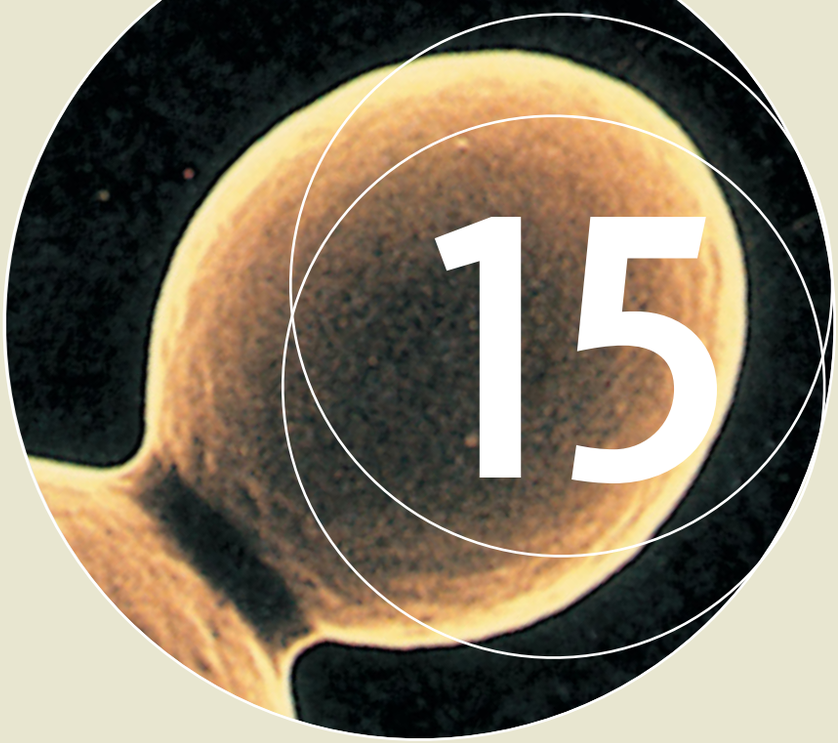


15

Commercial Products and Biotechnology

The common baker's yeast is an important tool for many of the commercial processes of both industrial microbiology and biotechnology.

Many commercial products are produced on a large scale by microorganisms, and this is the field of **industrial microbiology**. These products include antibiotics, of course, but also a wide variety of other products. A common thread that unites these products is the *scale* of their production, which is usually very large, and the fact that they sell for a relatively low price. The products typically originate from enhancements of metabolic reactions that the microorganisms were already capable of carrying out, with the main goal being the *overproduction* of the product of interest.

Industrial microbiology contrasts with **biotechnology**, in which microorganisms are altered by **genetic engineering** to produce substances they would otherwise not be able to produce, for example, human hormones such as insulin. In addition, products of the biotech industry are typically made in relatively small amounts and have high intrinsic value. Thus while penicillin is produced by the ton, insulin is produced by the kilogram. In this chapter we see how both industrial microbiology and biotechnology are done and describe a few common products of each commercial enterprise.

I Putting Microorganisms to Work

Humans have been putting microorganisms to work for thousands of years. In the first half of this chapter, our discussion of industrial microbiology touches on the earliest human uses, which are still important today. In the second half, we explore the most recent uses, achieved through genetic engineering.

15.1 Industrial Products and the Microorganisms That Make Them

Major products of industrial microbiology include the microbial cells themselves—for example, yeast cultivated for food, baking, or brewing, and substances produced by microbial cells. Examples of substances produced by cells include enzymes, antibiotics, amino acids, vitamins, other food additives, commodity chemicals, and alcoholic beverages (**Table 15.1**).

The major organisms used in industrial microbiology are fungi (yeasts and molds) (↻ Sections 20.13–20.18) and certain prokaryotes, in particular species of the genus *Streptomyces* (↻ Section 18.6). Industrial microorganisms can be thought of as metabolic specialists, capable of synthesizing one or more products in high yield. Industrial microbiologists often use classical genetic methods to select for high-yielding mutant strains; their goal is to increase the *yield* of the product to the point of being economically profitable. The genetics of the producing organism needs to be well understood. After selection, the metabolic behavior of the production strain may be far removed from that of the original wild-type strain.

Table 15.1 *Major products of industrial microbiology*

Product	*Example*
Antibiotics	Penicillin, tetracycline
Enzymes	Glucose isomerase, laundry proteases and lipases
Food additives	Vitamins, amino acids
Chemicals	Biofuels (alcohol and biodiesel), citric acid
Alcoholic beverages	Beer, wine, distilled spirits

A microorganism used in an industrial process must have other features in addition to being able to produce the substance of interest in high yield. First and foremost, the organism must be capable of growth and product formation in large-scale culture. Moreover, it should produce spores or some other reproductive cell so that it can be easily inoculated into the large vessels used to grow the producing organism on an industrial scale. It must also grow rapidly and produce the desired product in a relatively short period of time.

An industrially useful organism must also be able to grow in a liquid culture medium obtainable in bulk quantities at a low price. Many industrial microbiological processes use waste carbon from other industries as major or supplemental ingredients for large-scale culture media. These include *corn steep liquor* (a product of the corn wet-milling industry that is rich in nitrogen and growth factors) and *whey* (a waste liquid of the dairy industry containing lactose and minerals).

An industrial microorganism should not be pathogenic, especially to humans or economically important animals or plants. Because of the high cell densities in industrial microbial processes and the virtual impossibility of avoiding contamination of the environment outside the growth vessel, a pathogen would present potentially disastrous problems.

Finally, an industrial microorganism should be amenable to genetic analysis because the yields necessary to make an industrial process profitable typically demand the selection of high-yielding mutant derivatives of the original wild-type organism. Thus, an organism that can be genetically manipulated is a clear advantage for any potential industrial process.

MiniQuiz

- List three important products of industrial microbiology.
- List two desirable properties of an industrial microorganism.

15.2 Production and Scale

In Section 5.7 we considered microbial growth and described the various stages: *lag, exponential*, and *stationary*. Here we describe microbial growth and product formation in an industrial context. There are two types of microbial metabolites of interest to industrial microbiology, primary and secondary. A **primary metabolite** forms during the exponential growth phase of the microorganism. By contrast, a **secondary metabolite** forms near the end of growth, frequently at, near, or in the stationary phase of growth (**Figure 15.1**).

A typical primary metabolite is alcohol. Ethyl alcohol (ethanol) is a product of the fermentative metabolism of yeast and certain bacteria (↻ Section 4.8) and is formed as part of energy metabolism. Because organisms can grow only if they produce energy, ethanol forms in parallel with growth (Figure 15.1*a*). By contrast, secondary metabolites not coupled directly to growth are some of the

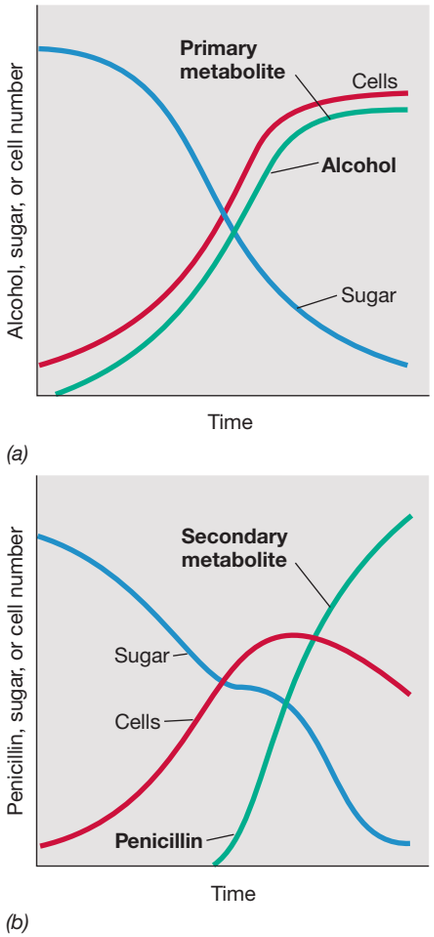


Figure 15.1 Contrast between production of primary and secondary metabolites. *(a)* Formation of alcohol by yeast—an example of a primary metabolite. *(b)* Penicillin production by the mold *Penicillium chrysogenum*—an example of a secondary metabolite. Note that penicillin is not made until after the exponential phase.

most complex and important metabolites of industrial interest (Figure 15.1*b*). Secondary metabolites typically share a number of characteristics. First, they are nonessential for growth and reproduction and their formation is highly dependent on growth conditions. Second, they are often produced as a group of closely related compounds and are often overproduced, sometimes in huge amounts. And finally, many secondary metabolites are the products of spore-forming microorganisms and production is linked to the sporulation process itself. Virtually all antibiotics, for example, are produced by either fungi or spore-forming prokaryotes.

Fermentors and the Characteristics of Large-Scale Fermentations

The vessel in which an industrial microbiology process is carried out is called a **fermentor**. In industrial microbiology, the term **fermentation** refers to *any* large-scale microbial process, whether or not it is, biochemically speaking, a fermentation. The size of fermentors varies from the small 5- to 10-liter laboratory scale to the enormous 500,000-liter industrial scale (**Figure 15.2**). The size of the fermentor used depends on the process and how it is operated. A summary of fermentor sizes for some common microbial fermentations is given in **Table 15.2**.

Table 15.2 *Fermentor sizes for various industrial fermentations*

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

Large-scale industrial fermentors are almost always constructed of stainless steel. Such a fermentor is essentially a large cylinder, closed at the top and bottom, into which various pipes and valves have been fitted (Figure 15.2*b*). Because sterilization of the culture medium and removal of heat are vital for successful operation, the fermentor is fitted with an external cooling jacket through which steam (for sterilization) or water (for cooling) can be run. For very large fermentors, sufficient heat cannot be transferred through the jacket and so internal coils must be provided through which either steam (for sterilization) or cooling water (for growth) can be piped (Figure 15.2).

A critical part of the fermentor is the aeration system. With large-scale equipment, transfer of oxygen throughout the growth medium is critical, and elaborate precautions must be taken to ensure proper aeration. Oxygen is poorly soluble in water, and in a fermentor with a high density of microbial cells, there is a tremendous oxygen demand by the culture. Because of this, two different devices are used to ensure adequate aeration: an aerator, called a *sparger*, and a stirring device, called an *impeller* (Figure 15.2*b*). The sparger is typically just a series of holes through which filter-sterilized air can be passed into the fermentor. The air enters the fermentor as a series of tiny bubbles from which the oxygen passes by diffusion into the liquid. Stirring of the fermentor with an impeller (Figure 15.2*c*) accomplishes two things: It mixes the gas bubbles generated by the sparger and mixes the organisms through the liquid, ensuring that the microbial cells have uniform access to the nutrients.

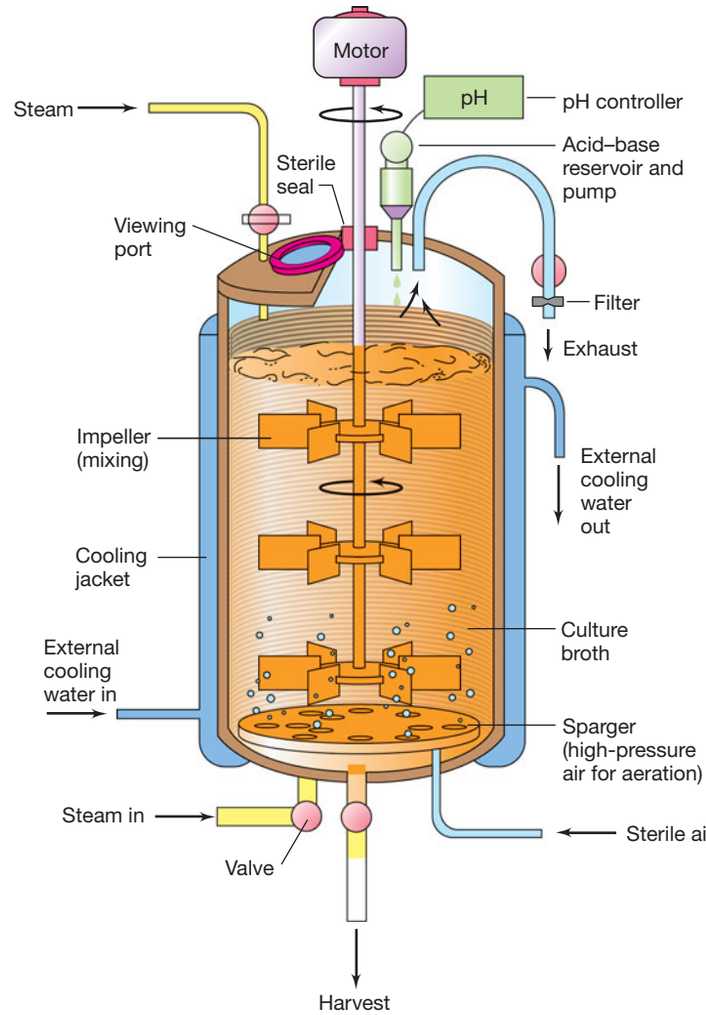
During an actual production run, fermentors are monitored in real time for temperature, oxygen, pH, and the levels of key nutrients, such as ammonia and phosphate. This is done because it is often necessary to alter the conditions in the fermentor as the fermentation progresses. Computers are used to process environmental data as the fermentation proceeds and are programmed to respond by signaling for nutrient additions, increases in the rate of cooling water, impeller speed or sparger pressure, or changes in pH or other parameters, at just the right time to maintain high product yield.

Scale-Up from Laboratory to Commercial Fermentor

An important aspect of industrial microbiology is the transfer of a process from small-scale laboratory equipment to large-scale commercial equipment, a process called **scale-up**. An understanding

(a)

(c)



(b)

Figure 15.2 **Fermentors.** *(a)* A small research fermentor with a volume of 5 liters. *(b)* Diagram of an industrial fermentor, illustrating construction and facilities for aeration and process control. *(c)* The inside of an industrial fermentor, showing the impeller and internal heating and cooling coils.

of scale-up is important because industrial processes rarely behave the same way in large-scale fermentors as in small-scale laboratory equipment (**Figure 15.3**). Many scale-up challenges arise from problems with aeration and mixing. Oxygen transfer is much more difficult to achieve in large fermentors than in small fermentors because the rich culture media used in industrial fermentations support high cell densities, and this leads to high oxygen demand. If oxygen levels become limiting, even for a short period, the culture may reduce—or even shut down—product formation.

In the development of an industrial process, everything begins in the laboratory flask. From here, a promising process is scaled-up to the laboratory fermentor, a small vessel, generally made of glass and 1 to 10 liters in size (Figures 15.2*a* and 15.3*a*). In the laboratory fermentor it is possible to test variations in culture media, temperature, pH, and other parameters, quickly and inexpensively. When these tests are successful, the process is scaled-up to the *pilot plant stage*, usually in fermentors of 300- to 3000-liter capacity. Here the conditions more closely approach those of the actual commercial fermentor, but cost is not yet an issue. Finally, the process moves to the commercial fermentor itself, 10,000–500,000 liters in volume (Table 15.2, and Figure 15.2*b, c*). In all stages of scale-up, aeration is the key variable that is closely monitored; as scale-up proceeds, oxygen dynamics are carefully measured to determine how increases in volume affect oxygen demand in the fermentation.

MiniQuiz

- Is penicillin a primary or a secondary metabolite? How can you tell by looking at Figure 15.1?
- What are the size differences among a laboratory fermentor, a pilot plant fermentor, and a commercial fermentor? How is proper aeration ensured in a large-scale fermentation?
- What parameters in an industrial fermentation are typically monitored and why would adjustments need to be made in real time by automated systems?

(a)

(b)

Figure 15.3 **Research and production fermentors.** *(a)* A bank of small research fermentors used in process development. The fermentors are the glass vessels with the stainless steel tops. The small plastic bottles collect overflow. *(b)* A large bank of outdoor industrial-scale fermentors (each 240 m^3) used in commercial production of alcohol in Japan.

II Drugs, Other Chemicals, and Enzymes

We now consider some products of industrial microbiology, beginning with antibiotics and continuing with amino acids, vitamins, and enzymes. Of the microbial products manufactured commercially, the most important for the health industry are the antibiotics. Antibiotic production is a huge industry worldwide and one in which many important aspects of large-scale microbial culture were perfected.

15.3 Antibiotics: Isolation, Yield, and Purification

Antibiotics are substances produced by microorganisms that kill or inhibit the growth of other microorganisms and are typical secondary metabolites (Section 15.2). Most antibiotics used in human and veterinary medicine are produced by filamentous fungi or bacteria of the *Actinobacteria* group (↻ Section 18.6). **Table 15.3** lists the most important antibiotics produced by large-scale industrial fermentations today.

Isolation of New Antibiotics

Modern drug discovery relies heavily on computer modeling of drug–target interactions (↻ Section 26.13). However, in the past, and to a more limited extent today, laboratory screening programs are the route to discovery of new antibiotics. In this approach, possible antibiotic-producing microorganisms are obtained from nature in pure culture and are then tested for antibiotic production by assaying for diffusible materials that inhibit the growth of test bacteria (**Figure 15.4**). The test bacteria are selected to be either representative of or related to bacterial pathogens against which the antibiotics would actually be used.

Antibiotic production can be assayed by the *cross-streak method* (Figure 15.4*b*). Those isolates that show evidence of antibiotic production are then studied further to determine if the

Table 15.3 *Some antibiotics produced commercially*[a]

Antibiotic	*Producing microorganism*[b]
Bacitracin	*Bacillus licheniformis* (EFB)
Cephalosporin	*Cephalosporium* spp. (F)
Cycloheximide	*Streptomyces griseus* (A)
Cycloserine	*Streptomyces orchidaceus* (A)
Erythromycin	*Streptomyces erythreus* (A)
Griseofulvin	*Penicillium griseofulvum* (F)
Kanamycin	*Streptomyces kanamyceticus* (A)
Lincomycin	*Streptomyces lincolnensis* (A)
Neomycin	*Streptomyces fradiae* (A)
Nystatin	*Streptomyces noursei* (A)
Penicillin	*Penicillium chrysogenum* (F)
Polymyxin B	*Bacillus polymyxa* (EFB)
Streptomycin	*Streptomyces griseus* (A)
Tetracycline	*Streptomyces rimosus* (A)

[a]See Chapter 26 for structures and more discussion of these antibiotics.
[b]EFB, endospore-forming bacterium; F, fungus; A, actinomycete.

