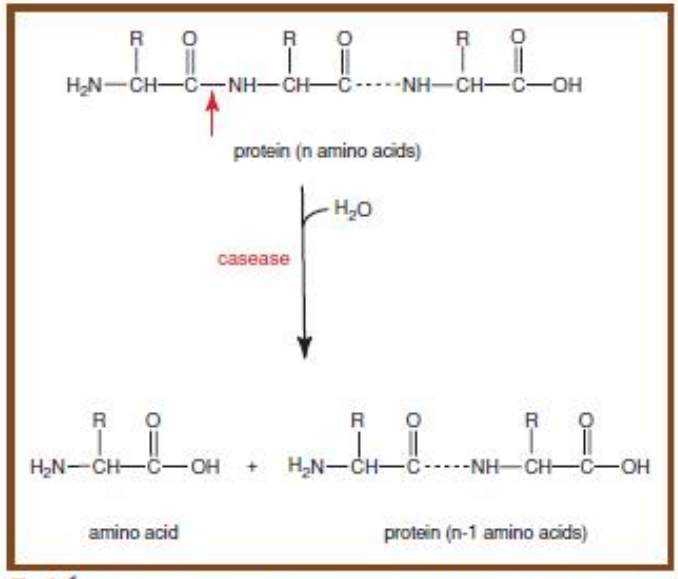
The Casein Hydrolysis Test is used to identify bacteria capable of hydrolyzing casein with the enzyme **casease**.

Principle

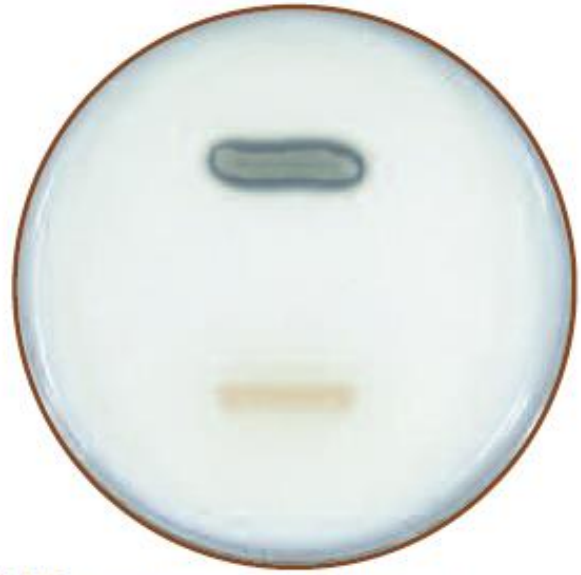
Many bacteria require proteins as a source of amino acids and other components for synthetic processes. Some bacteria have the ability to produce and secrete enzymes (exoenzymes) into the environment that catalyze the hydrolysis (break-down) of large proteins to smaller peptides or individual amino acids, thus enabling their uptake across the membrane.

Casease is an enzyme some bacteria produce to hydrolyze the milk protein **casein** (Figure 7-16), the molecule that gives milk its white color. When broken down into smaller fragments, the ordinarily white casein loses its opacity and becomes clear.

The presence of casease can be detected easily with the test medium Milk Agar (Figure 7-17). Milk Agar is an undefined medium containing pancreatic digest of casein, yeast extract, dextrose, and powdered milk. When plated Milk Agar is inoculated with a casease-positive organism, secreted casease will diffuse into the medium around the colonies and create a zone of clearing where the casein has been hydrolyzed. Casease-negative organisms do not secrete casease and, thus, do not produce clear zones around the growth.



7-16 Casein Hydrolysis Hydrolysis of any protein occurs by breaking peptide bonds (**red arrow**) between adjacent amino acids to produce short peptides or individual amino acids.



7-17 Casein Hydrolysis Test Results This Milk Agar plate was inoculated with *Bacillus megaterium* (casease-positive) above and *Micrococcus roseus* (casease-negative) below.

