Industrial Microbiology

Bio

Bio lectures

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MICROORGANISMS AND INDUSTRY

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

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

Substrate (raw material) + microorganisms → new products

Prerequisites to Practical Industrial Microbiological Processes

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

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

Major Classes of Products and Processes

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

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

1 ***Pharmaceutical chemicals.*** Most prominent in this category are the antibiotics and steroid drugs, but other substances, such as insulin and interferon, are now being produced by genetically engineered bacteria. Many other new products are likely through genetic biotechnology.

2 ***Commercially valuable chemicals.*** Solvents, enzymes, and intermediate compounds for the synthesis of other substances are representative of the kinds of substances produced commercially by microorganisms. Specific examples are provided later in this chapter.

3 ***Food supplements.*** Mass production of yeasts, bacteria, and algae from media containing inorganic nitrogen salts and other readily available and cheap nutrients provides a good source of protein and other organic nutrients useful as food supplements. Large-scale microbial production of amino acids is an attractive industrial process being employed in many parts of the world.

4 ***Alcoholic beverages.*** Brewing, wine making, and production of other alcoholic beverages constitute some of the oldest and largest microbiological industries.

5 ***Vaccines (immunizing antigens).*** Some microorganisms are grown in very large quantities for use as vaccines. The whole cell or some part or product of the cell is used for the preparation of vaccines.

6 ***Deterioration of materials by microorganisms.*** All kinds of material such as leather, textiles, wood, metals, and even optical equipment are subject to deterioration by contamination with and growth of microorganisms. The magnitude of potential destruction with resulting financial losses demands that methods for prevention of this destruction be developed. Industry is responsive to this need and produces many chemicals and treatment processes for this purpose.

7 ***Analytical microbiology.*** Microbiological techniques have been developed for assaying a variety of products like antibiotics, amino acids, and vitamins. Other microbiological procedures are available for evaluating wood and paint preservatives and for testing the efficacy of sterilization procedures.