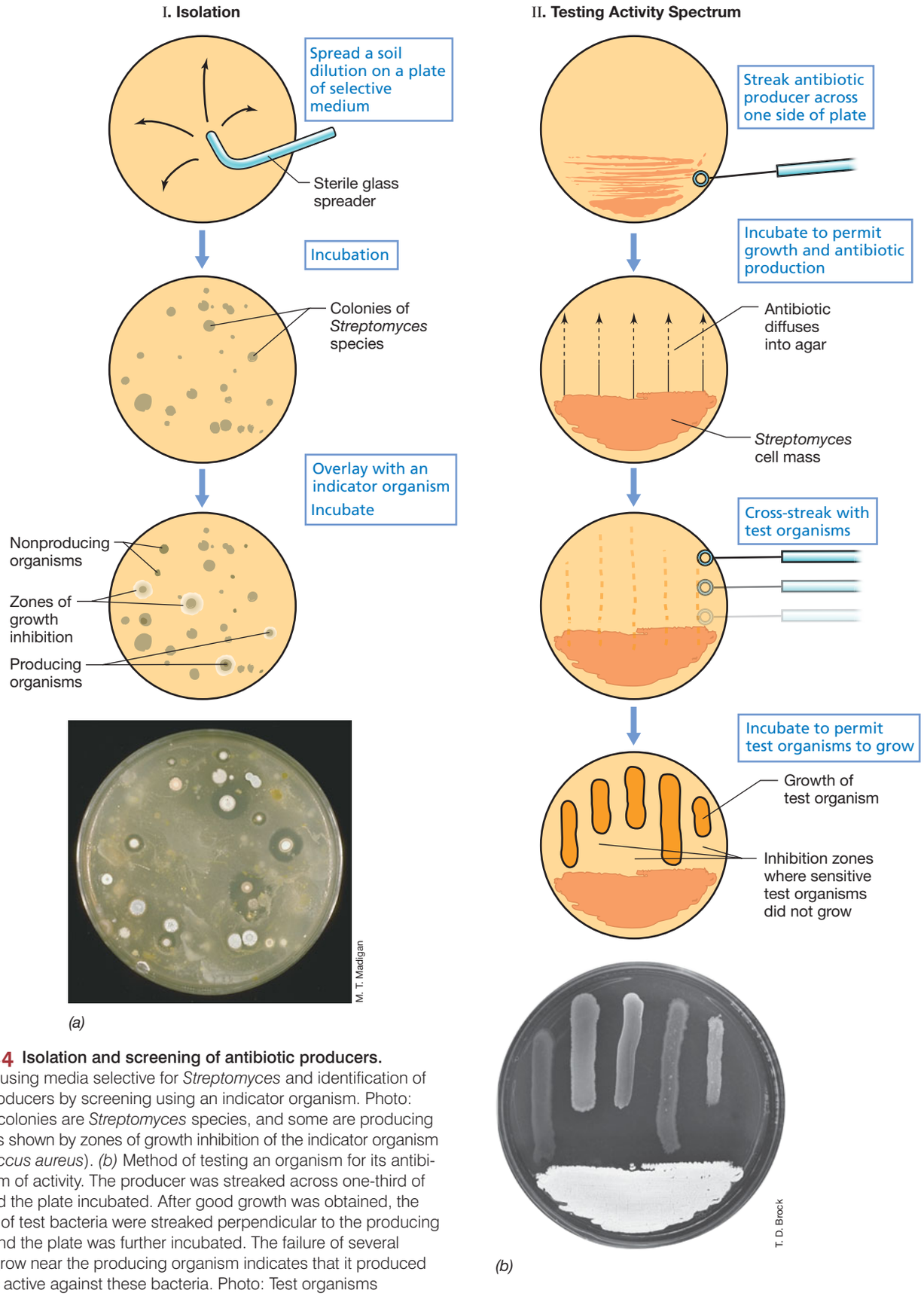


Figure 15.4 **Isolation and screening of antibiotic producers.** *(a)* Isolation using media selective for *Streptomyces* and identification of antibiotic producers by screening using an indicator organism. Photo: Most of the colonies are *Streptomyces* species, and some are producing antibiotics as shown by zones of growth inhibition of the indicator organism (*Staphylococcus aureus*). *(b)* Method of testing an organism for its antibiotic spectrum of activity. The producer was streaked across one-third of the plate and the plate incubated. After good growth was obtained, the five species of test bacteria were streaked perpendicular to the producing organism, and the plate was further incubated. The failure of several species to grow near the producing organism indicates that it produced an antibiotic active against these bacteria. Photo: Test organisms streaked vertically (left to right) include *Escherichia coli, Bacillus subtilis, S. aureus, Klebsiella pneumoniae, Mycobacterium smegmatis*.

compounds they produce are new. Most of the isolates obtained produce known antibiotics, but when a new antibiotic is discovered, it is produced in sufficient amounts for structural analyses and then tested for toxicity and therapeutic activity in animals. Unfortunately, most new antibiotics fail these tests. However, a few prove to be medically useful and go on to be produced commercially. The time and costs in developing a new antibiotic, from discovery to clinical usage, average 15 years and 1 billion ($US). This includes many phases of clinical trials, which alone can take several years to complete, analyze, and submit for United States Food and Drug Administration (FDA) approval.

Yield and Purification

Rarely do antibiotic-producing strains just isolated from nature produce an antibiotic at sufficiently high concentration that commercial production can begin immediately. So one of the major tasks of the industrial microbiologist is to isolate *high-yielding strains*. Strain selection may involve mutagenizing the wild-type organism to obtain mutant derivatives that are so altered that they overproduce the antibiotic of interest. Product yield is a central issue with virtually all pharmaceuticals, and even after commercial production of an antibiotic has begun, research often continues to obtain higher-yielding strains or a more efficient process.

The next challenge is to purify the antibiotic specifically and efficiently, and elaborate methods for extraction and purification of the antibiotic are often necessary. The goal is to eventually obtain a crystalline product of high purity. Depending on the process, further purification steps may be necessary to remove traces of microbial cells or cell products if they co-purify with the antibiotic. These substances, called *pyrogens*, can cause severe or even fatal reactions in patients treated with the drug, and thus the purified product ready to ship must be pyrogen-free.

www.microbiologyplace.com **Online Tutorial 15.1: Isolation and Screening of Antibiotic Producers**

MiniQuiz

- What are the major groups of microorganisms that produce antibiotics?
- What is meant by the word "screening" in the context of finding new antibiotics?

15.4 Industrial Production of Penicillins and Tetracyclines

Once a new antibiotic has been characterized and proven medically effective and nontoxic in tests on experimental animals, it is ready for clinical trials on humans. If the new drug proves clinically effective and passes toxicity and other tests, it is given FDA approval and is ready to be produced commercially. We focus here on the penicillins and tetracyclines, antibiotics that are produced by the ton for medical and veterinary use.

β-Lactam Antibiotics: Penicillin and Its Relatives

The penicillins, a class of **β-lactam antibiotics** characterized by the β-lactam ring (Figure 15.5), are produced by fungi of the genera *Penicillium* and *Aspergillus* and by certain prokaryotes. Commercial penicillin is produced in the United States using high-yielding strains of the mold *Penicillium chrysogenum*. Other important β-lactam antibiotics include the cephalosporins, produced commerically by the mold *Cephalosporium acremonium*. The penicillins and cephalosporins are covered in detail in Section 26.8.

Clinically useful penicillins are of several different types. The parent structure of all penicillins is the compound 6-aminopenicillanic acid (6-APA), which consists of a thiazolidine ring with a condensed β-lactam ring (Figure 15.5). The 6-APA carries a variable side chain in position 6. If the penicillin fermentation is carried out without addition of side-chain precursors, the **natural penicillins**, a group of related compounds, are produced. However, the final product can be specified by adding a side-chain precursor to the culture medium so that only one type of penicillin is produced in greatest amount. The product formed under these conditions is called **biosynthetic penicillin** (Figure 15.5).

To produce the most clinically useful penicillins, those with activity against gram-negative bacteria, researchers combined fermentation and chemical approaches, leading to the production of **semisynthetic penicillins**. To produce semisynthetic penicillins, a natural penicillin is treated to yield 6-APA that is then chemically modified by the addition of a side chain (Figure 15.5). Semisynthetic penicillins have many significant clinical advantages. These include in particular the fact that they are **broad-spectrum antibiotics**, meaning they are useful against a

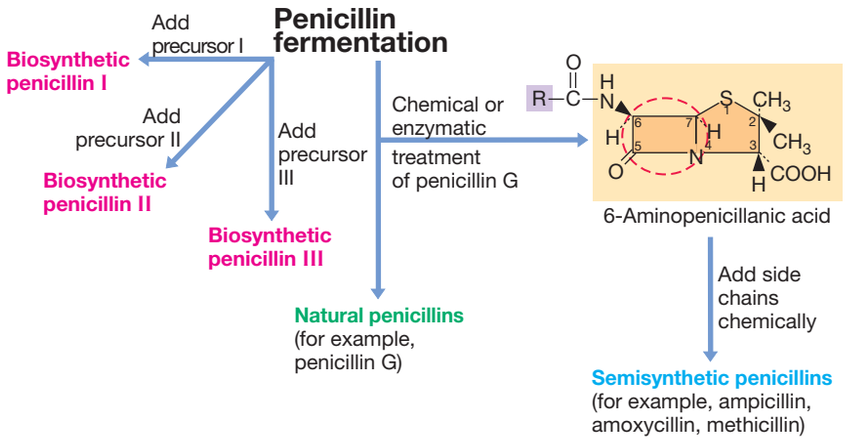


Figure 15.5 **Industrial production of penicillins.** The β-lactam ring is circled in red. The normal fermentation leads to the natural penicillins. If specific precursors are added during the fermentation, various biosynthetic penicillins are formed. Semisynthetic penicillins are produced by chemically adding a specific side chain to the 6-aminopenicillanic acid nucleus on the "R" group shown in purple. Semisynthetic penicillins are the most widely prescribed of all the penicillins today, primarily because of their broad spectrum of activity and ability to be taken orally.

UNIT 5

wide variety of bacterial pathogens, both gram-negative and gram-positive, and that most of them can be taken orally and thus do not require injection. The widely prescribed drug *ampicillin* is a good example of a semisynthetic penicillin. For these reasons, semisynthetic penicillins make up the bulk of the penicillin market today.

Production of Penicillins

Penicillin G is produced in fermentors of 40,000–200,000 liters. Penicillin production is a highly aerobic process, and efficient aeration is critical. Penicillin is a typical secondary metabolite. During the growth phase, very little penicillin is produced, but once the carbon source has been nearly exhausted, the penicillin production phase begins. By supplying additional carbon and nitrogen at just the right times, the production phase can be extended for several days (**Figure 15.6**).

A major ingredient of penicillin production media is corn steep liquor. This substance supplies the fungus with nitrogen and growth factors. High levels of glucose repress penicillin production, but high levels of lactose do not, so lactose (from whey) is added to the corn steep liquor in large amounts as a carbon source. As the lactose becomes limiting and cell densities in the fermentor become very high, "feedings" with low levels of glucose maximize penicillin yield (Figure 15.6). At the end of the production phase, the cells are removed by filtration and the pH is made acidic. The penicillin can then be extracted and concentrated into an organic solvent and, finally, crystallized.

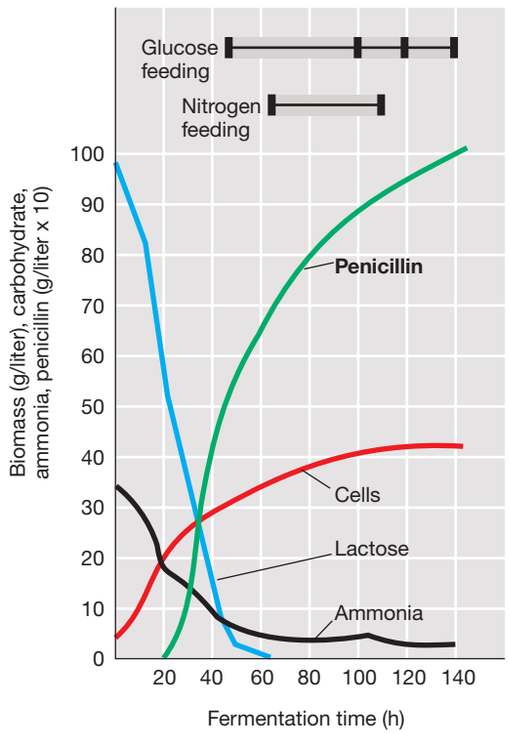


Figure 15.6 Kinetics of the penicillin fermentation with *Penicillium chrysogenum*. Note that penicillin is produced as cells are entering the stationary phase, when most of the carbon and nitrogen has been exhausted. Nutrient "feedings" keep penicillin production high over several days.

Production of Tetracyclines

The biosynthesis of **tetracyclines**, antibiotics containing the four-membered naphthacene ring, requires a large number of enzymatic steps. In the biosynthesis of chlortetracycline (**Figure 15.7**) for example, there are more than 72 intermediates. Genetic studies of *Streptomyces aureofaciens*, the producing organism in the chlortetracycline fermentation, have shown that a total of more than 300 genes are involved! With such a large number of genes, regulation of biosynthesis of this antibiotic is obviously quite complex. However, some key regulatory signals are known and are accounted for in the production scheme.

Chlortetracycline synthesis is repressed by both glucose and phosphate. Phosphate repression is especially significant, and so the medium used in commercial production contains relatively low phosphate concentrations. Figure 15.7 shows a tetracycline production scheme and the various stages in scale-up leading to the commercial fermentor. As in penicillin production, corn steep liquor is used, but sucrose rather than lactose is used as a carbon source. Glucose is avoided because glucose strongly represses antibiotic production through the transcriptional control mechanism known as catabolite repression (↻ Section 8.5).

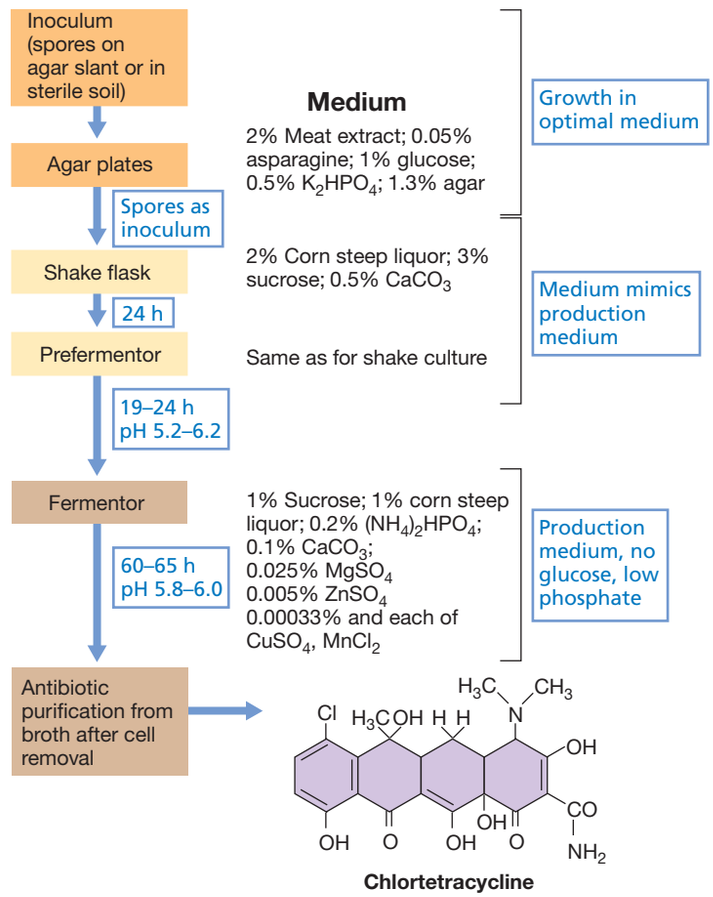


Figure 15.7 Production scheme for chlortetracycline using *Streptomyces aureofaciens*. The structure of chlortetracycline is shown on the bottom right. Glucose is used to grow the inoculum, but not for commercial production.

MiniQuiz

- What chemical structure is common to both the penicillins and the cephalosporins?
- In penicillin production, what is meant by the term semisynthetic? Biosynthetic?
- Why would corn syrup not be useful in the production of tetracyclines?

15.5 Vitamins and Amino Acids

Vitamins and amino acids are nutrients that are used in the pharmaceutical, nutraceutical (nutritional supplements), and food industries. Of these, several are produced on an industrial scale by microorganisms.

Vitamins

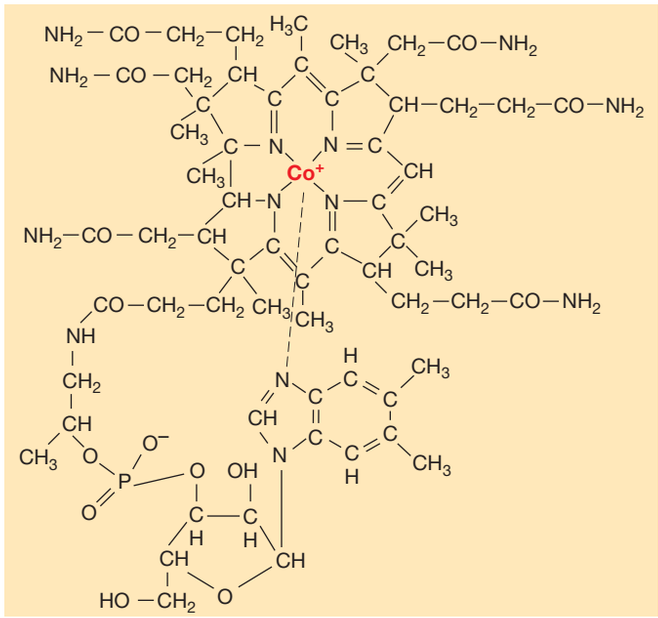
Vitamins are used as supplements for human food and animal feeds, and production of vitamins is second only to that of antibiotics in total sales of pharmaceuticals. Most vitamins are made commercially by chemical synthesis. However, a few are too complicated to be synthesized inexpensively but can be made in sufficient quantities relatively easily by microbial processes. Vitamin B_{12} and riboflavin are the most important of these vitamins. World production of B_{12} is on the order of 10,000 tons per year and of riboflavin about 1,000 tons per year.

Vitamin B_{12} (**Figure 15.8**) is synthesized in nature exclusively by microorganisms but is required as a growth factor by all animals. As a coenzyme, vitamin B_{12} plays an important role in microorganisms and animals in certain methyl transfers and related processes. In humans, a major deficiency of vitamin B_{12} leads to a debilitating condition called *pernicious anemia*, characterized by low production of red blood cells and nervous system disorders. For industrial production of vitamin B_{12}, microbial strains are employed that have been specifically selected for their high yields of the vitamin. Species of the bacteria *Propionibacterium* and *Pseudomonas* are the main commercial producers, especially *Propionibacterium freudenreichii*. The metal cobalt is present in vitamin B_{12} (Figure 15.8*a*), and commercial yields of the vitamin are greatly increased by addition of small amounts of cobalt to the culture medium.

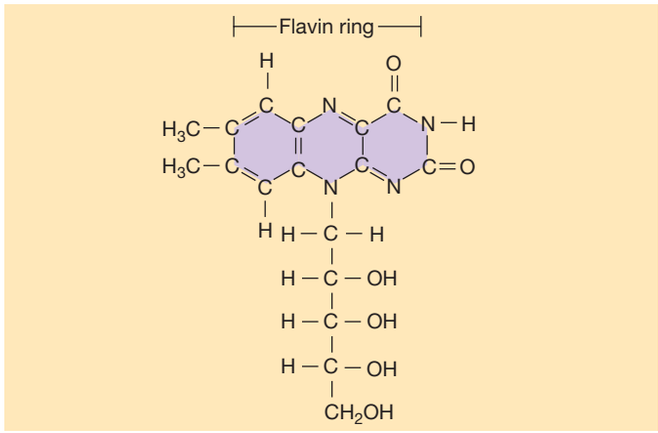
Riboflavin (Figure 15.8*b*) is the parent compound of the flavins FAD and FMN, coenzymes that play important roles in enzymes for oxidation–reduction reactions (↻ Section 4.9). Riboflavin is synthesized by many microorganisms, including bacteria, yeasts, and fungi. The fungus *Ashbya gossypii* naturally produces several grams per liter of riboflavin and is therefore the main organism used in microbial production. However, despite this good yield, there is significant economic competition between the microbiological process and strictly chemical synthesis.

Amino Acids

Amino acids are extensively used in the food and animal husbandry industries as additives, in the nutraceutical industry as nutritional supplements, and as starting materials in the chemical industry (**Table 15.4**). The most important commercial amino acid is glutamic acid, which is used as a flavor enhancer



(a) **B_{12}**



(b) **Riboflavin**

Figure 15.8 **Vitamins produced by microorganisms on an industrial scale.** *(a)* Vitamin B_{12}. Shown is the structure of cobalamin; note the central cobalt atom. The actual coenzyme form of vitamin B_{12} contains a deoxyadenosyl group attached to Co above the plane of the ring. *(b)* Riboflavin (vitamin B_2).

(monosodium glutamate, MSG). Over one million tons of this amino acid are produced annually by the gram-positive bacterium *Corynebacterium glutamicum*. To overproduce, the organism must be starved for the coenzyme biotin (↻ Section 4.1). Biotin is important in the synthesis of fatty acids and thus the cytoplasmic membrane. Starving the organism for biotin weakens the membrane and makes it leaky and susceptible to glutamate excretion.

Two other important microbially produced amino acids, aspartic acid and phenylalanine, are used to synthesize the artificial sweetener **aspartame**, a nonnutritive and noncarbohydrate sweetener of diet soft drinks and other foods sold as low-calorie or sugar-free products. The amino acid lysine is also produced on

Table 15.4 *Amino acids used in the food industry*[a]

Amino acid[b]	*Annual production worldwide (tons)*	*Uses*	*Purpose*
L-Glutamate (monosodium glutamate, MSG)	1,000,000	Various foods	Flavor enhancer; meat tenderizer
L-Aspartate and L-alanine	13,000	Fruit juices	"Round off" taste
Glycine	6,000	Sweetened foods	Improves flavor; starting point for organic syntheses
L-Cysteine	700	Bread	Improves quality
		Fruit juices	Antioxidant
L-Tryptophan + L-Histidine	400	Various foods, dried milk	Antioxidant, prevent rancidity; nutritive additives
Aspartame (made from L-phenylalanine + L-aspartic acid)	7,000	Soft drinks, chewing gum, many other "sugar-free" products	Low-calorie sweetener
L-Lysine	800,000	Bread, cereal, and feed additives	Nutritive additive
DL-Methionine	70,000	Soy products, feed additives	Nutritive additive

[a]Data from Glazer, A. N., and H. Mikaido. 2007. *Microbial Biotechnology*, 2nd edition, W. H. Freeman, New York.
[b]The structures of these amino acids are shown in Figure 6.29.

a large scale (Table 15.4). Lysine is an essential amino acid for humans and domestic animals and is also commercially produced by the bacterium *C. glutamicum* for use as a food additive. In cells, amino acids are used for the biosynthesis of proteins, and thus their production in bacteria is strictly regulated. However, for the overproduction necessary to make amino acids commercially from a microbial source, these regulatory mechanisms must be circumvented.