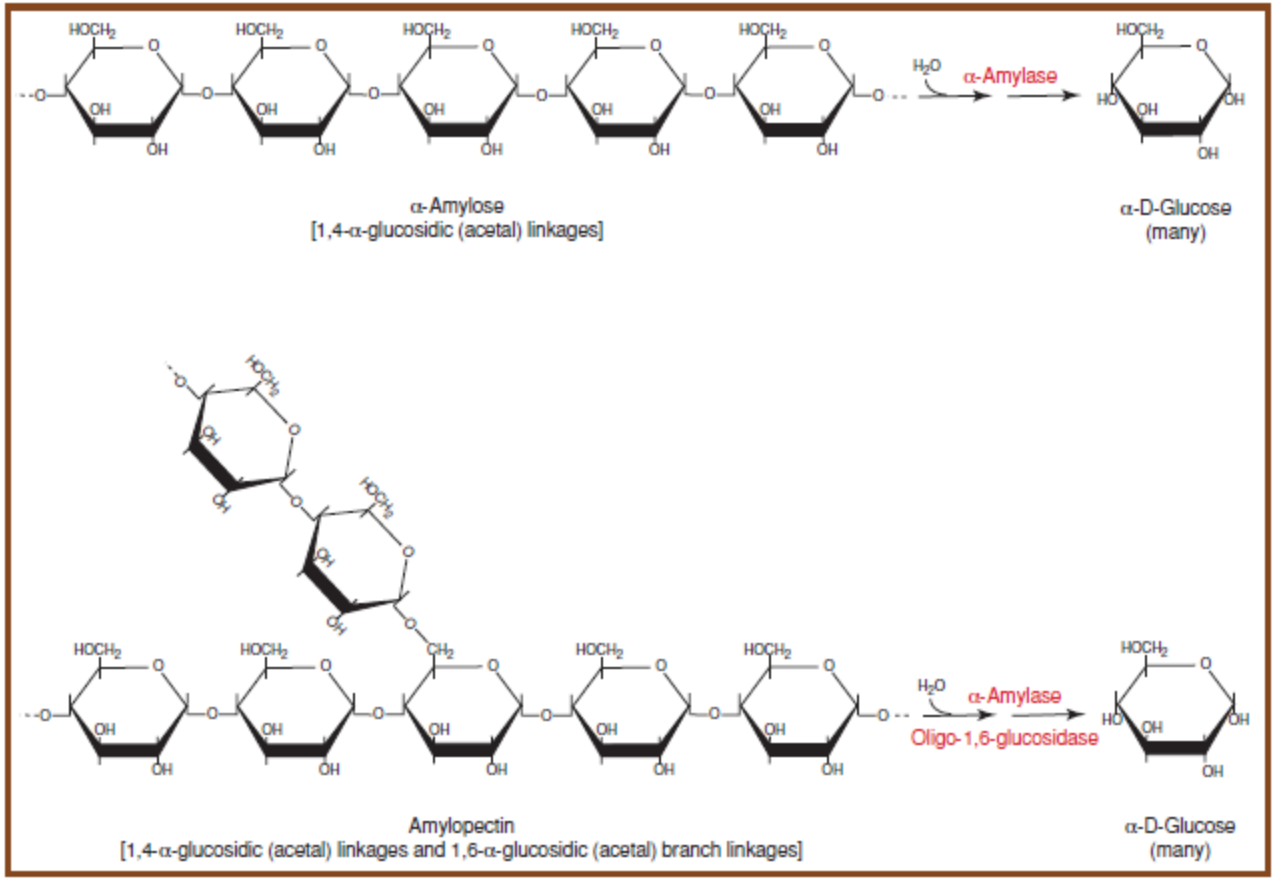
Starch Hydrolysis

Purpose

Starch Agar originally was designed for cultivating *Neisseria*. It no longer is used for this, but with pH indicators, it is used to isolate and presumptively identify *Gardnerella vaginalis*. It aids in differentiating species of the genera *Corynebacterium, Clostridium, Bacillus, Bacteroides, Fusobacterium*, and *Enterococcus*, most of which have positive and negative species.