Microorganisms Used in Industrial Processes

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

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

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

BIOENGINEERING OF MICROORGANISMS FOR INDUSTRIAL PURPOSES

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

Genetic Engineering of Microorganisms

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

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

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

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

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

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

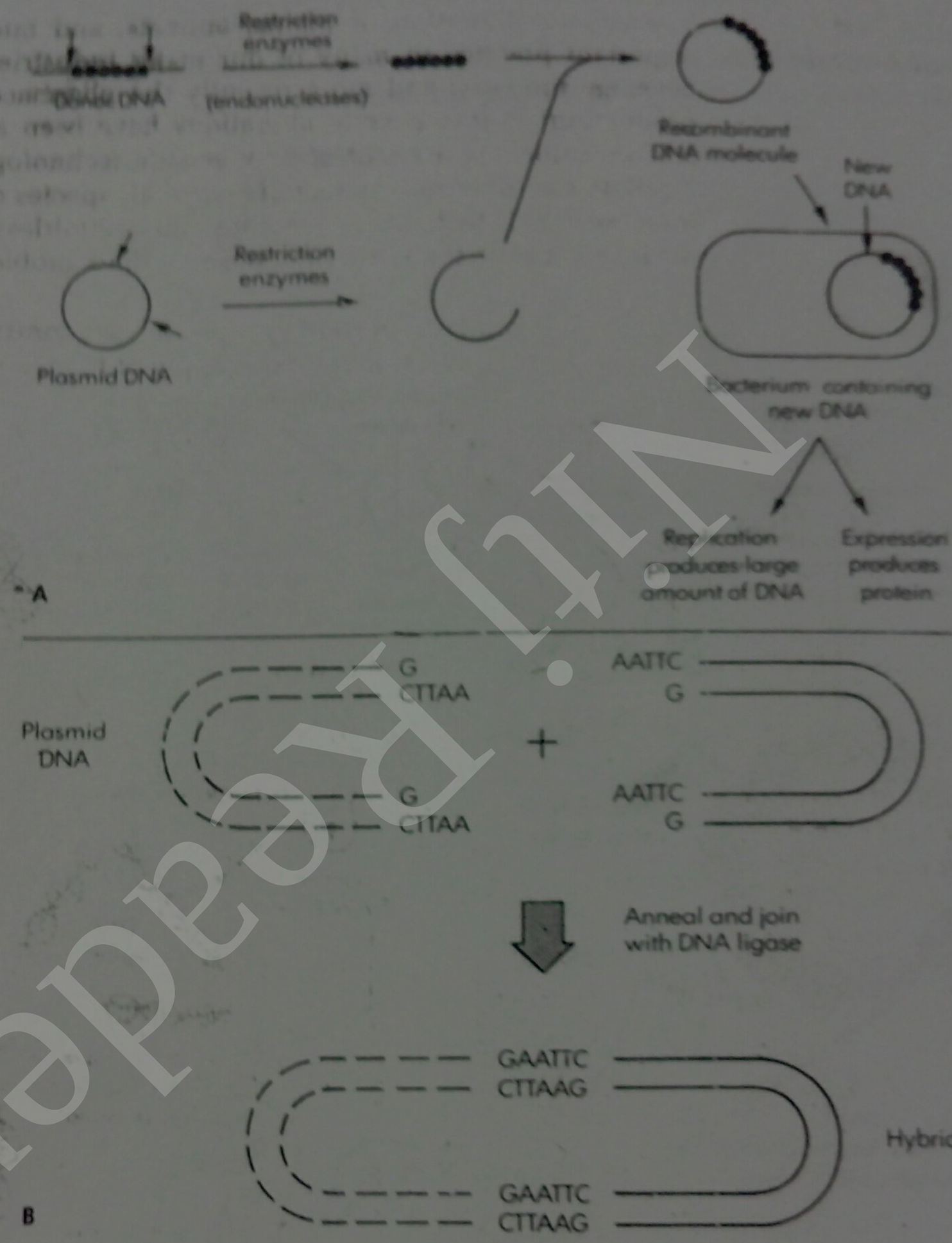


Figure 29-1. (A) The major steps in producing a "genetically engineered" bacterium. (B) Fragments of donor DNA and plasmid DNA excised by endonucleases and formation of hybrid DNA by joining these fragments using DNA ligase. *(Erwin F. Lessel, illustrator.)*