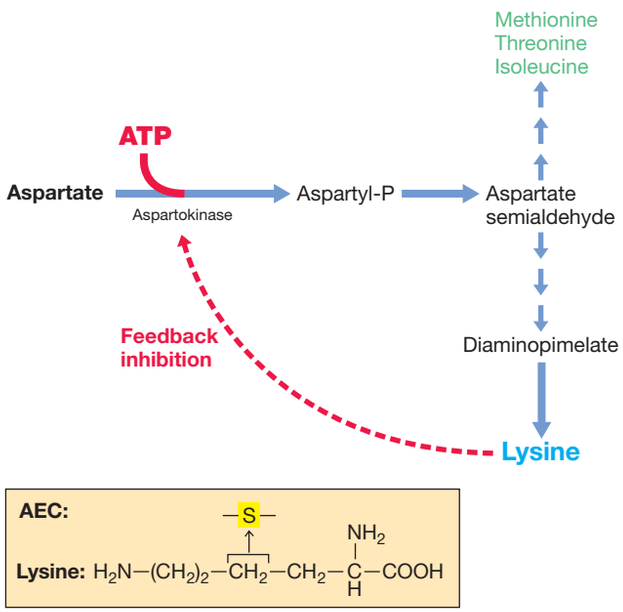


Figure 15.9 Industrial production of lysine using *Corynebacterium glutamicum*. Biochemical pathway leading from aspartate to lysine; note that lysine can feedback-inhibit activity of the enzyme aspartokinase, leading to cessation of lysine production. Shown also is the structure of lysine; the lysine analog *S*-aminoethylcysteine (AEC) is identical to lysine in structure except that a sulfur atom (S) replaces the CH_2 group shown. AEC normally inhibits growth, but AEC-resistant mutants of *C. glutamicum* have an altered allosteric site on their aspartokinase and grow and overproduce lysine because feedback inhibition no longer occurs.

The production of lysine in *C. glutamicum* is controlled at the level of the enzyme aspartokinase; excess lysine feedback inhibits the activity of this enzyme (**Figure 15.9**; the phenomenon of feedback inhibition was described in Section 4.16). However, overproduction of lysine can be obtained by isolating mutants of *C. glutamicum* in which aspartokinase is no longer subject to feedback inhibition; this is done by isolating mutants resistant to the lysine analog *S*-aminoethylcysteine (AEC). AEC binds to the allosteric site of aspartokinase and inhibits activity of the enzyme (Figure 15.9). However, AEC-resistant mutants can be obtained easily and synthesize a modified form of aspartokinase whose allosteric site no longer recognizes AEC or lysine. In such mutants, feedback inhibition of this enzyme by lysine is nearly eliminated. For example, typical AEC-resistant mutants of *C. glutamicum* can produce over 60 g of lysine per liter in industrial fermentors, a concentration sufficiently high to make the process commercially viable. Once produced in the commercial fermentor, the amino acid must be purified and crystallized before it is ready to enter the market.

MiniQuiz

- Which amino acid is commercially produced in the greatest amounts?
- Why is a mutant derivative of the bacterium *Corynebacterium glutamicum* required for commercial lysine production?

15.6 Enzymes as Industrial Products

Microorganisms produce many different enzymes, most of which are made in only small amounts and function within the cell. However, certain microbial enzymes are produced in much larger amounts and are excreted into the environment. These

Table 15.5 *Microbial enzymes and their applications*

Enzyme	*Source*	*Application*	*Industry*
Amylase (starch-digesting)	Fungi	Bread	Baking
	Bacteria	Starch coatings	Paper
	Fungi	Syrup and glucose manufacture	Food
	Bacteria	Cold-swelling laundry starch	Starch
	Fungi	Digestive aid	Pharmaceutical
	Bacteria	Removal of coatings (desizing)	Textile
	Bacteria	Removal of stains; detergents	Laundry
Protease (protein-digesting)	Fungi	Bread	Baking
	Bacteria	Spot removal	Dry cleaning
	Bacteria	Meat tenderizing	Meat
	Bacteria	Wound cleansing	Medicine
	Bacteria	Desizing	Textile
	Bacteria	Household detergent	Laundry
Invertase (sucrose-digesting)	Yeast	Soft-center candies	Candy
Glucose oxidase	Fungi	Glucose removal, oxygen removal	Food
		Test paper for diabetes	Pharmaceutical
Glucose isomerase	*Bacteria*	High-fructose corn syrup	Soft drink
Pectinase	Fungi	Pressing, clarification	Wine, fruit juice
Rennin	Fungi	Coagulation of milk	Cheese
Cellulase	*Bacteria*	Fabric softening, brightening; detergent	Laundry
Lipase	Fungi	Break down fat	Dairy, laundry
Lactase	Fungi	Breaks down lactose to glucose and galactose	Dairy, health foods
DNA polymerase	*Bacteria, Archaea*	DNA replication in polymerase chain reaction (PCR) technique (↻ Section 6.11)	Biological research; forensics

extracellular enzymes, called **exoenzymes**, digest insoluble polymers such as cellulose, protein, lipids, and starch, and because of this, have commercial applications in the food and health industries and in the laundry and textile industries (**Table 15.5**).

Proteases, Amylases, and High-Fructose Syrup

Enzymes are produced industrially from fungi and bacteria. The microbial enzymes produced in the largest amounts on an industrial basis are the bacterial *proteases*, used as additives in laundry detergents. Most laundry detergents contain enzymes, usually proteases, but also amylases and lipases (Table 15.5). These enzymes help remove stains from food, blood, and other organic-rich substances by degrading the polymers into water-soluble components that wash away in the laundry cycle. Many laundry enzymes are isolated from alkaliphilic bacteria, organisms that grow best at alkaline pH (↻ Section 5.15). The main producing organisms are species of *Bacillus*, such as *Bacillus licheniformis*. These enzymes, which have pH optima between 9 and 10, remain active at the alkaline pH of laundry detergent solutions.

Other important enzymes manufactured commercially are amylases and glucoamylases, which are used in the production of glucose from starch. The glucose is then converted by a second enzyme, glucose isomerase, to fructose, which is a much sweeter sugar than glucose. The final product is *high-fructose syrup* produced from glucose-rich starting materials, such as corn, wheat, or potatoes. High-fructose syrups are widely used in the food industry to sweeten soft drinks, juices, and many other products. Worldwide production of high-fructose syrups is over 10 billion kilograms per year.

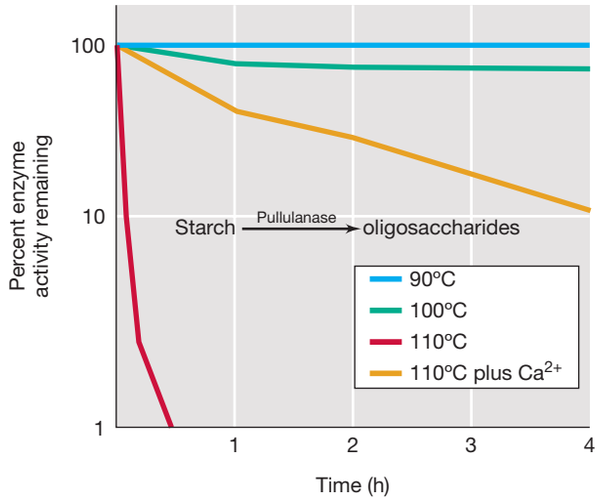
Extremozymes: Enzymes with Unusual Stability

In many chapters in this book we consider prokaryotes able to grow at extremely high temperatures, the *hyperthermophiles*. These remarkable organisms can grow at such high temperatures because they synthesize heat-stable macromolecules, including enzymes. The term **extremozyme** has been coined to describe enzymes that function at some environmental extreme, such as high or low temperature or pH (**Figure 15.10**). The organisms that produce extremozymes are called *extremophiles* (↻ Table 2.1).

Many industrial catalysts operate best at high temperatures, and so extremozymes from hyperthermophiles are widely used in both industry and research. Besides the *Taq* and *Pfu* DNA polymerases used in the polymerase chain reaction for amplifying specific DNA sequences (↻ Section 6.11), thermostable proteases, amylases, cellulases, pullulanases (Figure 15.10*b*), and xylanases have been isolated and characterized from one or another species of hyperthermophile. However, it is not only thermostable enzymes that have found a market. Cold-active

Finnfeeds International

(a)



(b)

Figure 15.10 **Examples of extremozymes, enzymes which function under environmentally extreme conditions.** *(a)* An acid-tolerant enzyme mixture used as a feed supplement for poultry. The enzymes function in the bird's stomach to digest fibrous materials in the feed, thereby improving the nutritional value of the feed and promoting more rapid growth. *(b)* Thermostability of the enzyme pullulanase from *Pyrococcus woesei*, a hyperthermophile whose growth temperature optimum is 100°C. At 110°C the enzyme denatures, but calcium improves the heat stability of this enzyme dramatically.

enzymes (obtained from psychrophiles), enzymes that function at high salinity (obtained from halophiles), and enzymes active at high or low pH (obtained from alkaliphiles and acidophiles, respectively) (Figure 15.10*a*) have been applied commercially.

Immobilized Enzymes

For some industrial processes it is desirable to attach an enzyme to a solid surface to form an **immobilized enzyme**. Immobilization not only makes it easier to carry out the enzymatic reaction

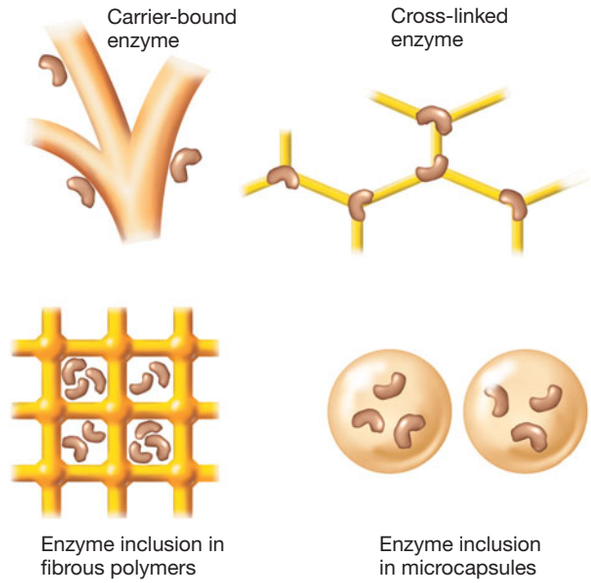


Figure 15.11 **Procedures for the immobilization of enzymes.** The procedure used varies with the enzyme, the product, and the production scale employed.

under large-scale continuous flow conditions, but also helps stabilize the enzyme to retard denaturation. Depending on the enzyme, the immobilized protein can remain active for up to several months.

A good example of the application of immobilized enzymes is in the starch-processing industry, mentioned previously. Starch is converted to high-fructose corn syrup by sequential treatment with amylase and glucose isomerase, but conventional treatment typically converts only about 50% of the glucose into fructose. However, this yield can be increased significantly by removing the fructose on a continuous basis and recycling the remaining glucose over an immobilized enzyme column of glucose isomerase.

Enzymes can be immobilized in three different ways (**Figure 15.11**). Enzymes can be bonded to a carrier made of cellulose, activated carbon, various minerals, or even glass beads through adsorption, ionic bonding, or covalent bonding. Enzyme molecules can also be linked to each other by chemical reaction with a cross-linking reagent such as dilute glutaraldehyde that reacts with amino acids in the enzyme and binds them together without affecting activity. And finally, enzymes can be enclosed in microcapsules, gels, semipermeable polymer membranes, or fibrous polymers such as cellulose acetate. Each of these methods for immobilizing enzymes has advantages and disadvantages, and the procedure used depends on the enzyme, the application, and the scale of the operation.

MiniQuiz

- How are enzymes of use in the laundry industry?
- What enzymes are needed to produce high-fructose corn syrup from starch?
- What is an extremozyme?

Alcoholic Beverages and Biofuels

Alcoholic beverages are a mainstay of human culture and have been produced on a large scale for centuries. Many different alcoholic beverages are known, with some having worldwide appeal and others more regional appeal. But all alcoholic beverages begin with a fermentation step in which some fermentable substance, typically a grain, vegetable, or fruit, is fermented by yeasts or bacteria to yield ethanol and carbon dioxide. The distinctive character of a given alcoholic beverage is the result of many factors, including natural flavors present in the fermentable substrate, chemicals other than alcohol produced during fermentation, and of course, the alcohol itself. We separate our coverage here into three sections, the first dealing with wine, the second with beer and distilled spirits, and the third with commodity alcohol and other biofuels.

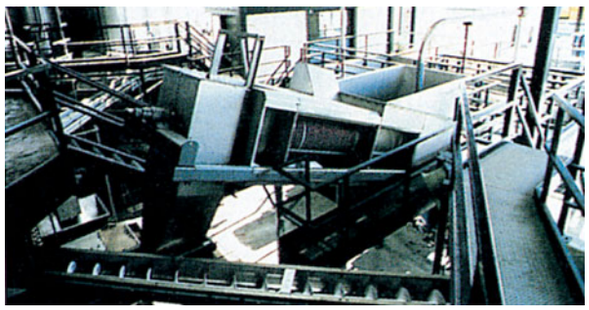
15.7 Wine

Fruit juices undergo a natural fermentation by wild yeasts present in them. From these, particular strains of yeasts have been selected through the years for use in the wine industry. Wine production is a major industry worldwide and one that is growing rapidly with the influx of small specialty wineries, especially in the United States.

Wine Varieties

Most wine is made from grapes, and thus most wine is produced in parts of the world where quality grapes can be grown economically. These include the United States (**Figure 15.12**), New Zealand and Australia, South America, and many countries of the European Union—in particular, France, Spain, Italy, and Germany. Wine can also be made from many other fruits and from some nonfruit sugars, such as honey.

There are a great variety of wines, and their quality and character vary considerably. Dry wines are wines in which the sugars



The Christian Brothers Winery

(a)

The Christian Brothers Winery

(b)

The Christian Brothers Winery

(c)

M.T. Madigan

(d)

Figure 15.12 **Commercial wine making.** *(a)* Equipment for transporting grapes to the winery for crushing. *(b)* Large tanks where the main wine fermentation takes place. *(c)* Large barrels used for aging wine in a large winery. *(d)* Smaller barrels used in a small French winery. Wine may be aged in these wooden casks for years. Red wines are almost always aged to some extent before being marketed, whereas white wines are rarely aged and can suffer in quality when aged significantly.

of the juice are almost completely fermented, whereas in sweet wines some of the sugar is left or additional sugar is added after the fermentation. A fortified wine is one to which brandy or some other alcoholic spirit is added after the fermentation; sherry and port are the best known fortified wines. A sparkling wine, such as champagne, is one in which considerable carbon dioxide (CO_2) is present, arising from a final fermentation by the yeast in the sealed bottle.

Wine Production

Wine production typically begins in the early fall with the harvesting of grapes. The grapes are crushed, and the juice, called *must*, is squeezed out. Depending on the grapes used and on how the must is prepared, either white or red wine may be produced (**Figure 15.13**). Typical varieties of white wine include Chablis, Rhine wine, sauterne, and chardonnay; typical red wines include burgundy, Chianti, claret, zinfandel, cabernet, and merlot. The yeasts that ferment wine are of two types: wild yeasts, which are present on the grapes as they are taken from the field and are transferred to the juice, and strains of the cultivated wine yeast, *Saccharomyces ellipsoideus*, which is added to the juice to begin the fermentation. Wild yeasts are less alcohol-tolerant than commercial wine yeasts and can also produce undesirable compounds affecting quality of the final product. Thus, it is the practice in most wineries to kill the wild yeasts present in the must by adding sodium metabisulfite ($Na_2S_2O_5$, labeled as "sulfites" on the bottle) at a level of 50–100 mg/l. Strains of *S. ellipsoideus* are resistant to this concentration of sulfite and are added to the must as a starter culture from a pure culture grown on sterilized grape juice.

The wine fermentation is carried out in fermentors of various sizes, from 200 to 200,000 liters, made of oak, cement, stone, or glass-lined metal (Figure 15.12). However, no matter what the construction, all fermentors must be designed so that the large amount of CO_2 produced during the fermentation can escape but air cannot enter, and this is accomplished by fitting the vessel with a special one-way valve.

Red and White Wines

A white wine is made either from white grapes or from the juice of red grapes from which the skins, containing the red coloring matter, have been removed. In the making of red wine, the skins, seeds, and pieces of stem, collectively called *pomace*, are left in during the fermentation. In addition to the color difference, red wine has a stronger flavor than white because of larger amounts of *tannins*, chemicals that are extracted into the juice from the grape skins during the fermentation.

In the production of a red wine, after about five days of fermentation, sufficient tannin and color have been extracted from the pomace that the wine can be drawn off for further fermentation in a new tank, usually for 1–2 weeks. The next step is called *racking;* the wine is separated from the sediment, which contains yeast cells and precipitate, and then stored at a lower temperature for aging, flavor development, and clarification. Clarification may be hastened by the addition of fining agents, materials such as casein, tannin, or bentonite clay that absorb particulates. Alternatively, the wine may be filtered through diatomaceous earth, asbestos, or membrane filters. The wine is then bottled and either stored for further aging, or sent to market.

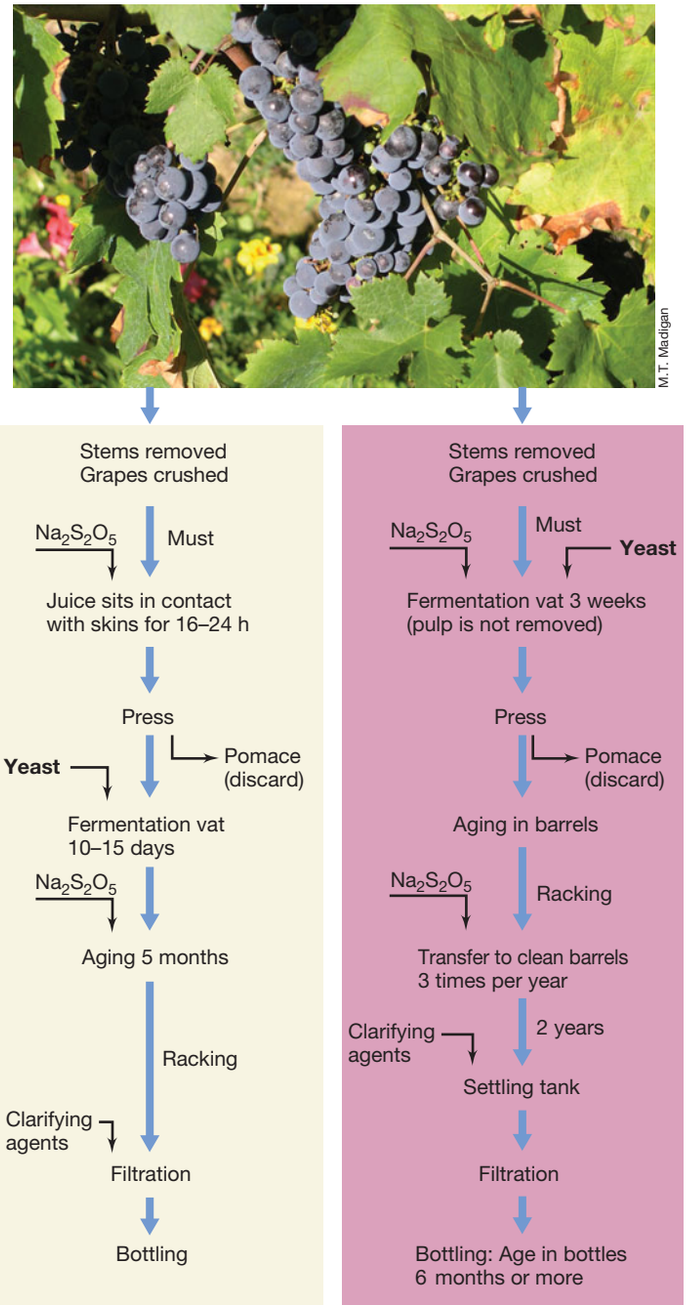


Figure 15.13 **Wine production.** *(a)* White wine. White wines vary from nearly colorless to straw-colored depending on the grapes used. *(b)* Red wine. Red wines vary in color from a faint red to a deep, rich burgundy. The background colors of parts a and b are those of chenin blanc, a typical white wine, and a rosé, a light red wine.