Principle

Starch is a polysaccharide made up of α-D-glucose subunits. It exists in two forms—linear (amylose) and branched (amylopectin)—usually as a mixture with the branched configuration being predominant. The α-D-glucose molecules in both amylose and amylopectin are bonded by 1,4-α-glycosidic (acetal) linkages (Figure 7-85). The two forms differ in that amylopectin contains polysaccharide side chains connected

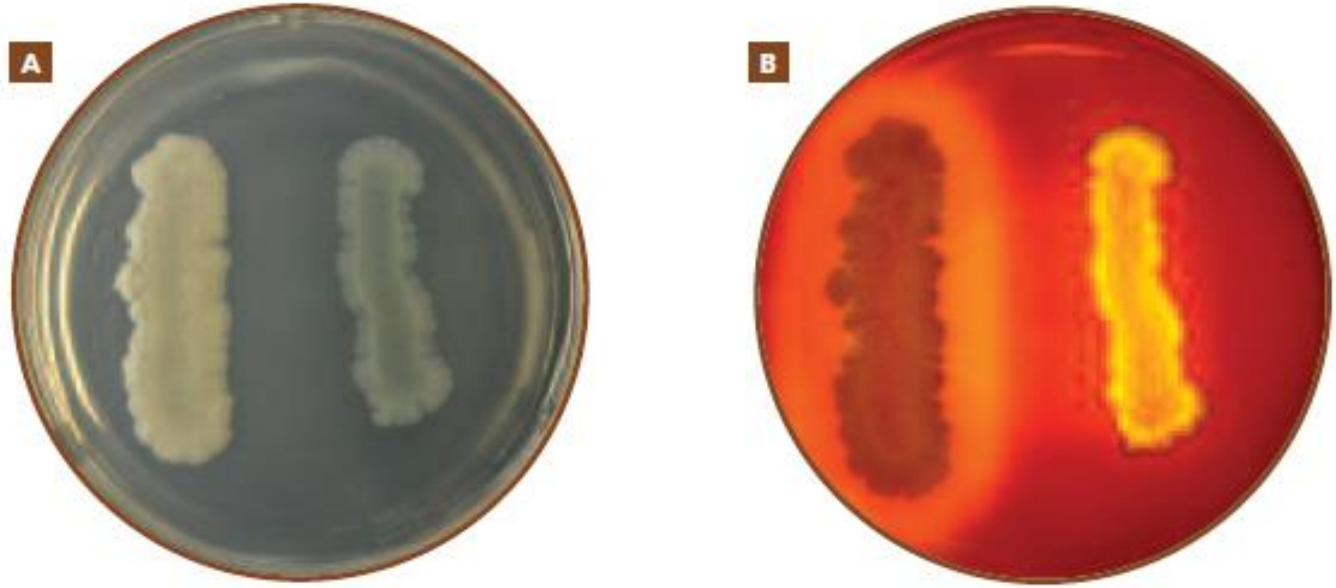


7-85 STARCH HYDROLYSIS BY α-AMYLASE AND OLIGO-1,6-GLUCOSIDASE

to approximately every 30th glucose in the main chain. These side chains are identical to the main chain except that the number 1 carbon of the first glucose in the side chain is bonded to carbon number 6 of the main chain glucose. The bond, therefore, is a 1,6-α-glycosidic linkage.

Starch is too large to pass through the bacterial cell membrane. Therefore, to be of metabolic value to the bacteria it must first be split into smaller fragments or individual glucose molecules. Organisms that produce and secrete the extracellular enzymes **α-amylase** and **oligo-1,6-glucosidase** are able to hydrolyze starch by breaking the glycosidic linkages between the sugar subunits. Although there usually are intermediate steps and additional enzymes utilized, the overall reaction is the complete hydrolysis of the polysaccharide to its individual α-glucose subunits (Figure 7-86).