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

The Potential and Problems of Genetic Engineering

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

Benefits from Genetic Engineering

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

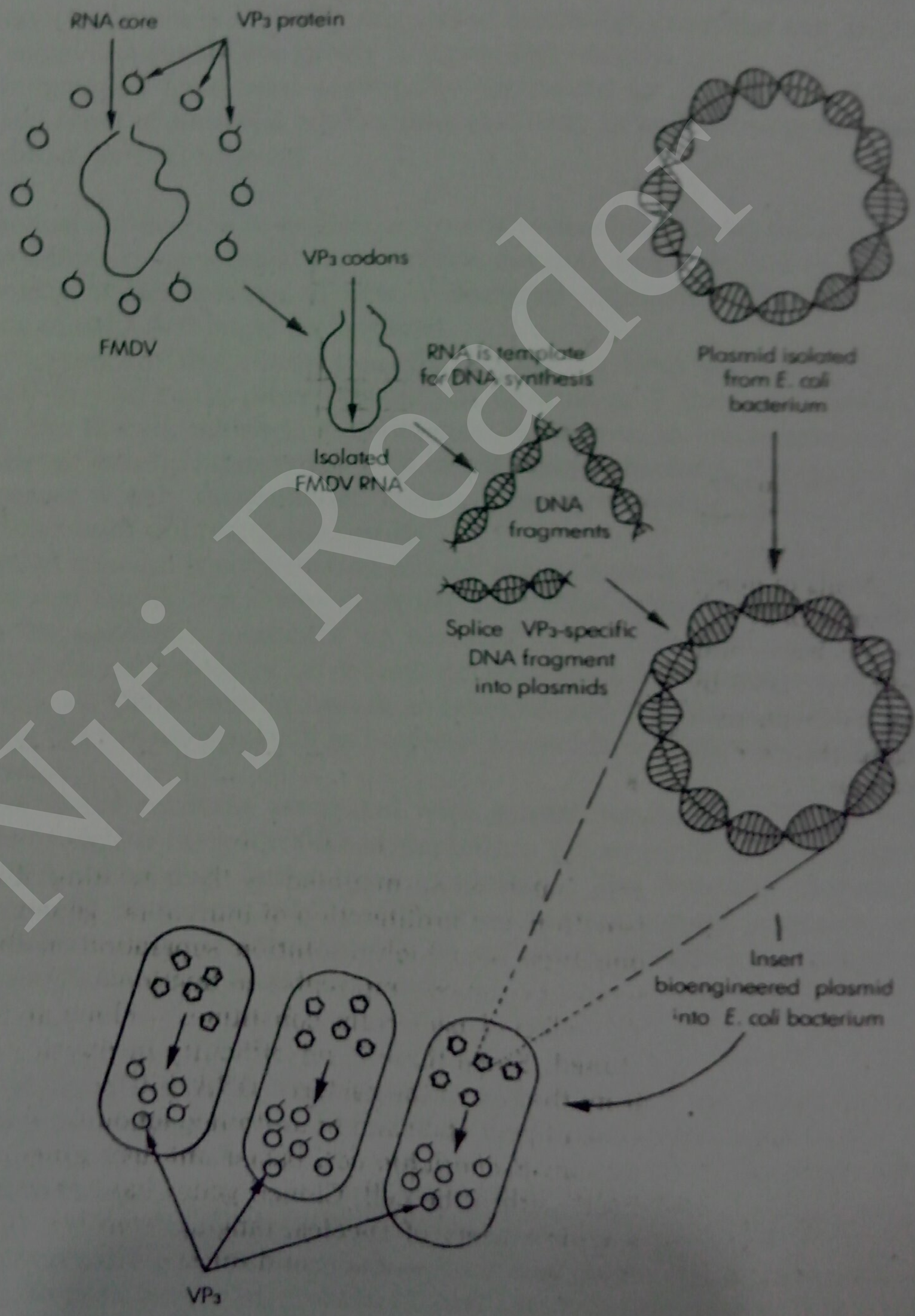


Figure 29-2. Recombinant DNA strategy for making foot-and-mouth disease vaccine. VP_3 is the protein from the shell of the foot-and-mouth disease virus (FMDV), which can act as a vaccine for immunizing livestock against foot-and-mouth disease. The idea is to make this VP_3 protein without making any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