Red wine is typically aged for months to several years (Figure 15.12*c, d*), but most white wine is sold without much aging. During aging, complex chemical changes occur, including the reduction of bitter components; this improves the flavor and odor, or *bouquet*, of the wine. The final alcohol content of wine varies

from about 8% to 16% depending on the sugar content of the grapes, length of the fermentation, and strain of wine yeast used.

Malolactic Fermentation

Many high-quality dry red wines and a few white wines such as the chardonnays are subjected to a secondary fermentation following the primary fermentation by yeast. This is done before bottling and is called the *malolactic fermentation*. Full-bodied dry red wines are the typical candidates for malolactic fermentation.

In grape varieties used for dry wines a considerable amount of malic acid can be present in the grapes. The malic acid content of the grape varies locally due to climatic and soil conditions. Malic acid is a sharp and rather bitter acid. During the malolactic fermentation, malic acid is fermented to lactic acid, a softer, smoother acid, and this makes the wine less acidic and fruity but more complex and palatable. Many other constituents are produced during the malolactic fermentation, including diacetyl (2,3-butanedione), a major flavoring ingredient in butter; this also helps to impart a soft, smooth character to the wine.

The malolactic fermentation is catalyzed by species of lactic acid bacteria (Section 18.1), including *Lactobacillus*, *Pediococcus*, and *Oenococcus*. These organisms are extremely acid-tolerant and can carry out the malolactic fermentation even if the initial pH of the wine is below pH 3.5. Commercial wineries typically use starter cultures of selected malolactic fermentation organisms and then store the wine in barrels especially for this purpose. Inocula for future rounds of malolactic fermentation come from lactobacilli that become attached to the insides of the barrel. The malolactic fermentation can take several weeks but is usually worth the wait, as the final product is often much smoother and far superior to the more sharp-tasting and bitter starting material.

MiniQuiz

- What production differences lead to red wine versus white wine?
- What occurs during the malolactic fermentation, and why is it carried out?

15.8 Brewing and Distilling

Beers and ales are popular alcoholic beverages produced worldwide from the fermentation of grains and other sources of starch. Although, like the wine industry, the brewing industry employs yeast to catalyze the fermentation itself, the amount of alcohol in brewed products is much lower than that in wine, and levels of CO_2 are typically much higher. Thus the two products—beer and wine—are quite different fermented beverages, and each has its own characteristic properties. And, like wine, the final brewed product can be greatly influenced by regional and cultural differences.

Making the Wort

Brewing is the production of alcoholic beverages from malted grains. Typical malt beverages include beer, ale, porter, and stout. *Malt* is prepared from germinated barley seeds, and it contains natural enzymes that digest the starch of grains and convert it to glucose. Because brewing yeasts are unable to digest starch, the malting process is essential for the generation of fermentable substrates.

The fermentable liquid for brewing is prepared by a process called *mashing*. The grain of the mash may consist only of malt, or other grains such as corn, rice, or wheat may be added. The mixture of ingredients in the mash is cooked and allowed to steep in a large mash tub at warm temperatures. During the heating period, enzymes from the malt cause digestion of the starches and liberate glucose, which will be fermented by the yeast. Proteins and amino acids are also liberated into the liquid, as are other nutrient ingredients necessary for the growth of yeast.

After mashing, the aqueous mixture, called *wort*, is separated by filtration. *Hops*, an herb derived from the female flowers of the hops plant, are added to the wort at this stage. Hops add flavors to the wort but also have antimicrobial properties, which help to prevent bacterial contamination in the subsequent fermentation. The wort is boiled for several hours, usually in large copper kettles (**Figure 15.14*a*,*b***), during which time desired ingredients are extracted from the hops, undesirable proteins present in the wort are coagulated and removed, and the wort is sterilized. The wort is filtered again, cooled, and transferred to the fermentation vessel.

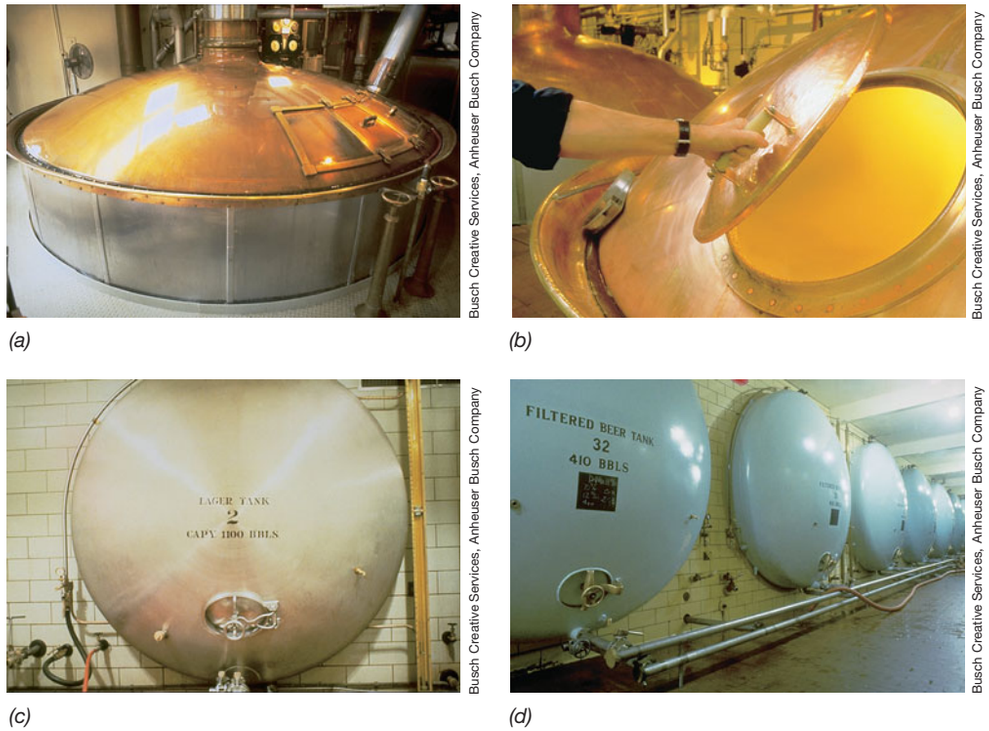
The Fermentation Process

Brewery yeast strains are of two major types: *top* fermenting and *bottom* fermenting. The main distinction between the two is that top-fermenting yeasts remain uniformly distributed in the fermenting wort and are carried to the top by the CO_2 gas generated during the fermentation, whereas bottom-fermenting yeasts settle to the bottom. Top yeasts are used in the brewing of *ales*, and bottom yeasts are used to make *lager beers*. Bottom yeasts have been given the species designation *Saccharomyces carlsbergensis*, whereas top yeasts are considered *Saccharomyces cerevisiae*. Top yeasts usually ferment at higher temperatures (14–23°C) than bottom yeasts (6–12°C) and thus complete the fermentation in a shorter time (5–7 days for top fermentation versus 8–14 days for bottom fermentation).

After bottom yeasts complete lager beer fermentation, the beer is pumped off into large tanks where it is stored in the cold (about −1°C) for several weeks (Figure 15.14*c*). Following this, the beer is filtered and placed in storage tanks (Figure 15.14*d*) from which it is packaged and sent to market. Top-fermented ale is stored for only short periods at a higher temperature (4–8°C), which assists in development of the characteristic ale flavor.

Home Brew

Amateur and small-scale commercial brewing has become popular in recent years, especially in the United States. Many styles of beer from English bitters and India pale ale to German bock and Russian Imperial stout can be made at home, and the character of a particular brew depends on many factors including the amounts and types of malt, sugar, hops, and grain used, the strain of yeast employed, the temperature and duration of the fermentation, and how the beer is aged. For home brewing only simple equipment is necessary such as a stainless steel container to prepare the wort, a 20-liter (5-gallon) fermentor fitted with a valve to



(a) (b) (c) (d)

Figure 15.14 **Brewing beer in a large commercial brewery.** *(a, b)* The copper brew kettle is where the wort is mixed with hops and then boiled. From the brew kettle, the liquid passes to large fermentation tanks where yeast ferments glucose to ethanol plus CO_2. *(c)* If the beer is a lager, it is stored for several weeks at low temperature in tanks where particulate matter, including yeast cells, settles. *(d)* The beer is then filtered and placed in storage tanks from which it is packaged into kegs, bottles, or cans.

allow CO_2 to escape, and glass bottles to store the final product (**Figure 15.15**).

Home brewing is much the same as commercial brewing except that hop-flavored malt extract is often used directly as the wort instead of preparing the wort in the traditional way. Using the same basic equipment, various beers can be made, each with its own distinctive taste and character. Dark beers, which typically contain more alcohol than lighter beers, require more malt for their production and are usually brewed from a combination of different malts such as those obtained from darker varieties of grain or those that have been roasted to caramelize the sugars and yield a darker color. Most beers contain 3–6% alcohol. A typical American-style lager (Figure 15.15*d*) contains about 3.5% alcohol (by volume), a Munich-style dark about 4.5%, and bock beers about 5%. Some specialty beers and ales can contain upwards of 12% alcohol.

(a) (b) (c) (d)

Figure 15.15 **Home brewing.** (*a*) A stainless steel pot to boil the wort. (*b*) The fermentation vessel. (*c*) Bottling and capping the beer. (*d*) Glasses of two common beers, a lager (pils) (left) and a common dark beer (bock) (right). The vessel is fitted with a fermentation lock that maintains anoxic conditions but allows CO_2 to escape.

The trend toward individuality in beer is evident not only by the growing number of home brewers, but also by the fact that many microbreweries are appearing. Total production by microbreweries in the United States is less than that of a major brewer, but the products often have their own distinctive character and local appeal. The particular properties of a given microbrew can be traced to the smaller scale on which the brewing is done, the unique sources of ingredients, water, and yeast strains employed, and to differences in times and temperatures used in the brewing process.

Distilled Alcoholic Beverages

Distilled alcoholic beverages are *distillates*, the products of heating fermented liquids to volatilize alcohol and other constituents. The distillate is condensed and collected, a process called *distilling*. A product much higher in alcohol content is obtained by distilling than is possible by fermentation alone. Virtually any alcoholic liquid can be distilled, and each yields a characteristic distilled beverage. The distillation of malt brews yields *whiskey*, distilled wine yields *brandy*, distilled fermented molasses yields *rum*, distilled fermented grain or potatoes yields *vodka*, and distilled fermented grain and juniper berries yields *gin* (**Figure 15.16**). Alcohol concentrations in distilled products vary from as little as 20% to as high as 95%. The "proof" rating, used primarily for labeling distilled spirits in the United States, is defined as twice the alcohol concentration. Thus a whiskey that is 80 proof contains 40% ethanol by volume.

The distillate contains not only alcohol but also other volatile products arising either from the yeast fermentation or released from the ingredients. Some of these other products add desirable flavor, whereas others are undesirable. To eliminate the latter, the distilled product is typically aged, usually in oak barrels. The aging removes undesirable products and allows desirable new flavors and aromatic ingredients to develop. The fresh distillate is typically colorless, whereas the aged product is often brown or yellow (Figure 15.16). The character of the final product is partly determined by the manner and length of aging; aging times of 5–10 years are common, but some very expensive distilled spirits are aged for 20 years or more.

MiniQuiz

- In brewing, why is the mashing process necessary?
- What are the major differences between a beer and an ale?
- How does whiskey differ from brandy?

15.9 Biofuels

Production of ethyl alcohol (ethanol) as a commodity chemical is a major industrial process, and today over 60 billion liters of ethanol are produced yearly worldwide from the fermentation of various feedstocks. In the United States most ethanol is obtained by yeast fermentation of glucose obtained from cornstarch. Ethanol is stripped from the fermentation broth by distillation (**Figure 15.17*a***). Various yeasts have been used in commodity ethanol production, including species of *Saccharomyces, Kluyveromyces*, and *Candida*, but most ethanol in the United States is produced by *Saccharomyces*.


Barton Spear

Figure 15.16 Distilled spirits. Aging in oak casks imparts a distinctive amber or yellow color to distilled spirits. Left to right, dark rum, brandy, whiskey. Gin and vodka (not shown) are not aged in oak and are colorless.

Ethanol as a Biofuel

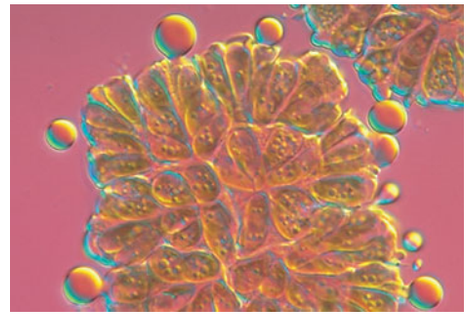
Ethanol is currently the most important global **biofuel**, a term indicating that the fuel was made from the fermentation of recently grown plant material rather than being of ancient origin (that is, fossil fuel). Other major biofuels include biodiesel, made from vegetable oils, and algal fuels, alcohols and oils produced from green algae. The feedstock used for ethanol production has been a major issue in the debate over whether biofuels are the wave of the future. In the United States, for example, the increased demand for corn as a biofuel feedstock has driven up the price of human foods and livestock feeds. In other countries, for example Brazil, which is a major ethanol producer, not only corn but also sugar cane, whey, sugar beets, and even wood chips and waste paper are used as feedstocks for the fermentation. For cellulosic materials, the cellulose must first be treated to release glucose, which is then fermented to alcohol. Alternative feedstocks showing great promise for ethanol production are grasses such as switchgrass (Figure 15.17*b*), a rapidly growing and easily harvestable grass whose cellulosic cell walls can be degraded to glucose and fermented to ethanol.

In the United States *gasohol* is produced by adding ethanol to gasoline; at a final concentration of 10% ethanol, it can be used in virtually all gasoline engines. The combustion of gasohol produces lower amounts of carbon monoxide and nitrogen oxides than pure gasoline, and hence gasohol is a cleaner-burning fuel. The production of more ethanol-rich fuels such as E-85 (85% ethanol and 15% gasoline) is also growing in the United States, but this fuel can only be used in modified engines. However, E-85 fuel reduces emission of nitrogen oxides by nearly 90% and is thus a means for reducing important pollutants in the atmosphere and reducing dependence on conventional sources of oil. Many major cities concerned about air pollution are retrofitting their public transportation systems, especially buses, to burn E-85.

Total ethanol production to meet fuel demands in the United States is scheduled to top 30 billion liters by 2012. The major downside to ethanol production is that at present it takes about 25% more energy to produce a liter of ethanol than is present in the ethanol itself. However, because bioethanol is a product of recently fixed carbon rather than of buried fossil fuel, its use is considered a more sustainable and environmentally friendly way to supply the liquid fuel needs of the foreseeable future.

(a)

(b)



(c)

Figure 15.17 **Biofuels.** (*a*) A bioethanol production plant in Nebraska (USA). In the plant, glucose obtained from cornstarch is fermented by *Saccharomyces cerevisiae* to ethanol plus CO_2. The large tank in the left foreground is the ethanol storage tank, and the tanks and pipes in the background are for distilling ethanol from the fermentation broth. (*b*) Switchgrass, a promising feedstock for bioethanol production. The cellulose from this rapidly growing plant can be treated to yield glucose that can then be fermented to ethanol or butanol. *(c)* The petroleum-producing colonial green alga, *Botryococcus braunii*. Note the excreted oil droplets that appear as bubbles along the margin of the cells.

Petroleum Biofuels

In addition to bioethanol production, green energy initiatives have spurred research on many other biofuels. This includes the production of longer-chain alcohols, such as butanol, from fermentative processes, but also the direct synthesis of petroleum by green algae. For example, during growth the colonial green alga *Botryococcus braunii* excretes long-chain (C_{30}–C_{36}) hydrocarbons that have the consistency of crude oil (Figure 15.17*c*). In *B. braunii* about 30% of the cell dry weight consists of petroleum, and there has been heightened interest in using this and other oil-producing algae as renewable sources of petroleum. There is even evidence from biomarker studies that some known petroleum reserves originated from green algae that grew in lakebeds in ancient times rather than having been formed from the microbial degradation of plant materials. Although it is a promising source of oil from a "green" perspective, the major problem with algal petroleum is scale: In a world that currently uses about 90 million barrels of oil per day, the logistics of growing oil-producing algae that could contribute significantly to global oil demand are daunting.

MiniQuiz

- How can yeast help to solve global energy problems?
- What is the difference between gasohol and E-85?

IV Products from Genetically Engineered Microorganisms

We now consider some products of the biotechnology industry, products synthesized by genetically engineered bacteria or other organisms. Compared with most of the products of industrial microbiology just considered, biotech drugs are inherently more valuable but are produced on a much smaller scale.

Before the era of biotechnology, diabetics relied on insulin extracted from animals to control their blood sugar levels. In most cases this worked well, but a small percentage of diabetics showed immune reactions to the foreign (porcine or bovine) version of insulin. Genetic engineering allowed genuine human insulin to be produced by bacteria. Indeed, human insulin was the first commercialized product from genetic engineering. Today several genetically engineered hormones and other human proteins are available for clinical use. These human proteins were originally produced by cloning human genes, inserting them into bacteria (typically *Escherichia coli*), and having the bacteria make the protein. However, several problems were encountered in this approach. Foreign proteins made in bacteria must be isolated by disrupting the bacterial cells and then purified. Traces of bacterial proteins that contaminate the desired protein may elicit an unwanted immune response. Moreover, traces of lipopolysaccharide from the gram-negative bacterial outer membrane are toxic (endotoxin, Section 27.12).

Other major problems revolved around the challenge of expressing eukaryotic genes in bacteria. These include the facts that (1) the genes must be placed under control of a bacterial promoter (Section 11.8); (2) the introns (Section 7.8) must be removed; (3) codon bias (Section 6.17) affects the efficiency of translation; and (4) many mammalian proteins are modified after translation, and bacteria lack the ability to perform most such modifications. As a result, recent efforts in biotechnology have been to express mammalian proteins using genetically modified eukaryotic host cells. Both eukaryotic cells in culture and whole transgenic animals have been used. For example, transgenic goats have been used that secrete the protein of interest in their milk. Most recently, plants are being engineered to express mammalian proteins. We consider some major topics in biotechnology in more detail now.

15.10 Expressing Mammalian Genes in Bacteria

The procedures for cloning and manipulating genes were covered in Chapter 11. Here we are concerned with expressing cloned genes to manufacture a useful product. Expression vectors (Section 11.8) are needed to express eukaryotic genes in bacteria. However, there are still obstacles to be faced, even if the mammalian gene has been cloned into an expression vector. One major issue is the presence of introns that disrupt the coding sequence of many eukaryotic genes, especially in higher organisms such as mammals (Section 7.8). Introns must be spliced out for the genes to function; however, prokaryotic hosts lack the machinery to do so. To skirt this problem, introns are removed during the cloning process, before the genes are inserted into the host used for production. Typically, cloned mammalian genes no longer contain their original introns but consist of an uninterrupted coding sequence. The two major ways of achieving this are now described.

Cloning the Gene via mRNA

The standard way to obtain an intron-free eukaryotic gene is to clone it via its messenger RNA (mRNA). Because introns are removed during the processing of mRNA, the mature mRNA carries an uninterrupted coding sequence. Tissues expressing the gene of interest often contain large amounts of the corresponding mRNA, although other mRNAs are also present. In certain situations, however, a single mRNA dominates in a tissue type, and extraction of bulk mRNA from that tissue provides a useful starting point for gene cloning.

In a typical mammalian cell, about 80–85% of the RNA is ribosomal RNA, 10–15% is transfer RNA, and only 1–5% is mRNA. However, eukaryotic mRNA is unique because of the poly(A) tails found at the 3′ end (Section 7.8), and this makes it easy to isolate, even though it is scarce. If a cell extract is passed over a chromatographic column containing strands of poly(T) linked to a cellulose support, most of the mRNA separates from other RNAs by the specific pairing of A and T bases. The RNA is released from the column by a low-salt buffer, which gives a preparation greatly enriched in mRNA.