Starch agar is a simple plated medium of beef extract, soluble starch, and agar. When organisms that produce α-amylase and oligo-1,6-glucosidase are cultivated on starch agar they hydrolyze the starch in the area surrounding their growth. Because both the starch and its sugar subunits are soluble and virtually invisible in the medium, the reagent iodine is used to detect the presence or absence of starch in the vicinity around the bacterial growth. Iodine reacts with starch and produces a blue or dark brown color; therefore, any microbial starch hydrolysis will be revealed as a clear zone surrounding the growth (Figure 7-86).



7-86 STARCH AGAR **A** *Bacillus subtilis* is on the left. *Escherichia coli* is on the right. Notice the wavy margin of *E. coli*. **B** After iodine has been added, the clearing in the medium around *B. subtilis* demonstrates a positive result for starch hydrolysis. *E. coli*, with no clearing, is negative. Note that the wavy margin of *E. coli* produced a lighter region around the growth that might be misinterpreted as clearing. To prevent reading a false positive, it is a good idea to establish the margin of growth for each tested organism prior to adding iodine.

Winogradsky Column

Purpose

The Winogradsky column is a method for growing a variety of microbes with uniquely microbial metabolic abilities. Bacterial photoautotrophs, chemolithotrophs, and photoheterotrophs may be found in a mature column. And more "typical" chemoheterotrophs and photoautotrophs also are likely to be found. A mature Winogradsky column is a good source for studying these organisms in the laboratory.

Principle

The Winogradsky column bears the name of its developer, Sergei Winogradsky (1856–1953), a Russian microbiologist and pioneer in microbial ecology. He studied sulfur bacteria because of their ease of handling and cultivation, and then moved on to bacteria associated with the nitrogen cycle. One of his major discoveries was finding microorganisms (*Beggiatoa*) capable of the unheard of type of metabolism that came to be known as chemolithotrophic autotrophy (see below). Until he made his discovery, only photoautotrophs—those performing photosynthesis—were known to be autotrophs.

As a result of his work and the work of others, metabolic categories of microorganisms have been identified based on their carbon, energy, and electron sources. These are listed below. Note that in practice, terms are combined to describe the organism more fully.

Autotroph: an organism capable of obtaining all of its carbon from CO_2.

Heterotroph: an organism that can only get its carbon from organic molecules.

Chemotroph: an organism that gets its energy from the oxidation of chemicals.

Phototroph: an organism that gets its energy from light ($h\nu$).

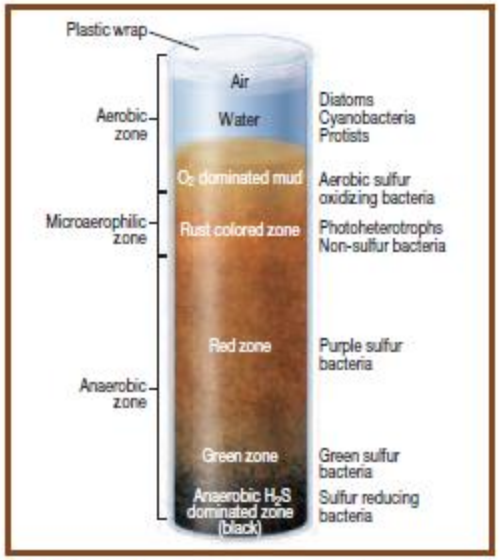
Organotroph: an organism that gets its electrons from an organic molecule.

Lithotroph: an organism that gets its electrons from an inorganic molecule.

Winogradsky pioneered this method of growing microbes in the late 19th century. It was (and is) used as a convenient laboratory source to supply for study a variety of **anaerobic, microaerophilic,** and **aerobic** bacteria, including purple

nonsulfur bacteria, purple sulfur bacteria, green sulfur bacteria, chemoheterotrophs, and many others (Figure 19-22).