Once mRNA has been isolated, the genetic information is converted into complementary DNA (cDNA). This is done by the enzyme reverse transcriptase. This enzyme, essential for retroviral replication (Section 21.11), copies information from RNA into DNA, a process called **reverse transcription** (Figure 15.18). Reverse transcriptase needs a primer to start DNA synthesis. When making DNA using mRNA as a template, a primer is used that is complementary to the poly(A) tail of the mRNA. This primer is hybridized to the mRNA, and reverse transcriptase is added.

Reverse transcriptase makes DNA that is complementary to the mRNA. As seen in Figure 15.18, the newly synthesized cDNA has a hairpin loop at its end. The loop forms because, after the enzyme completes copying the mRNA, it starts to copy the newly made DNA. This hairpin loop provides a convenient primer for synthesis of the second (complementary) strand of DNA by DNA polymerase I and is later removed by a single-strand-specific nuclease. The result is a linear, double-stranded DNA molecule, one strand of which is complementary to the original mRNA (Figure 15.18).

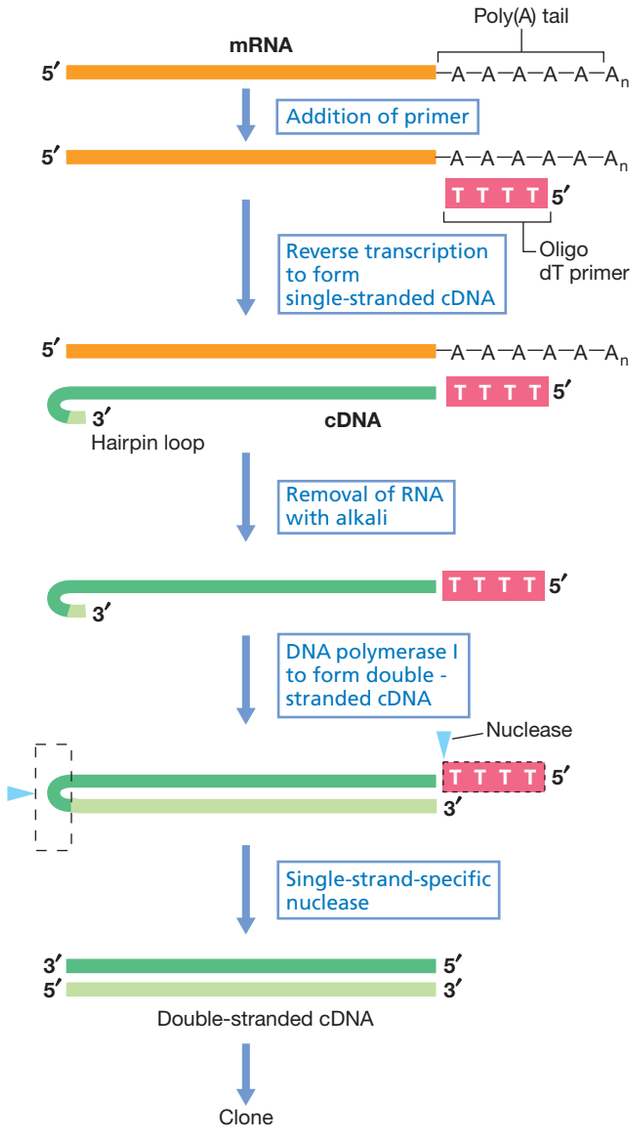


Figure 15.18 Complementary DNA (cDNA). Steps in the synthesis of cDNA from an isolated mRNA using the retroviral enzyme reverse transcriptase. The poly(A) tail is typical of eukaryotic mRNA.

This double-stranded cDNA contains the coding sequence but lacks introns. It can be inserted into a plasmid or other vector for cloning. However, because the cDNA corresponds to the mRNA, it lacks a promoter and other upstream regulatory sequences that are not transcribed into RNA. Special expression vectors with bacterial promoters and ribosome-binding sites are used to obtain high-level expression of genes cloned in this way (Section 11.8).

A cDNA library is a gene library (Section 11.3) consisting of cDNA versions of genes made from mRNA extracted from a eukaryotic cell. The library reflects only those genes expressed in the particular tissue under the existing conditions.

Finding the Gene via the Protein

Knowing the sequence of a gene allows the synthesis of a cDNA molecule to use as a probe. This can be used to find the gene by screening a gene library (↻ Section 11.3). Knowledge of the amino acid sequence of a protein can also be used to construct a probe or even to synthesize a whole gene.

The amino acid sequence of a protein can be used to design and synthesize an oligonucleotide probe that encodes it. This process is illustrated in **Figure 15.19**. Unfortunately, degeneracy of the genetic code complicates this approach. Most amino acids are encoded by more than one codon (↻ Table 6.5), and codon usage varies from organism to organism. Thus, the best region of a gene to synthesize as a probe is one that encodes part of the protein rich in amino acids specified by only a single codon (for example, methionine, AUG; tryptophan, UGG) or at most two codons (for example, phenylalanine, UUU, UUC; tyrosine, UAU, UAC; histidine, CAU, CAC). This strategy increases the chances that the probe will be nearly complementary to the mRNA or gene of interest. If the complete amino acid sequence of the protein is not known, partial sequence data may be used.

For certain small proteins there may be good reason to synthesize the entire gene. Many mammalian proteins (including high-value peptide hormones) are made by protease cleavage of larger precursors. Thus, to produce a short peptide hormone such as insulin, it may be more efficient to construct an artificial gene that encodes just the final hormone rather than the larger precursor protein from which it is derived naturally. Chemical synthesis also allows synthesis of modified genes that may make useful new proteins. Artificial synthesis of DNA is now routine, and it is possible to synthesize genes encoding proteins over 200 amino acid residues in length (600 nucleotides). The synthetic approach was first used in a major way for production of the human hormone insulin in bacteria. Moreover, constructed genes are free of introns and thus the mRNA does not need processing. Also, promoters and other regulatory sequences can easily be built into the gene upstream of the coding sequences, and codon bias (↻ Section 6.17) can be accounted for.

With these techniques many human and viral proteins have been expressed at high yield under the control of bacterial regulatory systems. These include insulin, somatostatin, viral capsid proteins, and interferon.

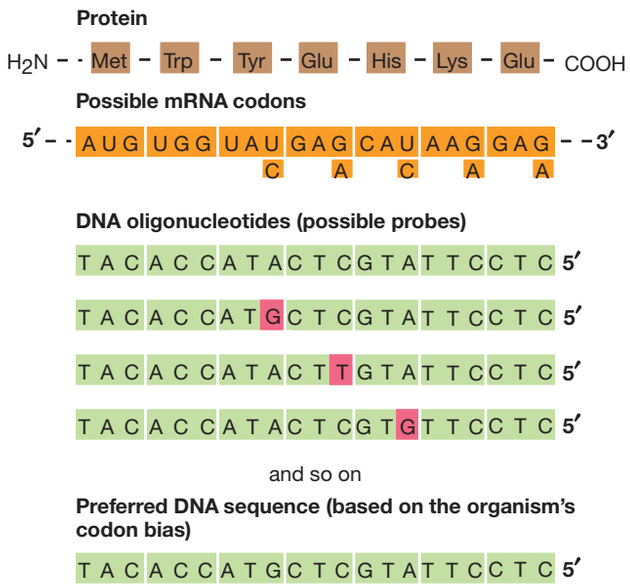


Figure 15.19 Deducing the best sequence of an oligonucleotide probe from the amino acid sequence of a protein. Because many amino acids are encoded by multiple codons, many nucleic acid probes are possible for a given polypeptide sequence. If codon usage by the target organism is known, a preferred sequence can be selected. Complete accuracy is not essential because a small amount of mismatch can be tolerated, especially with long probes.

Protein Folding and Stability

The synthesis of a protein in a new host may bring additional problems. For example, some proteins are susceptible to degradation by intracellular proteases and may be destroyed before they can be isolated. Moreover, some eukaryotic proteins are toxic to prokaryotic hosts, and the host cell may be killed before a sufficient amount of the product is synthesized. Further engineering of either the host or the vector may eliminate these problems.

Sometimes when foreign proteins are massively overproduced, they form inclusion bodies inside the host. Inclusion bodies consist of aggregated insoluble protein that is often misfolded or partly denatured, and they are often toxic to the host cell. Although inclusion bodies are relatively easy to purify because of their size, the protein they contain is often difficult to solubilize and may be inactive. One possible solution to this problem is to use a host that overproduces molecular chaperones that aid in folding (↻ Section 6.21).

Fusion Proteins for Improved Purification

Protein purification can often be made much simpler if the target protein is made as a **fusion protein** along with a carrier protein encoded by the vector. To do this, the two genes are fused to yield a single coding sequence. A short segment that is recognized and cleaved by a commercially available protease is included between them. After transcription and translation, a single protein is made. This is purified by methods designed for the carrier protein. The fusion protein is then cleaved by the protease to release the target protein from the carrier protein. Fusion proteins simplify purification of the target protein because the carrier protein can be chosen to have ideal properties for purification.

Several fusion vectors are available to generate fusion proteins. **Figure 15.20** shows an example of a fusion vector that is also an expression vector. In this example, the carrier protein is the *Escherichia coli* maltose-binding protein, and the fusion protein is easily purified by methods based on its affinity for maltose. Once purified, the two portions of the fusion protein are separated by a specific protease (factor Xa, a protease whose natural role is in blood clotting). In some cases the target protein is released from the carrier protein by specific chemical treatment, rather than by protease cleavage.

Fusion systems are also used for other purposes. One advantage of making a fusion protein is that the carrier protein can be chosen to contain the bacterial *signal sequence*, a peptide rich in hydrophobic amino acids that enables transport of the protein

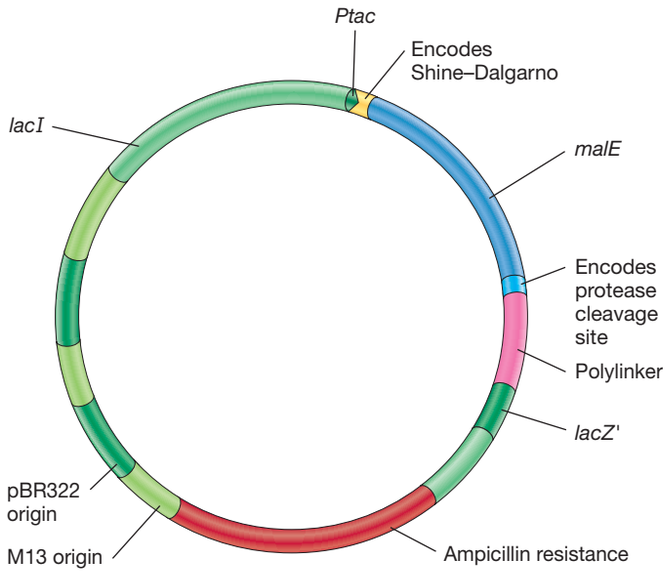


Figure 15.20 **An expression vector for fusions.** The gene to be cloned is inserted into the polylinker so it is in frame with the *malE* gene, which encodes maltose-binding protein. The insertion inactivates the gene for the alpha fragment of *lacZ*, which encodes β-galactosidase. The fused gene is under control of the hybrid *tac* promoter (*Ptac*). The plasmid also contains the *lacI* gene, which encodes the *lac* repressor. Therefore, an inducer must be added to turn on the *tac* promoter. The plasmid contains a gene conferring ampicillin resistance on its host. In addition to the plasmid origin of replication, there is a bacteriophage M13 origin. Thus, this vector is a phagemid and can be propagated either as a plasmid or as a phage. This vector was developed by New England Biolabs (Ipswich, MA).

across the cytoplasmic membrane (↻ Section 6.21). This makes possible a bacterial expression system that not only makes mammalian proteins, but also secretes them. When the right strains and vectors are employed, the desired protein can make up as much as 40% of the protein molecules in a cell.

MiniQuiz

- What major advantage does cloning mammalian genes from mRNA or using synthetic genes have over PCR amplification and cloning of the native gene?
- How is a fusion protein made?

15.11 Production of Genetically Engineered Somatotropin

One of the most economically profitable areas of biotechnology today is the production of human proteins. Many mammalian proteins have high pharmaceutical value but are typically present in very low amounts in normal tissue, and it is therefore extremely costly to purify them. Even if the protein can be produced in cell culture, this is much more expensive and difficult than growing microbial cultures that produce the protein in high yield. Therefore, the biotechnology industry has genetically engineered microorganisms to produce many different mammalian proteins. Although insulin was the first human protein to be produced in this manner, the procedure had several unusual complications, because insulin consists of two short polypeptides held together by disulfide bonds. A more typical example is somatotropin, and we focus on this here.

Growth hormone, or *somatotropin*, consists of a single polypeptide encoded by a single gene. Somatotropin from one mammalian species usually functions reasonably well in other species; indeed, transgenic animals have been made expressing foreign somatotropin genes, as discussed below. Lack of somatotropin results in hereditary dwarfism. Because the human somatotropin gene was successfully cloned and expressed in bacteria, children showing stunted growth can be treated with recombinant human somatotropin. However, dwarfism may also be caused by lack of the somatotropin receptor, and in this case administration of somatotropin has no effect. (People of the African Pygmy tribes have normal levels of human somatotropin, but most of them are no taller than 4 feet, 10 inches because they have defective growth hormone receptors.)

The somatotropin gene was cloned as complementary DNA (cDNA) from mRNA as described in Section 15.10 (**Figure 15.21**).

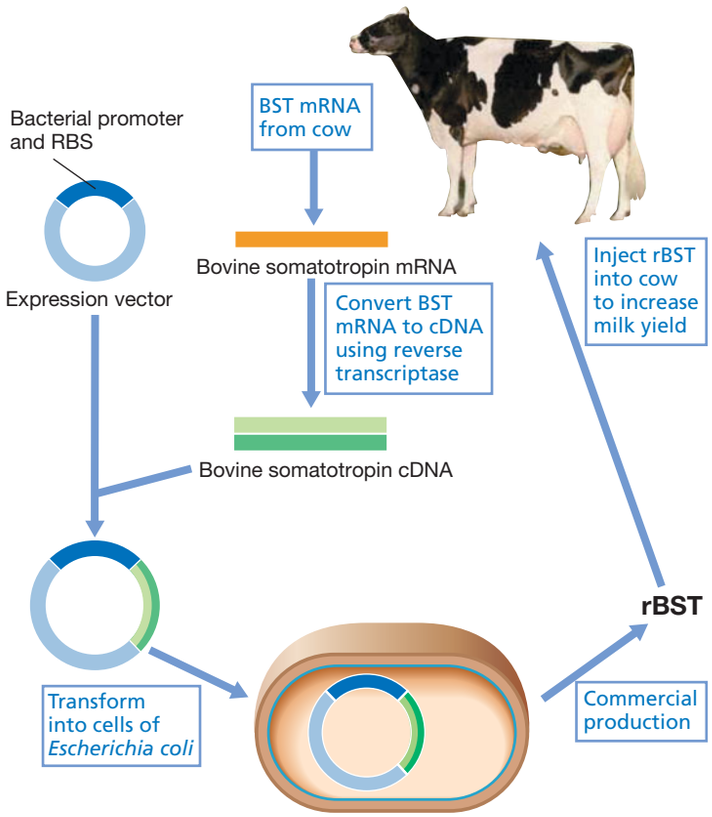


Figure 15.21 **Cloning and expression of bovine somatotropin.** The mRNA for bovine somatotropin (BST) is obtained from an animal. The mRNA is converted to cDNA by reverse transcriptase. The cDNA version of the somatotropin gene is then cloned into a bacterial expression vector that has a bacterial promoter and ribosome-binding site (RBS). The construct is transformed into cells of *Escherichia coli*, and recombinant bovine somatotropin (rBST) is produced. Milk production increases in cows treated with rBST.

The cDNA was then expressed in a bacterial expression vector. The main problem with producing relatively short polypeptide hormones such as somatotropin is their susceptibility to protease digestion. This problem can be countered by using bacterial host strains defective for several proteases.

Recombinant bovine somatotropin (rBST) is used in the dairy industry (Figure 15.21). Injection of rBST into cows does not make them grow larger but instead stimulates milk production. The reason for this is that somatotropin has two binding sites. One binds to the somatotropin receptor and stimulates growth, the other to the prolactin receptor and promotes milk production. However, excessive milk production by cows causes some health problems in the animals, including an increased frequency of infections of the udder and decreased reproductive capability.

When somatotropin is used to remedy human growth defects, it is desirable to avoid side effects from the hormone's prolactin activity (prolactin stimulates lactation). Site-directed mutagenesis (↻ Section 11.4) of the somatotropin gene was used to generate somatotropin that no longer binds the prolactin receptor. To accomplish this, several amino acids needed for binding to the prolactin receptor were altered by changing their coding sequences. Thus it is possible not merely to make genuine human hormones, but also to alter their specificity and activity to make them better pharmaceuticals.

MiniQuiz

- What is the advantage of using genetic engineering to make insulin?
- What are the major problems when manufacturing proteins in bacteria?
- How has biotechnology helped the dairy industry?

15.12 Other Mammalian Proteins and Products

Many other mammalian proteins are produced by genetic engineering (**Table 15.6**). These include, in particular, an assortment of hormones and proteins for blood clotting and other blood processes. For example, tissue plasminogen activator (TPA) is a blood protein that scavenges and dissolves blood clots that may form in the final stages of the healing process. TPA is primarily used in heart patients or others suffering from poor circulation to prevent the development of clots that can be life-threatening. Heart disease is a leading cause of death in many developed countries, especially in the United States, so microbially produced TPA is in high demand.

In contrast to TPA, the blood clotting factors VII, VIII, and IX are critically important for the *formation* of blood clots. Hemophiliacs suffer from a deficiency of one or more clotting factors and can therefore be treated with microbially produced clotting factors. In the past hemophiliacs have been treated with clotting factor extracts from pooled human blood, some of which was contaminated with viruses such as HIV and hepatitis C, putting hemophiliacs at high risk for contracting these diseases. Recombinant clotting factors have eliminated this problem.

Table 15.6 *A few therapeutic products made by genetic engineering*

Product	*Function*
Blood proteins	
Erythropoietin	Treats certain types of anemia
Factors VII, VIII, IX	Promotes blood clotting
Tissue plasminogen activator	Dissolves blood clots
Urokinase	Promotes blood clotting
Human hormones	
Epidermal growth factor	Wound healing
Follicle-stimulating hormone	Treatment of reproductive disorders
Insulin	Treatment of diabetes
Nerve growth factor	Treatment of degenerative neurological disorders and stroke
Relaxin	Facilitates childbirth
Somatotropin (growth hormone)	Treatment of some growth abnormalities
Immune modulators	
α-Interferon	Antiviral, antitumor agent
β-Interferon	Treatment of multiple sclerosis
Colony-stimulating factor	Treatment of infections and cancer
Interleukin-2	Treatment of certain cancers
Lysozyme	Anti-inflammatory
Tumor necrosis factor	Antitumor agent, potential treatment of arthritis
Replacement enzymes	
β-Glucocerebrosidase	Treatment of Gaucher disease, an inherited neurological disease
Therapeutic enzymes	
Human DNase I	Treatment of cystic fibrosis
Alginate lyase	Treatment of cystic fibrosis

Some mammalian proteins made by genetic engineering are enzymes rather than hormones (Table 15.6). For instance, *human DNase I* is used to treat the buildup of DNA-containing mucus in patients with cystic fibrosis. The mucus forms because cystic fibrosis is often accompanied by life-threatening lung infections by the bacterium *Pseudomonas aeruginosa*. The bacterial cells form biofilms (↻ Section 23.4) within the lungs that make drug treatment difficult. DNA is released when the bacteria lyse, and this fuels mucus formation. DNase digests the DNA and greatly decreases the viscosity of the mucus. There are more than 30,000 patients with cystic fibrosis in the United States alone. Treatment of cystic fibrosis with DNase was approved in 1994, and sales today of this life-saving enzyme exceed $100 million. A second enzyme, *alginate lyase*, also produced by genetic engineering, shows promise in treating cystic fibrosis because it degrades the polysaccharide produced by *P. aeruginosa* cells. Like DNA from lysed cells, this polymer also contributes to lung mucus, and thus its hydrolysis relieves respiratory symptoms.

Not all the enzymes produced by genetic engineering have therapeutic uses. Many commercial enzymes (Section 15.6) are now produced in this way. Sometimes the benefits of genetic

engineering are quite unexpected. Rennet, which is an enzyme used to make cheese, is the product of slaughtered animals and thus cannot be consumed by strict vegetarians (vegans). However, "vegetarian cheese" containing recombinant rennet produced in a microorganism is being marketed and has found wide acceptance.

Further applications come from using site-directed mutagenesis (↻ Section 11.4) on existing cloned genes to generate new products with new properties. Certain molecules, such as many antibiotics, are synthesized in cells by biochemical pathways that use a series of enzymes (Section 15.4). These enzymes can be modified by genetic engineering to produce modified forms of the antibiotics.

MiniQuiz

- Contrast the activity of TPA and blood factors VII, VIII, and IX.
- Explain how a DNA-degrading enzyme can be useful in treating a bacterial infection, such as that which occurs with cystic fibrosis.

15.13 Genetically Engineered Vaccines

Vaccines are substances that elicit immunity to a particular disease when injected into an animal (↻ Section 28.7). Typically, vaccines are suspensions of killed or modified pathogenic microorganisms or viruses (or parts isolated from them). Often the part that elicits the immune response is a surface protein, for instance, a viral coat protein. Genetic engineering can be applied in many different ways to the production of vaccines.

Recombinant Vaccines

Recombinant DNA techniques can be used to modify the pathogen itself. For instance, one can delete pathogen genes that encode virulence factors but leave those whose products elicit an immune response. This yields a recombinant, live, attenuated vaccine. Conversely, one can add genes from a pathogenic virus to another, relatively harmless virus, referred to as a carrier virus. Such vaccines are called **vector vaccines**. This approach induces immunity to the pathogenic viral disease. Indeed, one can even combine the two approaches. For example, a recombinant vaccine is used to protect poultry against both fowlpox (a disease that reduces weight gain and egg production) and Newcastle disease (a viral disease that is often fatal). The fowlpox virus (a typical pox virus; ↻ Section 21.15) was first modified by deleting genes that cause disease, but not ones that elicit immunity. Then immunity-inducing genes from the Newcastle virus were inserted. This resulted in a **polyvalent vaccine**, a vaccine that immunizes against two different diseases.

Vaccinia virus is widely used to prepare live recombinant vaccines for human use (↻ Section 21.15). Vaccinia virus itself is generally not pathogenic for humans and has been used for over 100 years as a vaccine against the related smallpox virus. However, cloning genes into vaccinia virus requires a selective marker, which is provided by the gene for thymidine kinase. Vaccinia is unusual for a virus in carrying its own thymidine kinase, an enzyme that normally converts thymidine into thymidine triphosphate. However, this enzyme also converts the base analog

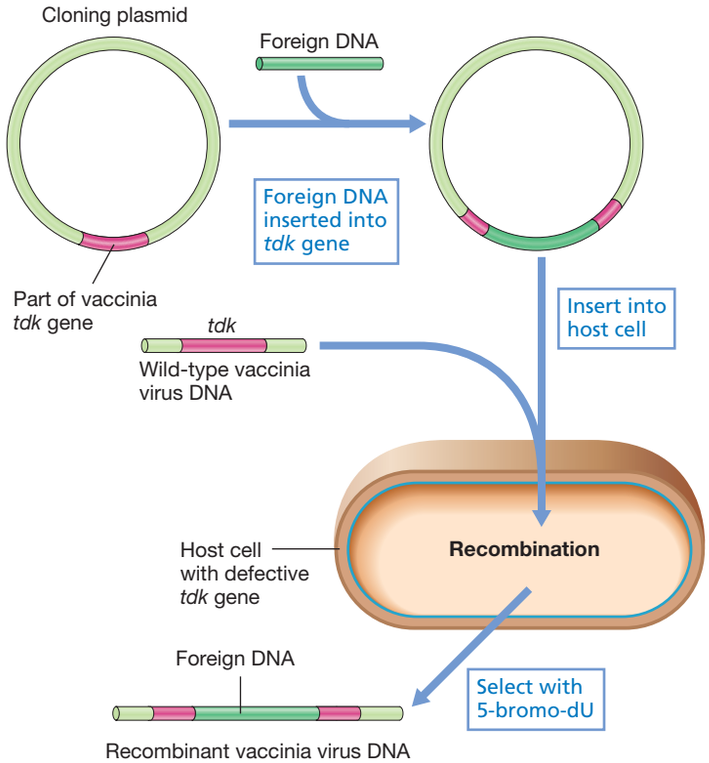


Figure 15.22 Production of recombinant vaccinia virus. Foreign DNA is inserted into a short segment of the thymidine kinase gene (*tdk*) from vaccinia virus carried on a plasmid. The plasmid with the insert and wild-type vaccinia virus are both put into the same host cell where they recombine. The cells are treated with 5-bromodeoxyuridine (5-bromo-dU), which kills cells with active thymidine kinase. Only recombinant vaccinia viruses whose *tdk* gene is inactivated by insertion of foreign DNA survive.

5-bromodeoxyuridine to a nucleotide that is incorporated into DNA, which is a lethal reaction. Therefore, cells that express thymidine kinase (whether from the host cell genome or from a virus genome) are killed by 5-bromodeoxyuridine.

Genes to be put into vaccinia virus are first inserted into an *Escherichia coli* plasmid that contains part of the vaccinia thymidine kinase (*tdk*) gene (**Figure 15.22**). The foreign DNA is inserted into the *tdk* gene, which is therefore disrupted. This recombinant plasmid is then transformed into animal cells whose own *tdk* genes have been inactivated. These cells are also infected with wild-type vaccinia virus. The two versions of the *tdk* gene—one on the plasmid and the other on the virus—recombine. Some viruses gain a disrupted *tdk* gene plus its foreign insert (Figure 15.22). Cells infected by wild-type virus, with active thymidine kinase, are killed by 5-bromodeoxyuridine. Cells infected by recombinant vaccinia virus with a disrupted *tdk* gene grow long enough to yield a new generation of viral particles (Figure 15.22). In other words, the protocol selects for viruses whose *tdk* gene contains a cloned insert of foreign DNA.

Vaccinia virus does not actually need thymidine kinase to survive. Consequently, recombinant vaccinia viruses can still infect human cells and express any foreign genes they carry. Indeed, vaccinia viruses can be engineered to carry genes from multiple viruses (that is, they are polyvalent vaccines). Currently, several

vaccinia vector vaccines have been developed and licensed for veterinary use, including one for rabies. Many other vaccinia vaccines are at the clinical trial stage. Vaccinia vaccines are relatively benign, yet highly immunogenic in humans, and their use will likely increase in the coming years.

Subunit Vaccines

Recombinant vaccines need not include every protein from the pathogenic organism. Subunit vaccines may contain only a specific protein or two from a pathogenic organism. For a virus this protein is often the coat protein because coat proteins are typically highly immunogenic. The coat proteins are purified and used in high dosage to elicit a rapid and high level of immunity. Subunit vaccines are currently very popular because they can be used to produce large amounts of immunogenic proteins without the possibility that the purified products contain the entire pathogenic organism, even in minute amounts.

The steps in preparing a viral subunit vaccine are as follows: fragmentation of viral DNA by restriction enzymes; cloning viral coat protein genes into a suitable vector; providing proper promoters, reading frame, and ribosome-binding sites; and reinsertion and expression of the viral genes in a microorganism. Sometimes only certain portions of the protein are expressed rather than the entire protein, because immune cells and antibodies typically react with only small portions of the protein. (When this approach is used against an RNA virus, the viral genome must be converted to a cDNA copy first.)

When *E. coli* is used as the cloning host, viral subunit vaccines are often poorly immunogenic and fail to protect in experimental tests of infection. The problem is that the recombinant proteins produced by bacteria are nonglycosylated, and glycosylation is necessary for the proteins to be immunologically active. Glycosylation is accomplished in an animal cell infected by the virus when, in the course of viral replication, viral coat proteins are modified after translation by the addition of sugar residues (glycosylation). To solve the problem with the vaccine, a eukaryotic cloning host is used. For example, the first recombinant subunit vaccine approved for use in humans (against hepatitis B) was made in yeast. The gene encoding a surface protein from hepatitis B virus was cloned and expressed in yeast. The protein produced was glycosylated and formed aggregates very similar to those found in patients infected with the virus. These aggregates were purified and used to effectively vaccinate humans against infection by hepatitis B virus.

Subunit vaccines against many viruses and other pathogens are currently being developed. Cultured insect or mammalian cells are often used as hosts to prepare such recombinant vaccines. As noted, to obtain the correct glycosylation or other modifications of the immunogenic protein, it is often important to use a eukaryotic host. However, vaccines with correct glycosylation can often be produced in eukaryotic hosts relatively unrelated to humans, such as plants or insect cells. Recently, both yeast and insect cells have themselves been genetically engineered by the insertion of human genes that catalyze glycosylation. The resulting host cells add human-type glycosylation patterns to the proteins they produce.

The Future of Recombinant Vaccines

Genetically engineered recombinant vaccines will likely become increasingly common for several reasons. They are safer than normal attenuated or killed vaccines because it is impossible to transmit the disease in the vaccine. They are also more reproducible because their genetic makeup can be carefully monitored.

In addition, recombinant vaccines can usually be prepared much faster than those made by more traditional methods. For preparing vaccines for some diseases such as influenza, time is of the essence. Recombinant vaccines using cloned influenza virus hemagglutinin genes can be made in just 2 or 3 months. This contrasts with the 6–9 months needed to make an attenuated intact ("live") influenza virus vaccine. Preparation time is important in responding to an epidemic caused by a new strain of virus, a common situation with influenza outbreaks. Finally, recombinant vaccines are typically less expensive than those produced by traditional methods.

DNA Vaccines

Although vaccines have been extremely successful in the fight against infectious diseases, in some cases vaccines are difficult to produce. However, a conceptually new approach to vaccine production is possible—**DNA vaccines**—also known as *genetic vaccines*. DNA vaccines use the genome of the pathogen itself to immunize the individual. Defined fragments of the pathogen's genome or specific genes that encode immunogenic proteins are used. The key genes are cloned into a plasmid or viral vector and delivered by injection. When DNA is taken up by animal cells it may be degraded or it may be transcribed and translated. If it is translated and the protein produced is immunogenic, the animal will be effectively immunized against the pathogen. Thus, the immune response is made against the protein encoded by the vaccine DNA. The DNA itself is not immunogenic.

Several DNA vaccines, for example, vaccines against HIV, hepatitis B, and several cancers, have undergone clinical trials. For unknown reasons, the DNA vaccines so far tested have not proved potent enough to provide protective immunity to humans. It is hoped that future improvements will permit clinical use. Nonetheless, DNA vaccines have been licensed for use in animals (for example, a vaccine against West Nile virus for horses).

Unlike viral vaccines, DNA vaccines escape surveillance by the host immune system because nucleic acids themselves are poorly immunogenic. This prevents the animal from suffering autoimmune effects in which antibodies and immune cells attack host cells (Section 28.9). DNA vaccines have the advantage that they are both safe and inexpensive. In addition, DNA is more stable than live vaccines, which avoids the need for refrigeration—an important practical point for using vaccines in developing countries.

MiniQuiz

- Explain why recombinant vaccines might be safer than some vaccines produced by traditional methods.
- What are the important differences among a recombinant live attenuated vaccine, a vector vaccine, a subunit vaccine, and a DNA vaccine?

15.14 Mining Genomes

Just as the total genetic content of an organism is its *genome*, so the collective genomes of an environment is known as its *metagenome*. Complex environments, such as fertile soil, contain vast numbers of uncultured bacteria and other microorganisms together with the viruses that prey on them (↻ Section 12.6). Taken together, these contain correspondingly vast numbers of novel genes. Indeed, most of the genetic information on Earth exists in microorganisms and their viruses that have not been cultured.

Environmental Gene Mining

Gene mining is the process of isolating potentially useful novel genes from the environment without culturing the organisms that carry them. Instead of being cultured, DNA (or RNA) is isolated directly from environmental samples and cloned into suitable vectors to construct a metagenomic library (**Figure 15.23**). The nucleic acid includes genes from uncultured organisms as well as DNA from dead organisms that has been released into the environment but has not yet been degraded. If RNA is isolated, it must be converted to a DNA copy by reverse transcriptase (Figure 15.18). However, isolating RNA is more time consuming and limits the metagenomic library to those genes that are transcribed and therefore active in the environment sampled.

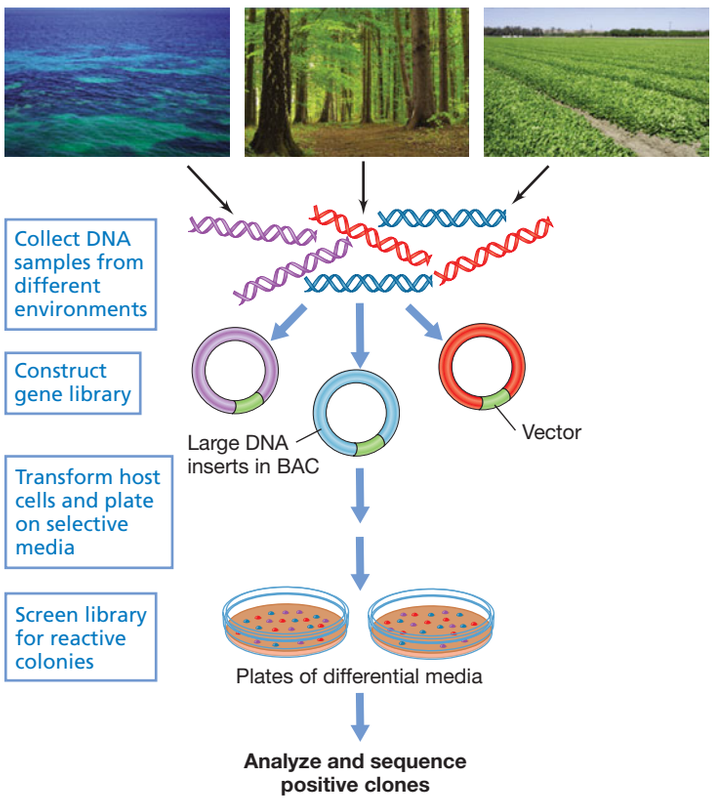


Figure 15.23 Metagenomic search for useful genes in the environment. DNA samples are obtained from different sites, such as seawater, forest soil, and agricultural soil. A clone library is constructed using bacterial artificial chromosomes (BACs) and screened for genes of interest. Possibly useful clones are analyzed further.

The metagenomic library is screened by the same techniques as any other clone library. Metagenomics has identified novel genes encoding enzymes that degrade pollutants and enzymes that make novel antibiotics. So far several lipases, chitinases, esterases, and other degradative enzymes with novel substrate ranges and other properties have been isolated by this approach. Such enzymes are used in industrial processes for various purposes (Section 15.6). Enzymes with improved resistance to industrial conditions, such as high temperature, high or low pH, and oxidizing conditions, are especially valuable and sought after.

Discovery of genes encoding entire metabolic pathways, such as for antibiotic synthesis, as opposed to single genes, requires vectors such as bacterial artificial chromosomes (BACs) that can carry large inserts of DNA (↻ Section 11.10). BACs are especially useful for screening samples from rich environments, such as soil, where vast numbers of unknown genomes are present and correspondingly large numbers of genes are available to screen.

Targeted Gene Mining

Metagenomics can screen directly for enzymes with certain properties. Suppose one needed an enzyme or entire pathway capable of degrading a certain pollutant at a high temperature. The first step would be to find a hot environment polluted with the target compound. Assuming that microorganisms capable of degradation were present in the environment, a reasonable hypothesis, DNA from the environment would then be isolated and cloned. Host bacteria containing the clones would be screened for growth on the target compound. For convenience, this step is usually done in an *Escherichia coli* host, on the assumption that thermostable enzymes will still show some activity at 40°C (this is typically the case). Once suspects have been identified, enzyme extracts can be tested *in vitro* at high temperatures. Recently, thermophilic cloning systems have been developed that allow direct selection at high temperature. These rely on expression vectors that can replicate in both *E. coli* and the hot spring thermophile *Thermus thermophilus*.

MiniQuiz

- Explain why metagenomic cloning gives large numbers of novel genes.
- What are the advantages and disadvantages of isolating environmental RNA as opposed to DNA?

15.15 Engineering Metabolic Pathways

Although proteins are large molecules, expressing large amounts of a single protein that is encoded by a single gene is relatively simple. By contrast, small metabolites are typically made in biochemical pathways employing several enzymes. In these cases, not only are multiple genes needed, but their expression must be regulated in a coordinated manner as well.

Pathway engineering is the process of assembling a new or improved biochemical pathway using genes from one or more organisms. Most efforts so far have modified and improved existing pathways rather than creating entirely new ones (but see the Microbial Sidebar, "Synthetic Biology and Bacterial Photography").

Synthetic Biology and Bacterial Photography

The term "synthetic biology" refers to the use of genetic engineering to create novel biological systems out of available biological parts, often from several different organisms. An ultimate goal of synthetic biology is to synthesize a viable cell from component parts, a feat that will likely be accomplished in the near future. A major start in this direction was made in 2007 when a team of synthetic biologists transferred the entire chromosome of one species of bacterium into another species of bacterium. The latter species then took on all of the properties of the species whose genome it had acquired. In 2010, the team extended their work and placed a *laboratory-synthesized* chromosome of one bacterial species into the cell of a second bacterial species and got the synthetic chromosome to function normally and direct the activities of the recipient cell.

An interesting example of synthetic biology on a smaller scale is the use of genetically modified *Escherichia coli* cells to produce photographs. The engineered bacteria are grown as a lawn on agar plates. When an image is projected onto the lawn, bacteria in the dark make a dark pigment whereas bacteria in the light do not. The result is a primitive black-and-white photograph of the projected image.

Construction of the photographic *E. coli* required the engineering and insertion of three genetic modules: (1) a light detector and signaling module; (2) a pathway to convert heme (already present in *E. coli*) into the photoreceptor pigment phycocyanobilin; and (3) an enzyme encoded by a gene whose transcription can be switched on and off to make the dark pigment (Figure 1*a*). The light detector is a fusion protein. The outer half is the light-detecting part of the phytochrome protein from the cyanobacterium *Synechocystis*. This needs a special light-absorbing pigment, phycocyanobilin, which is not made by *E. coli*, hence the need to install the pathway to make phycocyanobilin.

The inner half of the light detector is the signal transmission domain of the EnvZ sensor protein from *E. coli*. EnvZ is part of a two-component regulatory system, its partner being OmpR (Section 8.7). Normally, EnvZ activates the DNA-binding protein OmpR. Activated OmpR in turn activates target genes by binding to the promoter. In the present case, the hybrid protein was designed to activate OmpR in the dark but not in the light. This is because phosphorylation of OmpR is required for activation, and red light converts the sensor to a state in which phosphorylation is inhibited. Consequently the target gene is off in the light and on in the dark. When a mask is placed over the Petri plate containing a lawn of the engineered *E. coli* cells (Figure 1*b*), cells in the dark make a pigment that cells in the light do not, and in this way a "photograph" of the masked image develops (Figure 1*c*).

The pigment made by the *E. coli* cells results from the activity of an enzyme naturally found in this organism that functions in lactose metabolism, β-galactosidase. The target gene, *lacZ*, encodes this enzyme. In the dark, the *lacZ* gene is expressed and β-galactosidase is made. The enzyme cleaves a lactose analog called *S-gal* present in the growth medium to release galactose and a colored dye. In the light, the *lacZ* gene is not expressed, no β-galactosidase is made, and so no black dye is released. The difference in contrast between cells producing the dye and cells that are not generates the bacterial photograph (Figure 1*c*).