The basis for the Winogradsky column is threefold. The first two factors involve opposing gradients that impact the types of organisms that can grow. The first is the oxygen gradient, which decreases (gets more and more anaerobic) toward the bottom. As a result, obligate aerobes, microaerophiles, facultative anaerobes, and obligate anaerobes are found in different locations in the column. The second is the H_2S gradient, which runs opposite in direction to the O_2 gradient. The third factor is the diffuse light shined upon the column. This promotes growth of phototrophic organisms at levels where they are adapted to the opposing gradients of O_2 and H_2S. These layers of phototrophs occur in natural ecosystems but are extremely thin because light doesn't penetrate mud sediments very far. But with the transparent column, thicker layers develop and it is easier to obtain samples for cultures or microscopy (Figures 19-23 and 19-24).



19-22 An Artist's Rendition of a Winogradsky Column What you put into a Winogradsky column dictates what you grow. Any well-constructed column has an oxygen gradient from top to bottom, with the aerobic zone penetrating perhaps only as much as 20% of the total depth. The remaining portion of the mud column becomes progressively more anaerobic. The different amounts of oxygen lead to layering of microbial communities adapted to that specific environment. This illustration is a generalized picture of the layering that you might see in a mature column. (The column often produces intermixed patches rather than distinct layers.) Starting at the top and working downward, the layers are: air, water (containing algae and cyanobacteria), aerobic mud (sulfur oxidizing bacteria), microaerophilic mud (nonsulfur photosynthetic bacteria), red/purple zone (purple sulfur photosynthetic bacteria), green zone (green sulfur photosynthetic bacteria), and black anaerobic zone (sulfur reducing bacteria).

19-23 A Freshly Made Winogradsky Column The black layer comprising the majority of the column is the unenriched mud. The lighter gray area at the bottom contains mud, $CaCO_3$, $CaSO_4$, and shredded paper mixed into a slurry. Note the absence of air spaces.

19-24 A Mature Winogradsky Column at Eight Weeks Notice the layers and colors! Also notice that the layers are not as well defined as in Figure 19-22. In fact, some look mixed (*e.g.*, the rust and red portions appear mixed in some regions). But the dark, anaerobic zone above the whitish layer at the bottom is well defined. The remainder is—pardon the expression—clear as mud.

Sulfur Cycle—Introduction

Sulfur is one of the most abundant elements on Earth. Having oxidation states from −2 to +6, it is able to form many different compounds usable by living things. Most of the sulfur compounds used by microorganisms are inorganic molecules, used strictly for energy or to be incorporated into organic molecules in biosynthetic processes. Table 19-3 summarizes some important sulfur compounds and their oxidation states.

The sulfur microorganisms are a diverse group and include both *Bacteria* and *Archaea*. They live in habitats as diverse as freshwater ponds, lakes, and rivers (especially where there is sewage contamination), water-saturated soils, saltwater lagoons, sulfur solfaturas as in Yellowstone National Park, and in and around deep ocean hydrothermal vents. This vast group includes photoautotrophs, photoheterotrophs, chemolithoautotrophs, chemolithoheterotrophs, obligate aerobes, facultative anaerobes, and obligate anaerobes.

Many sulfur oxidizers and reducers live **syntrophically** in mutually dependent communities, in which sulfur is converted back and forth between reduced and oxidized forms. Conversely, sulfur oxidizers living in and around hydrothermal vents, although still part of a complex community, have an abundant source of reduced sulfur flowing up from the vents. These microbes, receiving no biologically reduced sulfur, thrive in the ecosystem and produce large living mats that cover surrounding surfaces.