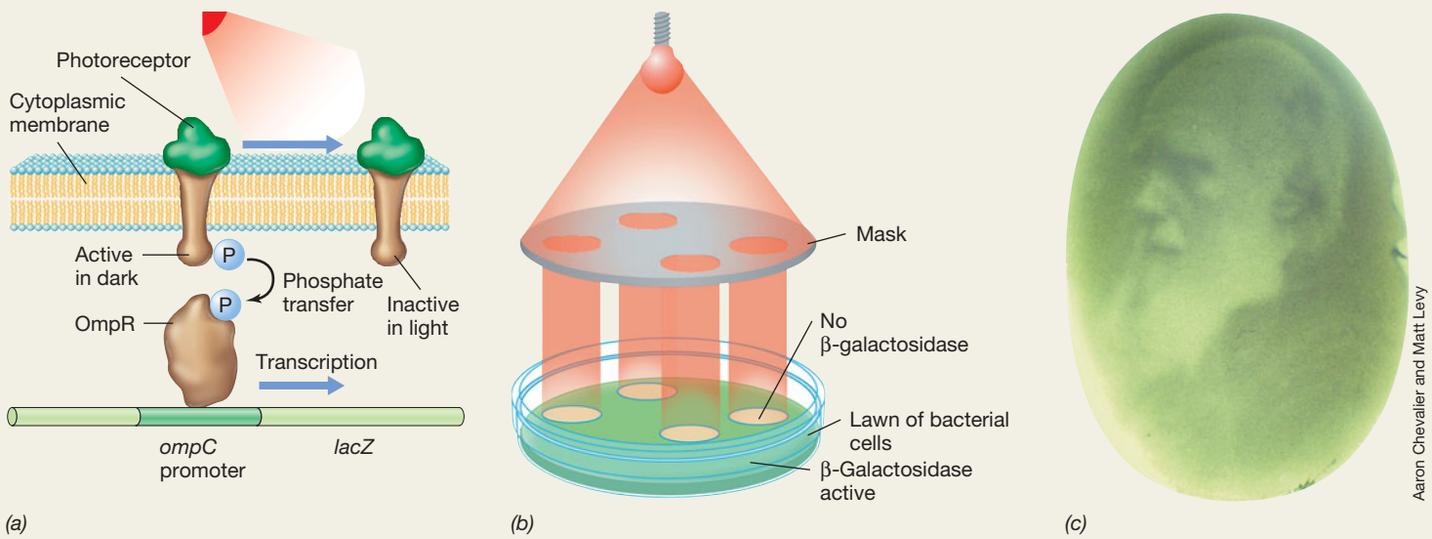


Figure 1 **Bacterial photography.** *(a)* Light-detecting *Escherichia coli* cells were genetically engineered using components from cyanobacteria and *E. coli* itself. Red light inhibits phosphate (P) transfer to the DNA-binding protein OmpR; phosphorylated OmpR is required to activate *lacZ* transcription (*lacZ* encodes β-galactosidase). *(b)* Set-up for making a bacterial photograph. The opaque portions of the mask correspond to zones where β-galactosidase is active and thus to the dark regions of the final image. *(c)* A bacterial photograph of a portrait of Charles Darwin.

Because genetic engineering of bacteria is simpler than that of higher organisms, most pathway engineering has been done with bacteria. Engineered microorganisms are used to make products, including alcohols, solvents, food additives, dyes, and antibiotics. They may also be used to degrade agricultural waste, pollutants, herbicides, and other toxic or undesirable materials.

An example of pathway engineering is the production of indigo by *Escherichia coli* (**Figure 15.24**). Indigo is an important dye used for treating wool and cotton. Blue jeans, for example, are made of cotton dyed with indigo. In ancient times indigo and related dyes were extracted from sea snails. More recently, indigo was extracted from plants, but today it is synthesized chemically. The demand for indigo by the textile industry has spawned new approaches for its synthesis, including a biotechnological one.

Because the structure of indigo is very similar to that of naphthalene, enzymes that oxygenate naphthalene also oxidize indole to its dihydroxy derivative, which then oxidizes spontaneously in air to yield indigo, a bright blue pigment. Enzymes for oxygenating naphthalene are present on several plasmids found in *Pseudomonas* and other soil bacteria. When genes from such plasmids were cloned into *E. coli*, the cells turned blue due to production of indigo; the blue cells had picked up the genes for the enzyme naphthalene oxygenase.

Although only the gene for naphthalene oxygenase was cloned during indigo pathway engineering, the indigo pathway consists of four steps, two enzymatic and two spontaneous (Figure 15.24). *E. coli* synthesizes the enzyme tryptophanase that carries out the first step, the conversion of tryptophan to indole. For indigo production, tryptophan must be supplied to the recombinant *E. coli* cells. This is accomplished by affixing the cells to a solid support in a bioreactor and trickling a tryptophan solution from waste protein or other sources over them. Recirculating the material over the cells several times, as is typically done in these types of immobilized cell industrial processes (Section 15.6), steadily increases indigo levels until the dye can be harvested.

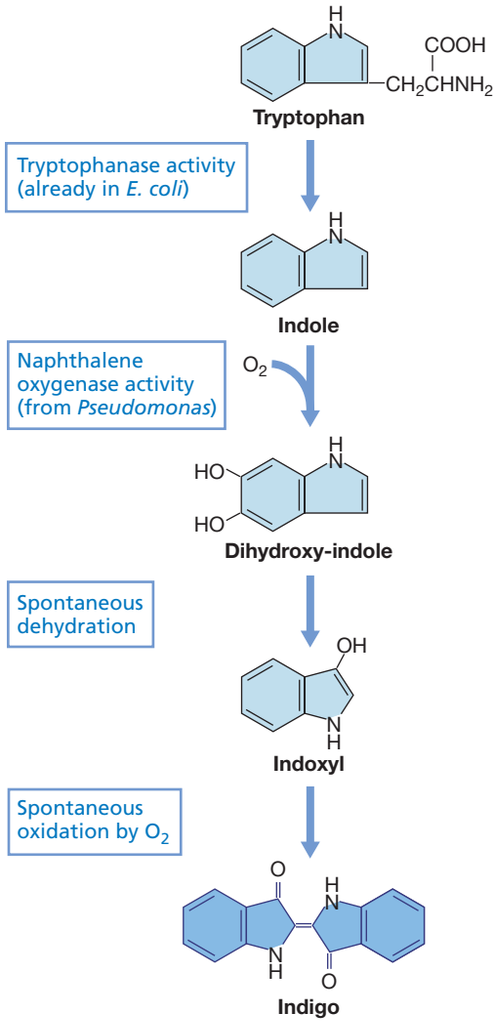


Figure 15.24 **Engineered pathway for production of the dye indigo.** *Escherichia coli* naturally expresses tryptophanase, which converts tryptophan into indole. Naphthalene oxygenase (originally from *Pseudomonas*) converts indole to dihydroxy-indole, which spontaneously dehydrates to indoxyl. Upon exposure to air, indoxyl dimerizes to form indigo, which is blue.

MiniQuiz

- Explain why pathway engineering is more difficult than cloning and expressing a human hormone.
- How was *Escherichia coli* modified to produce indigo?

UNIT 5

V Transgenic Eukaryotes

A *transgene* is a gene from one organism that has been inserted into a different organism. Hence, a **transgenic organism** is one that contains a transgene. A related term is **genetically modified organism (GMO)**. Strictly speaking, this refers to genetically engineered organisms whether or not they contain foreign DNA. However, in common usage, especially in agriculture, "GMO" is often used interchangeably with "transgenic organism."

On the one hand, the genetic engineering of higher organisms is not truly microbiology. On the other hand, much of the DNA manipulation is carried out using bacteria and their plasmids long before the engineered transgene is finally inserted into the plant or animal. Furthermore, vectors based on viruses are widely used in the genetic engineering of higher organisms. Therefore we emphasize the microbial systems that have contributed to the genetic manipulation of plants and animals.

15.16 Genetic Engineering of Animals

Many foreign genes have been incorporated and expressed in laboratory research animals and in commercially important animals. The genetic engineering uses microinjection to deliver cloned genes to fertilized eggs; genetic recombination then incorporates the foreign DNA into the genomes of the eggs. The first transgenic animals were mice that were engineered as model systems for studying mammalian physiology. Genes for growth hormone from rats or humans were engineered for expression and inserted into eggs that developed into mice that expressed the growth hormone. The result, of course, was larger mice. More recently, farm animals have been genetically modified, not only to improve yields, but also so that their waste is less polluting.

Transgenic Animals in Pharming

Transgenic animals can be used to produce proteins of pharmaceutical value—a process called *pharming*. Transgenic animals are particularly useful for producing human proteins that require specific posttranslational modifications for activity, such as blood-clotting enzymes. Proteins of this type are not made in an active form by microorganisms or by plants.

Some proteins have been genetically engineered to be secreted in high yield in animal milk. This is convenient for several reasons. First, this allows larger volumes of material to be made more simply and cheaply than by bacterial culture. Second, a milk-processing industry already exists, so little new technology is needed to purify the protein. Third, milk is a natural product that most humans can tolerate, so purification to remove possibly toxic bacterial proteins is unnecessary. Goats have proven useful for making several human proteins including tissue plasminogen activator, which is used to dissolve blood clots (Section 15.12).

Transgenic Animals in Medical Research

Transgenic animals have become increasingly important in basic biomedical research for studying gene regulation and developmental biology. For example, so-called "knockout" mice, which have had both copies of a particular gene inactivated by genetic engineering, are used to analyze genes active in animal physiology. For instance, knockout mice that lack both copies of the gene for myostatin, a protein that slows muscle growth, develop massive muscles. In contrast, transgenic mice that overproduce myostatin show reduced muscle mass. Many other strains of knockout mice have been developed for use in medical research, and a 2007 Nobel Prize was awarded for the development of this important genetic tool.

Improving Livestock and Other Food Animals

Livestock may be engineered to increase their productivity, nutritional value, and disease resistance. Occasionally, transgenic livestock are produced that do not have increased commercial value but that demonstrate the feasibility of certain genetic techniques. For example, pigs genetically engineered to express the reporter gene that encodes the green fluorescent protein (↻ Section 11.5) are, as expected, green (**Figure 15.25*a***).

One scheme to improve the nutrition of livestock is to insert entire metabolic pathways from bacteria into the animals. For example, genes that encode the enzymes of the metabolic pathway for making methionine, a required amino acid, could remove the need for this amino acid in the animals' diet. A notable technical success has been the insertion into pigs of a gene from *Escherichia coli* that helps degrade organic phosphate. The resulting Enviropig™ no longer needs phosphate supplements in its feed. However, most importantly, the manure from these animals is low in phosphate, and this prevents phosphate runoff from pig-manure waste ponds into freshwater; such an influx of inorganic nutrients can trigger algal blooms and fish die-offs (↻ Section 23.8).

Pigs have also been genetically engineered to increase their levels of omega-3 fatty acids. These fatty acids reduce heart disease but are found in significant amounts only in cold-water fish, such as salmon, and a few other rare foods. To create transgenic pigs with an altered fatty acid profile, a gene from the roundworm *Caenorhabditis elegans*, called *fat1*, was inserted into the pigs. The enzyme encoded by *fat1* converts the less healthy but more common omega-6 fatty acids into omega-3 fatty acids. Such animals should be healthier for consumers, especially those who have dietary restrictions on fat or who are at high risk for heart disease. It will be several years before the omega-3-enriched pigs reach the consumer market, assuming they receive government approval.

Another interesting practical example of a transgenic animal is the "fast-growing salmon" (Figure 15.25*b*). These transgenic salmon do not grow to be larger than normal salmon but simply reach market size much faster. The gene for growth hormone in natural salmon is activated by light. Consequently, salmon grow rapidly only during the summer months. In the genetically engineered salmon, the promoter for the growth hormone gene was replaced with the promoter from another fish that grows at a more or less constant rate all year round. The result was salmon that make growth hormone constantly and thus grow faster.

Wu-Shinn Chih

(a)

Aqua Bounty Technologies

(b)

Figure 15.25 **Transgenic animals.** *(a)* A piglet (left), seen under blue light, that has been genetically engineered to express the green fluorescent protein. Control piglets are shown in the center and right. *(b)* Fast-growing salmon. The *AquAdvantage*™ Salmon (top) was engineered by Aqua Bounty Technologies (St. Johns, Newfoundland, Canada). The transgenic and the control fish are 18 months old and weigh 4.5 kg and 1.2 kg, respectively.

MiniQuiz

- What is pharming?
- Why are knockout mice useful in investigating human gene function?
- What environmental advantage does Enviropig™ have over normal pigs?

15.17 Gene Therapy in Humans

A large number of human genetic diseases are known. A list can be found in the OMIM (Online Mendelian Inheritance in Man) database, available online at http://www.ncbi.nlm.nih.gov/. Because conventional genetic experiments cannot be done with humans as they can with other animals, our understanding of human genetics has lagged behind that of many other organisms. However, by using recombinant DNA technology, coupled with conventional genetic studies (following family inheritance, and so on), it is possible to localize particular genetic defects to specific regions of particular chromosomes. Moreover, the human genome has been sequenced (↻ Section 12.1). Consequently, if the region encoding a presumed genetic defect is cloned and sequenced, the base sequence of the defective gene may be compared with that of the normal gene. Even without knowledge of the enzyme defect, it is possible to obtain information about the genetic changes causing many hereditary defects.

Human Hereditary Diseases

Many genes, including those for Huntington's disease, hemophilia types A and B, cystic fibrosis, Duchenne muscular dystrophy, multiple sclerosis, and breast cancer have been located with these techniques, and the mutations in the defective genes have been identified. With this in mind, how can genetic engineering be used to treat or cure these diseases?

The use of genetic engineering to treat human genetic diseases, including attacking cancer cells, is known as **gene therapy**. In the gene therapy procedure, a nonfunctional or dysfunctional gene in a person is "replaced" by a functional gene. Strictly speaking, it is not the defective *gene* that is replaced, but instead its *function*. The therapeutic wild-type gene is inserted elsewhere in the genome and its gene product corrects the genetic disorder. Major obstacles to this approach exist in targeting the correct cells for gene therapy and in successfully inserting the required gene into cell lines that will perpetuate the genetic alteration.

The first genetic disease for which the use of gene therapy was approved is a form of severe combined immune deficiency (SCID). This disease, caused by the absence of adenosine deaminase (ADA), an enzyme of purine metabolism in bone marrow cells, leads to a crippled immune system. The gene therapy approach uses a retrovirus as a vector to carry a wild-type copy of the ADA gene. T cells (part of the immune system; ↻ Section 28.1) are removed from the patient and infected with the retrovirus carrying the ADA gene. The retrovirus also carries a marker gene, encoding resistance to the antibiotic neomycin, so that T cells carrying the inserted retrovirus may be selected and identified. The engineered T cells are then placed back in the body. However, because T cells have a limited life span, the therapy must be repeated every few months. Consequently, for newborn infants diagnosed with defects in the ADA gene, treatment protocols for SCID have been developed in which the ADA gene is inserted into stem cells obtained from the umbilical cord blood of the infants. The engineered stem cells are then returned to the infants. Because stem cells continue to divide and provide a fresh supply of new T cells, this effects a long-term cure.

Several other gene therapy treatments, some using other virus vectors, are currently being tested with various levels of success. After the first gene therapy experiment with ADA in 1990, there were no striking breakthroughs until 2000. Another form of SCID, caused by defects in a different gene, was successfully treated in several patients. It seems likely that this very rare form of the disease can now be successfully treated using gene therapy.

Technical Problems with Gene Therapy

Although gene therapy has tremendous potential, most applications remain distant prospects. Some current difficulties are related to the vectors being used. Although using retroviral vectors gives stable integration of the transgene, the site of insertion is unpredictable and expression of the cloned gene is often transient. The vectors also have limited infectivity and are rapidly inactivated in the host. Many nonretroviral vectors, such as the adenovirus vector, have similar problems, and adverse reactions to the vector itself can also be a severe problem. However, some promising new vectors for gene therapy have emerged, including human artificial chromosomes (↻ Section 11.10) and highly modified retroviral vectors.

It is important to recall that in the gene therapy protocols being tested, the defective copy of the gene is not actually replaced; rather, its *defective function* is replaced. The retrovirus (containing the good copy of the gene) simply integrates somewhere into the human genome of the target cells. Actual gene replacements in germ line cells (cells that give rise to gametes) can be accomplished in experimental animals, although the techniques of isolating individual animals with these changes cannot readily be applied to humans. Moreover, attempts to change the germ cells of humans would also raise ethical questions that will likely keep these types of procedures, even if they have great medical promise, only a very long-range possibility.

MiniQuiz

- Why is SCID such a serious disease, and how can gene therapy help someone afflicted with SCID?
- What problems arise from using a retrovirus as a vector in gene therapy?
- A person treated successfully by gene therapy will still have a defective copy of the gene. Explain.

15.18 Transgenic Plants in Agriculture

Genetic improvement of plants by traditional selection and breeding has a long history, but recombinant DNA technology has led to revolutionary changes. Plant DNA can be modified by genetic engineering and then transformed into plant cells by either electroporation or the particle gun (↻ Section 11.7). Alternatively,

one can use plasmids from the bacterium *Agrobacterium tumefaciens*, which naturally transfers DNA directly into the cells of certain plants (↻ Section 25.7).

Plants differ from animals in having no real separation of the germ line from the somatic cells. Consequently plants can often be regenerated from just a single cell. Moreover, it is possible to culture plant cells *in vitro*. Therefore plant genetic engineering is mostly done with plant cells growing in culture. After genetically altered clones have been selected, the cells are induced by treatment with plant hormones to grow into whole plants.

Many successes in plant genetic engineering have already been achieved, and several transgenic plants are in agricultural production. The public knows these plants as *genetically modified* (*GM*) plants. In this section we discuss how foreign genes are inserted into plant genomes and how transgenic plants may be used.

The Ti Plasmid and Transgenic Plants

The gram-negative plant pathogen *A. tumefaciens* contains a large plasmid, called the **Ti plasmid**, that is responsible for its virulence. This plasmid contains genes that mobilize DNA for transfer to the plant, which as a result contracts crown gall disease (↻ Section 25.7). The segment of the Ti plasmid DNA that is actually transferred to the plant is called **T-DNA**. The sequences at the ends of the T-DNA are essential for transfer, and the DNA to be transferred must be included between these ends.

One common Ti-vector system that has been used for the transfer of genes to plants is a two-plasmid system called a *binary vector*, which consists of a cloning vector plus a helper plasmid. The cloning vector contains the two ends of the T-DNA flanking a multiple cloning site, two origins of replication so that it can replicate in both *Escherichia coli* (the host for cloning) and *A. tumefaciens*, and two antibiotic resistance markers, one for selection in plants and the other for selection in bacteria. The foreign DNA is inserted into the vector, which is transformed into *E. coli* and then moved to *A. tumefaciens* by conjugation (**Figure 15.26**).

This cloning vector lacks the genes needed to transfer T-DNA to a plant. However, when placed in an *A. tumefaciens* cell that contains a suitable helper plasmid, the T-DNA can be transferred to a plant. The "disarmed" helper plasmid, called *D-Ti*, contains the virulence (*vir*) region of the Ti plasmid but lacks the T-DNA. It lacks the genes that cause disease but supplies all the functions needed to transfer the T-DNA from the cloning vector. The cloned DNA and the kanamycin resistance marker of the vector are mobilized by D-Ti and transferred into a plant cell where they enter the nucleus (Figure 15.26*d*). Following integration into a plant chromosome, the foreign DNA can be expressed and confer new properties on the plant.

A number of transgenic plants have been produced using the Ti plasmid of *A. tumefaciens*. The Ti system works well with broadleaf plants (dicots), including crops such as tomato, potato, tobacco, soybean, alfalfa, and cotton. It has also been used to produce transgenic trees, such as walnut and apple. The Ti system does not work with plants from the grass family (monocots, including the important crop plant, corn), but other methods of introducing DNA, such as the particle gun (↻ Section 11.7), have been used successfully for them.

Herbicide and Insect Resistance

Major areas targeted for genetic improvement in plants include herbicide, insect, and microbial disease resistance as well as improved product quality. The first GM crop to be grown commercially was tobacco grown in China in 1992 that was engineered for resistance to viruses. By the year 2005, the area planted worldwide with GM crops was estimated to exceed 1 billion acres (440 million hectares). The main GM crops today are soybeans, corn, cotton, and canola. Almost all the GM soybeans and canola planted were herbicide resistant, whereas the corn and cotton were herbicide resistant or insect resistant, or both. In 2005 the United States grew over half the world's total of GM crops. Argentina, Canada, Brazil, and China were the other major producers, with the rest of the world accounting for less than 5% of the total.

Herbicide resistance is genetically engineered into a crop plant to protect it from herbicides applied to kill weeds. Many herbicides inhibit a key plant enzyme or protein necessary for growth.