Sulfur bacteria fall into three major categories—photoautotrophs, chemolithoautotrophs, and the sulfur reducers. Table 19-4 lists the major groups of sulfur bacteria and some

TABLE 19-3 Sulfur Compounds and Sulfur Organisms That Use Them

Sulfur Compound	Chemical Formula	Oxidation State	Used By Oxidizers	Used By Reducers
Organic sulfur	R–SH	−2	+	+
Sulfide	H_2S, HS^-, S^{2-}	−2	+	
Elemental sulfur	S^0	0	+	+
Thiosulfate	$S_2O_3^{2-}$	+2 per S	+	+
Sulfur dioxide	SO_2	+4		
Sulfite	SO_3^{2-}	+4		+
Sulfate	SO_4^{2-}	+6		+

TABLE 19-4 Major Sulfur Bacteria Reactions

Microbial groups	Representative Organisms	Habitat	Reactions	Representative Summary Reactions
Photoautotrophs	*Chromatium, Chlorobium*	Anoxic	Anoxygenic photosynthesis	$H_2S + CO_2 \rightarrow S^0 + [CH_2O]$ $S^0 + CO_2 \rightarrow SO_4^{2-} + [CH_2O]$
Chemolithoautotrophs	*Beggiatoa, Macromonas, Thiobacillus, Thiobacterium*	Anoxic/oxic interface where H_2S and O_2 meet	Sulfur/sulfide/ thiosulfate oxidation	$HS^- + ½\ O_2 + H^+ \rightarrow S^0 + H_2O$ $H_2S + 2\ O_2 \rightarrow SO_4^{2-} + 2H^+$ $S^0 + 1½\ O_2 + H_2O \rightarrow H_2SO_4$ $S_2O_3^{2-} + H_2O + 2\ O_2 \rightarrow 2\ SO_4^{2-} + 2H^+$
Sulfate/sulfur reducers	*Desulfovibrio, Desulfobulbus Desulfobacter, Desulfuromonas*	Either oxic or anoxic	Assimilatory sulfate reduction	$SO_4^{2-} \rightarrow S^{2-}$ + O-acetyl-L-serine $\rightarrow$ L- cysteine + acetate + H_2O
		Anoxic	Dissimilatory sulfate reduction	$SO_4^{2-} \rightarrow S^0$ $S^0 \rightarrow H_2S$
Many groups	Many organisms	Either oxic or anoxic	Desulfuration	Organic sulfur compounds (R–SH) + $H_2O \rightarrow$ R–OH + H_2S

summary reactions they perform. Figure 19-31 illustrates the major biogeochemical transformations.

The photoautotrophs (Figures 19-32 and 33) are anoxygenic photosynthesizers, that is, they perform a type of photosynthesis that does not produce oxygen. These organisms reside in the anoxic zone of a pond or other aquatic ecosystem close enough to the surface to use the sun's energy to fix carbon from CO_2. Rather than chloroplasts, as in green plants, anoxygenic phototrophs contain membrane-bound bacteriochlorophyll. In sulfur bacteria, bacteriochorophyll traps light energy and converts it to ATP, which ultimately is used to fix CO_2. Oxidation of H_2S or elemental sulfur provides electrons for CO_2 reduction. These reactions are analogous to the oxygenic photosynthetic reactions performed by cyanobacteria and eukaryotes.

photosynthetic eukaryotes:

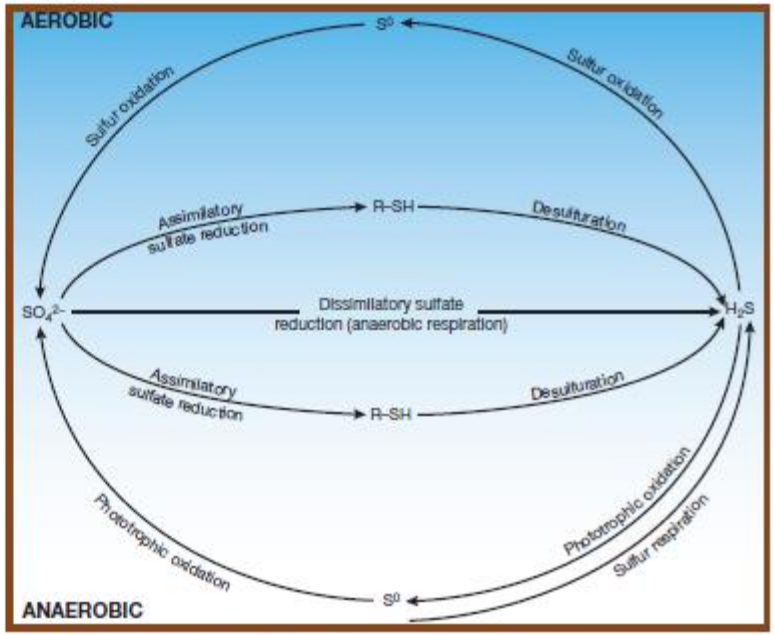
$$CO_2 + H_2O \longrightarrow [CH_2O] + O_2$$

photosynthetic sulfur bacteria:

$$CO_2 + H_2S \longrightarrow [CH_2O] + S^0$$

The chemolithoautotrophs (Figures 19-32 and 19-34) are aerobic organisms that oxidize reduced sulfur compounds as an energy source and use it to fix CO_2. Because the most common form of reduced sulfur is sulfide gas (S^{2-}, HS^-, H_2S) produced in anoxic sediments, oxidation by these bacteria must occur as the gaseous sulfur rises and meets the oxic zone.

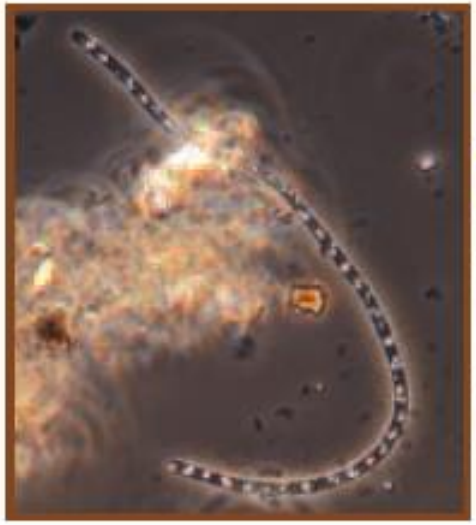
Sulfur reducers (Figure 19-35) perform two important reduction reactions: dissimilatory and assimilatory sulfate reduction. Assimilatory sulfate reduction is the production of sulfide in the form of the –SH groups of biochemicals. Dissimilatory sulfate reduction is a purely energy releasing respiratory reaction, where sulfate acts as a final electron acceptor in anaerobic respiration. Finally, desulfuration (sulfur mineralization) is the reversal of assimilatory reduction and involves the release of H_2S to the environment.



19-31 Biogeochemical Sulfur Transformations These are the major sulfur transformations in the sulfur cycle. Refer to Tables 19-3 and 19-4 for details.

19-32 Microbial Mat of Sulfur Bacteria The layers of this microbial mat formed at the edge of the Salton Sea contain sulfur bacteria. The chemoautotroph *Beggiatoa* is found in the lighter, surface mat. A portion of the surface mat has been removed to reveal the black mud less than 1 cm below in which sulfur reducing bacteria reside. Green sulfur bacteria (photoautotrophs) are found in between.

19-33 Photoautotrophic Sulfur Bacterium This organism was provisionally identified as *Allochromatium*, purple sulfur bacterium. Note the evenly distributed sulfur granules in the cytoplasm.



19-34 Chemo-autotrophic Sulfur Oxidizing Bacterium (Phase Contrast) This gliding bacterium, provisionally identified as *Beggiatoa*, shows numerous sulfur granules in the cytoplasm. These are the by-product of H_2S oxidation, the method by which *Beggiatoa* obtains energy.

19-35 Phase-Contrast Image of a Sulfur Reducer This is a phase-contrast photomicrograph of unknown sulfur reducers recovered from black (anoxic) pond sediment.