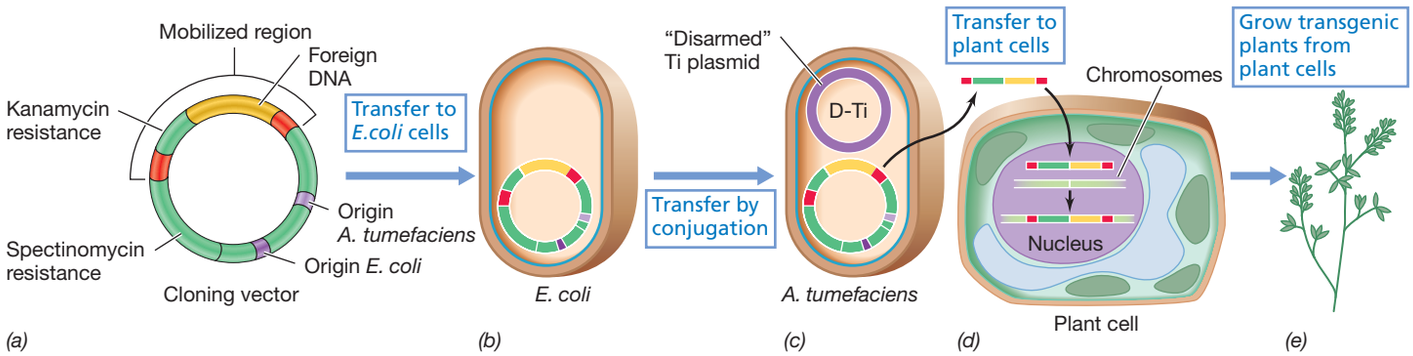


Figure 15.26 Production of transgenic plants using a binary vector system in *Agrobacterium tumefaciens*. *(a)* Plant cloning vector containing ends of T-DNA (red), foreign DNA, origins of replication, and resistance markers. *(b)* The vector is put into cells of *Escherichia coli* for cloning and then *(c)* transferred to *A. tumefaciens* by conjugation. The resident Ti plasmid (D-Ti) has been genetically engineered to remove key pathogenesis genes. *(d)* D-Ti can still mobilize the T-DNA region of the vector for transfer to plant cells grown in tissue culture. *(e)* From the recombinant plant cell, a whole plant can be grown.

Stephen R. Padgette, Monsanto Company

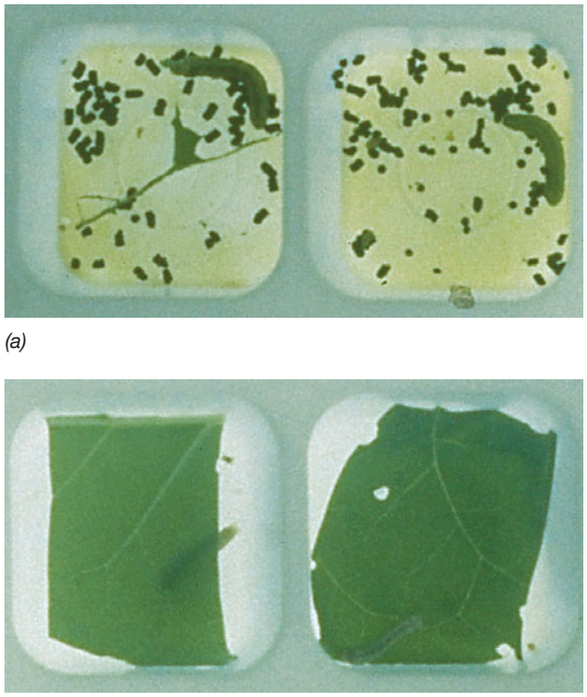
Figure 15.27 **Transgenic plants: herbicide resistance.** The photograph shows a portion of a field of soybeans that has been treated with Roundup™, a glyphosate-based herbicide manufactured by Monsanto Company (St. Louis, MO). The plants on the right are normal soybeans; those on the left have been genetically engineered to be glyphosate resistant.

For example, the herbicide glyphosate (Roundup™) kills plants by inhibiting an enzyme necessary for making aromatic amino acids. Some bacteria contain an equivalent enzyme and are also killed by glyphosate. However, mutant bacteria were selected that were resistant to glyphosate and contained a resistant form of the enzyme. The gene encoding this resistant enzyme from *A. tumefaciens* was cloned, modified for expression in plants, and transferred into important crop plants, such as soybeans. When sprayed with glyphosate, plants containing the bacterial gene are not killed (**Figure 15.27**). Thus glyphosate can be used to kill weeds that compete for water and nutrients with the growing crop plants. Herbicide-resistant soybeans are now widely planted in the United States.

Insect Resistance: Bt Toxin

Insect resistance has also been genetically introduced into plants. One widely used approach is based on introducing genes encoding the toxic proteins of *Bacillus thuringiensis* into plants. *B. thuringiensis* produces a crystalline protein called *Bt toxin* (↻ Section 18.2) that is toxic to moth and butterfly larvae. Many variants of Bt toxin exist that are specific for different insects. Certain strains of *B. thuringiensis* produce additional proteins toxic to beetle and fly larvae and mosquitoes.

Several different approaches are used to enhance the action of Bt toxin for pest control in plants. One approach is to use a single set of Bt toxins that is effective against many different insects. This is possible because the protein consists of separate structural regions (domains) that are responsible for specificity and toxicity. The toxic domain is highly conserved in all the various Bt toxins. Genetic engineers have made hybrid genes that encode the toxic domain and one of several different specificity domains to yield a suite of toxins, each best suited for a particular plant or pest situation.



(a)

(b)

Kevin McBride, Calgene, Inc.

Figure 15.28 **Transgenic plants: insect resistance.** *(a)* The results of two different assays to determine the effect of beet armyworm larvae on tobacco leaves from normal plants. *(b)* The results of similar assays using tobacco leaves from transgenic plants that express Bt toxin in their chloroplasts.

The Bt transgene is normally inserted directly into the plant genome. For example, a natural Bt toxin gene was cloned into a plasmid vector under control of a chloroplast ribosomal RNA promoter and then transferred into tobacco plant chloroplasts by microprojectile bombardment (↻ Section 11.7). This yielded transgenic plants that expressed Bt toxin at levels that were extremely toxic to larvae from a number of insect species (**Figure 15.28**).

Although transgenic Bt toxin looked at first to be a great agricultural success, some problems have arisen, in particular, the selection of insects resistant to Bt toxin. Resistance to insecticides and herbicides is a common problem in agriculture, and the fact that a product has been produced by genetic engineering does not exempt it from this problem. In addition, Bt toxin often kills nontarget insects, some of which may be helpful. Many approaches must be used for pest control in agriculture, and Bt toxin is just one of many. Nevertheless, transgenic crops with Bt toxin are widely planted in the United States.

Bt toxin is harmless to mammals, including humans, for several reasons. First, cooking and food processing destroy the toxin. Second, any toxin that is ingested is digested and therefore inactivated in the mammalian gastrointestinal tract. Third, Bt toxin works by binding to specific receptors in the insect intestine that are absent from the intestines of other groups of organisms. Binding promotes a change in conformation of the toxin, which then generates pores in the intestinal lining of the insect that disrupt the insect digestive system and kill the insect.

Other Uses of Plant Biotechnology

Not all genetic engineering is directed toward making plants disease resistant. Genetic engineering can also be used to develop GM plants that are more nutritious or that have more desirable consumer-oriented characteristics. For instance, the first GM food grown for sale in the United States market was a tomato in which spoilage was delayed, increasing the shelf life. In addition, transgenic plants can be genetically engineered to produce commercial or pharmaceutical products, as has been done with microorganisms and animals. For example, crop plants such as tobacco and tomatoes have been engineered to produce a number of products, such as the human protein *interferon*. Transgenic crop plants can also be used to produce human antibodies efficiently and inexpensively. These antibodies, called *plantibodies*, have potential as anticancer or antiviral drugs, and some are undergoing clinical trials. For example, transgenic tobacco plants have been used to make an antibody known as CaroRx that blocks bacteria that cause dental caries from attaching to teeth. CaroRx is made in high levels in tobacco leaves and is relatively easy to purify. So far clinical trials have shown it to be safe and effective. Plants are useful in producing these types of products because they typically modify proteins correctly and because crop plants can be efficiently grown and harvested in large amounts.

Crop plants are also being developed for the production of vaccines. For instance, a recombinant tobacco mosaic virus (Section 21.7) has been engineered whose coat contains surface proteins of *Plasmodium vivax*, one of the microbial parasites that cause malaria (Section 34.5). The *P. vivax* proteins elicit an immune response in humans. Hence, this recombinant virus could be used to produce a malaria vaccine cheaply in large amounts by simply harvesting infected tobacco or tomato plants and processing them for the immunogenic proteins. Another interesting approach is to produce a vaccine in an edible plant product. Such *edible vaccines* now under development could immunize humans against diseases caused by enteric bacteria, including cholera and diarrhea (Section 35.5).

A rather different kind of transgenic plant is the *Amflora potato*, developed by BASF, a German chemical company. The Amflora potato is not intended for eating. Unlike normal potatoes, which produce two types of starch, amylopectin and amylose, the Amflora potato makes only amylopectin, a raw material in the paper and adhesives industries. Use of Amflora potatoes will avoid the expensive and energy-consuming purification that removes amylose from amylopectin. Approval of this transgenic crop is presently under consideration by the European Union.

Although public acceptance of GM crops remains high in the United States, there have been some concerns over the contamination of human food with GM corn, so far approved only for animal food. In some European Union countries there has been considerable public concern over GM organisms. Most concerns center around either the perception of adverse effects of foreign genes on humans or domesticated animals or the potential "escape" of transgenes from transgenic plants into native plants. At present, supporting evidence for either of these scenarios is not strong; however, there are indications that glyphosate resistance genes are beginning to spread into the weed plant population. Thus, concerns remain about GM plants and have served to control the rate at which new transgenic plants enter the marketplace.

MiniQuiz

- What is a transgenic plant?
- Give an example of a genetically modified plant and describe how its modification benefits agriculture.
- What advantages do plants have as vehicles for making antibodies?

Big Ideas

15.1
An industrial microorganism must synthesize a product in high yield, grow rapidly on inexpensive culture media available in bulk, be amenable to genetic analysis, and be nonpathogenic. Industrial products include both cells and substances made by cells.

15.2
Primary metabolites are produced during the exponential phase and secondary metabolites are produced later. Large-scale aerobic industrial fermentations require stirring and aeration, and the process must be continuously monitored to ensure high product yields. Scale-up is the process of converting an industrial fermentation from laboratory scale to production scale.

15.3
Industrial production of antibiotics begins with screening for antibiotic producers. Once new producers are identified, chemical analyses of the antimicrobial agent are performed. If the new antibiotic is biologically active and nontoxic in both experimental animals and humans, high-yielding strains are sought for more cost-efficient commercial production.

15.4
The β-lactam antibiotics penicillin and cephalosporin and the tetracyclines are major drugs of medical and veterinary importance. Biosynthetic and semisynthetic derivatives of natural penicillins are the most widely used penicillins today.

15.5
Vitamins produced by industrial microbiology include vitamin B_{12} and riboflavin, and the major amino acids produced commercially are glutamate and lysine. High yields of amino acids are obtained by modifying regulatory signals that control their synthesis.

15.6
Many microbial enzymes are used in the laundry industry to remove stains from clothing, and thermostable and alkali-stable enzymes have many advantages for this application. When an enzyme is used in a large-scale process, it is often immobilized by being bonded to an inert substrate.

15.7
Most wine is made by fermenting the juice of grapes. Complex chemical changes occur during the wine fermentation due to a suite of chemicals in addition to alcohol. The malolactic fermentation is used to remove bitterness and produce a smoother final product.

15.8
Brewed products are made from malted grains, and distilled spirits are made by the distillation of ethanol and other flavor ingredients from brews, other fermented products, and wines.

15.9
Ethanol for use in biofuels can be made by the fermentation of glucose from starch or cellulose. Gasohol is the major biofuel in the United States and usually consists of a blend of gasoline (90%) and ethanol (10%).

15.10
To achieve very high levels of expression of eukaryotic genes in prokaryotes, the expressed gene must be free of introns. This can be accomplished by synthesizing cDNA from the mature mRNA encoding the protein of interest or by making an entirely synthetic gene. Protein fusions are often used to stabilize or solubilize the cloned protein.

15.11
The first human protein made commercially using engineered bacteria was human insulin. Recombinant bovine somatotropin is widely used in the United States to increase milk yield in dairy cows.

15.12
Many proteins are extremely expensive to obtain by traditional purification methods because they are found in human or animal tissues in only small amounts. Many of these can now be made in large amounts from a cloned gene in a suitable expression system.

15.13
Many recombinant vaccines have been produced or are under development. These include live recombinant, vector, subunit, and DNA vaccines.

15.14
Genes for useful products may be cloned directly from DNA or RNA in environmental samples without first isolating the organisms that carry them.

15.15
In pathway engineering, genes that encode the enzymes for a metabolic pathway are assembled. These genes may come from one or more organisms, but the engineering must achieve regulation of the coordinated sequence of expression required in the pathway.

15.16
Genetic engineering can make transgenic animals that produce proteins of pharmaceutical value and animal models of human diseases for medical research. Most recently, attempts are being made to improve livestock for human consumption and to reduce harmful environmental effects of mass-produced livestock.

15.17
A major hope of genetic engineering is in human gene therapy, a process whereby functional copies of a gene are inserted into a person to treat a genetic disease.

15.18
Genetic engineering can make plants resistant to disease, improve product quality, and make crop plants a source of recombinant proteins and vaccines. The Ti plasmid of the bacterium *Agrobacterium tumefaciens* can transfer DNA into plant cells. Genetically engineered commercial plants are called genetically modified (GM) organisms.

Review of Key Terms

Aspartame a nonnutritive sweetener composed of the amino acids aspartate and phenylalanine, the latter as a methyl ester
β-Lactam antibiotic a member of a group of antibiotics including penicillin that contain the four-membered heterocyclic β-lactam ring
Biofuel a fuel made by microorganisms from the fermentation of carbon-rich feedstocks
Biosynthetic penicillin a form of penicillin produced by supplying the synthesizing microorganism with a specific side-chain precursor
Biotechnology the use of genetically engineered organisms in industrial, medical, or agricultural applications
Brewing the manufacture of alcoholic beverages such as beer from the fermentation of malted grains
Broad-spectrum antibiotic an antimicrobial drug useful in treating a wide variety of bacterial diseases caused by both gram-negative and gram-positive bacteria
Distilled alcoholic beverage a beverage containing alcohol concentrated by distillation
DNA vaccine a vaccine that uses the DNA of a pathogen to elicit an immune response
Exoenzyme an enzyme produced by a microorganism and then excreted into the environment
Extremozyme an enzyme able to function in one or more chemical or physical extremes, for example, high temperature or low pH
Fermentation in an industrial context, any large-scale microbial process, whether carried out aerobically or anaerobically
Fermentor a tank in which an industrial fermentation is carried out

Fusion protein a genetically engineered protein made by fusing two DNA sequences encoding different proteins together into a single gene

Gene therapy the treatment of a disease caused by a dysfunctional gene by introducing a functional copy of the gene

Genetically modified organism (GMO) an organism whose genome has been altered using genetic engineering; the abbreviation GM is also used in terms such as GM crops and GM foods

Genetic engineering the use of *in vitro* techniques in the isolation, manipulation, alteration, and expression of DNA (or RNA), and in the development of genetically modified organisms

Immobilized enzyme an enzyme attached to a solid support over which substrate is passed and converted to product

Industrial microbiology the large-scale use of microorganisms to make products of commercial value

Malolactic fermentation a secondary fermentation used to remove bitterness in the production of some wines by the conversion of malic acid to lactic acid

Natural penicillin the parent penicillin structure produced by cultures of *Penicillium* not supplemented with side-chain precursors

Pathway engineering the assembly of a new or improved biochemical pathway using genes from one or more organisms

Polyvalent vaccine a vaccine that immunizes against more than one disease

Primary metabolite a metabolite excreted during a microorganism's exponential growth phase

Protease an enzyme that degrades proteins by hydrolysis

Reverse transcription the conversion of an RNA sequence into the corresponding DNA sequence

Scale-up the conversion of an industrial process from a small laboratory setup to a large commercial fermentation

Secondary metabolite a metabolite excreted from a microorganism at the end of its exponential growth phase and into the stationary phase

Semisynthetic penicillin a penicillin produced using components derived from both microbial fermentation and chemical syntheses

T-DNA the segment of the *Agrobacterium tumefaciens* Ti plasmid that is transferred into plant cells

Tetracycline a member of a class of antibiotics containing the four-membered naphthacene ring

Ti plasmid a plasmid in *Agrobacterium tumefaciens* capable of transferring genes from bacteria to plants

Transgenic organism a plant or an animal with foreign DNA inserted into its genome

Vector vaccine a vaccine made by inserting genes from a pathogenic virus into a relatively harmless carrier virus

Review Questions

1. In what ways do industrial microorganisms differ from microorganisms in nature? In what ways are they similar (Section 15.1)?
2. List three major types of industrial products that can be obtained with microorganisms, and give two examples of each (Section 15.1).
3. Compare and contrast primary and secondary metabolites, and give an example of each. List at least two molecular explanations for why some metabolites are secondary rather than primary (Section 15.2).
4. How does an industrial fermentor differ from a laboratory culture vessel? How does a fermentor differ from a fermenter (Section 15.2)?
5. List three examples of antibiotics that are important products of industrial microbiology. For each of these antibiotics, list the producing organisms and the general chemical structure (Sections 15.3 and 15.4).
6. Compare and contrast the production of natural, biosynthetic, and semisynthetic penicillins (Section 15.4).
7. What unusual characteristics must an organism have if it is to overproduce and excrete an amino acid such as lysine (Section 15.5)?
8. What is high-fructose syrup, how is it produced, and what is it used for in the food industry (Section 15.6)?
9. What are extremozymes? What industrial uses do they have (Section 15.6)?
10. In what way is the manufacture of beer similar to the manufacture of wine? In what ways do these two processes differ? How does the production of distilled alcoholic beverages differ from that of beer and wine (Sections 15.7 and 15.8)?
11. What is the major liquid biofuel made worldwide? How is it currently being made in the United States? Why is it necessary for new feedstocks to be developed (Section 15.9)?
12. What is the significance of reverse transcriptase in the cloning of animal genes for expression in bacteria (Section 15.10)?
13. What classes of mammalian proteins are produced by biotechnology? How are the genes for such proteins obtained (Sections 15.11 and 15.12)?
14. What is a subunit vaccine and why are subunit vaccines considered a safer way of conferring immunity to viral pathogens than attenuated virus vaccines (Section 15.13)?
15. How has metagenomics been used to find novel useful products (Section 15.14)?
16. What is pathway engineering? Why is it more difficult to produce an antibiotic than to produce a single enzyme via genetic engineering (Section 15.15)?
17. What is a knockout mouse? Why are knockout mice important for the study of human physiology and hereditary defects (Section 15.16)?
18. How has genetic engineering benefited the treatment of SCID and cystic fibrosis (Sections 15.12 and 15.17)?
19. What is the Ti plasmid and how has it been of use in genetic engineering (Section 15.18)?
20. List several examples in which crop plants have been improved by genetic engineering. How have genetically engineered plants helped human medicine (Section 15.18)?

Application Questions

1. As a researcher in a pharmaceutical company you are assigned the task of finding and developing an antibiotic effective against a new bacterial pathogen. Outline a plan for this process, starting from isolation of the low-yield producing organism to high-yield industrial production of the new antibiotic.
2. You wish to produce high yields of the amino acid phenylalanine for use in production of the sweetener aspartame. The overproducing organism you wish to use is not subject to feedback inhibition by phenylalanine, but is subject to typical repression of phenylalanine biosynthesis enzymes by excess phenylalanine. Applying the principles of enzyme regulation studied in Chapter 8 and microbial genetics in Chapter 10, describe two classes of mutants you could isolate that would overcome this problem, and detail the genetic lesions each would have.
3. You have just discovered a protein in mice that may be an effective cure for cancer, but it is present only in tiny amounts. Describe the steps you would use to produce this protein in therapeutic amounts. Which host would you want to clone the gene into and why? Which host would you use to express the protein in and why?
4. Gene therapy is used to treat people who have a genetic disease and, if successful, it will cure them. However, such people will still be able to pass on the genetic disease to their offspring. Explain. Why do you believe this might be an area of research that is not attracting as much attention as treatment of the individual?
5. Compare the advantages and disadvantages of using transgenic crops (such as Bt corn) as a source of human food. Consider various viewpoints, including that of the farmer, the environmentalist, and the consumer.

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