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

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

Potential Problems of Genetic Engineering

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

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

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

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

INDUSTRIAL USES OF BACTERIA

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

Lactic Acid Production

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

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

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

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

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

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

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

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

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

Vinegar Production

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

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

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

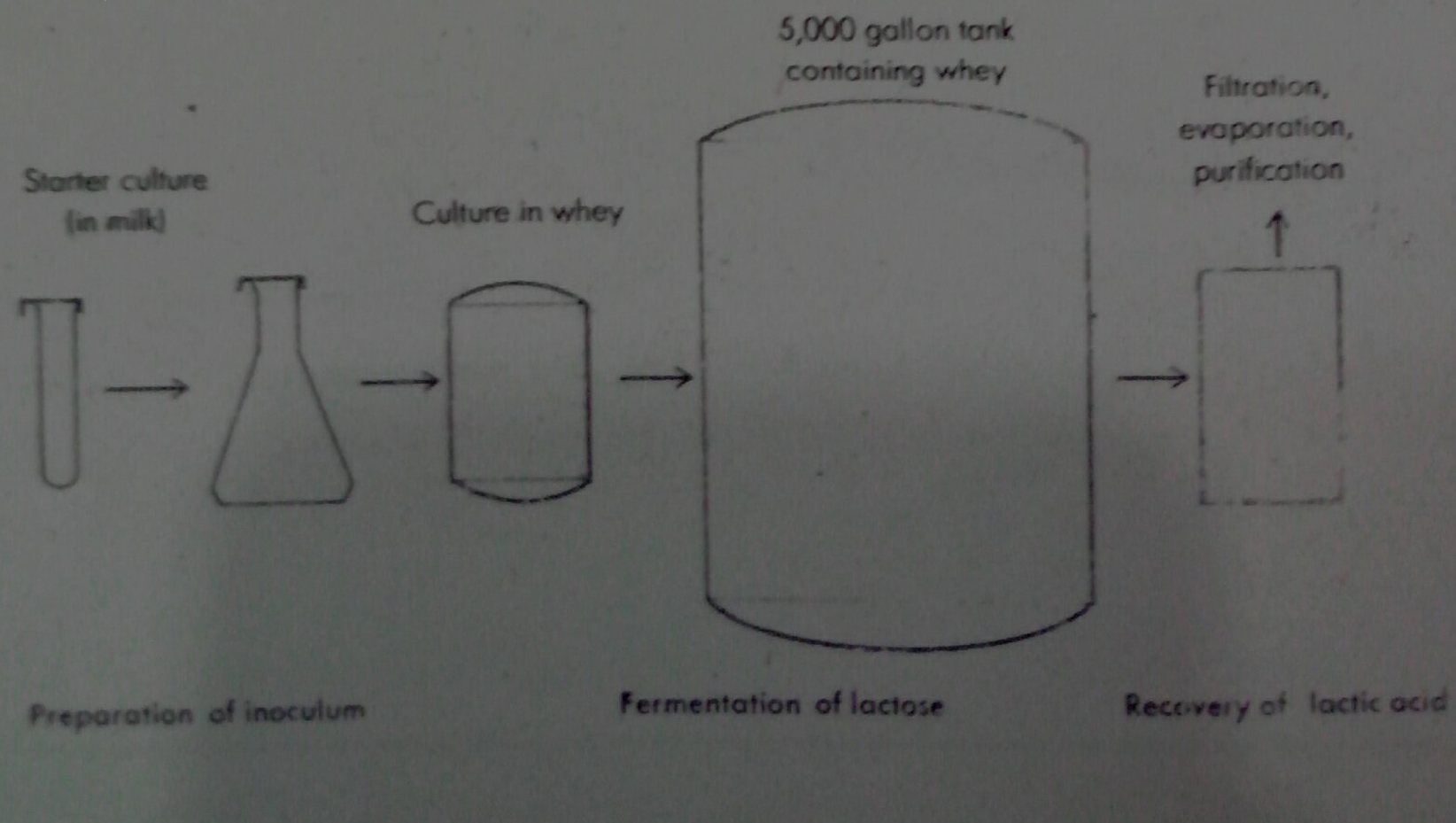


Figure 29-3. Lactic acid production from whey by *Lactobacillus bulgaricus*.

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

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

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

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

Amino Acid Production

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

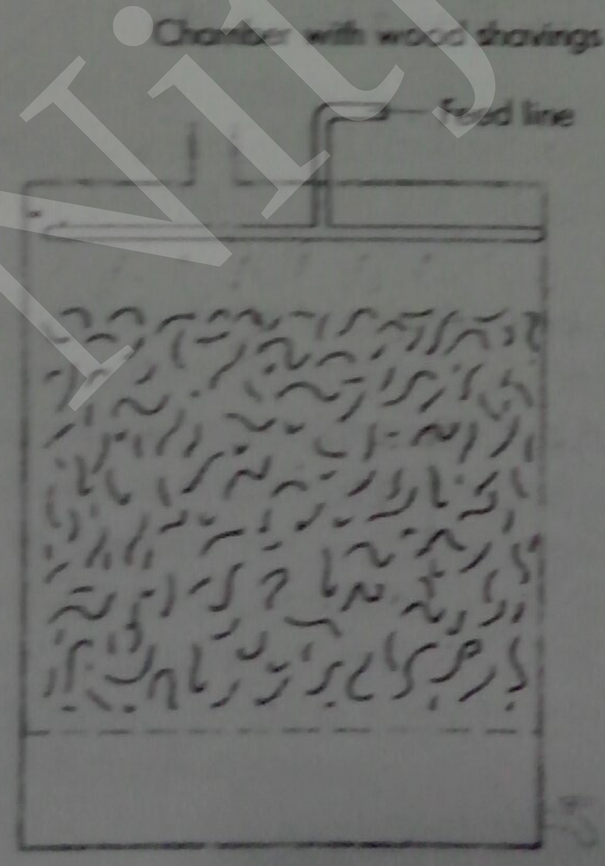
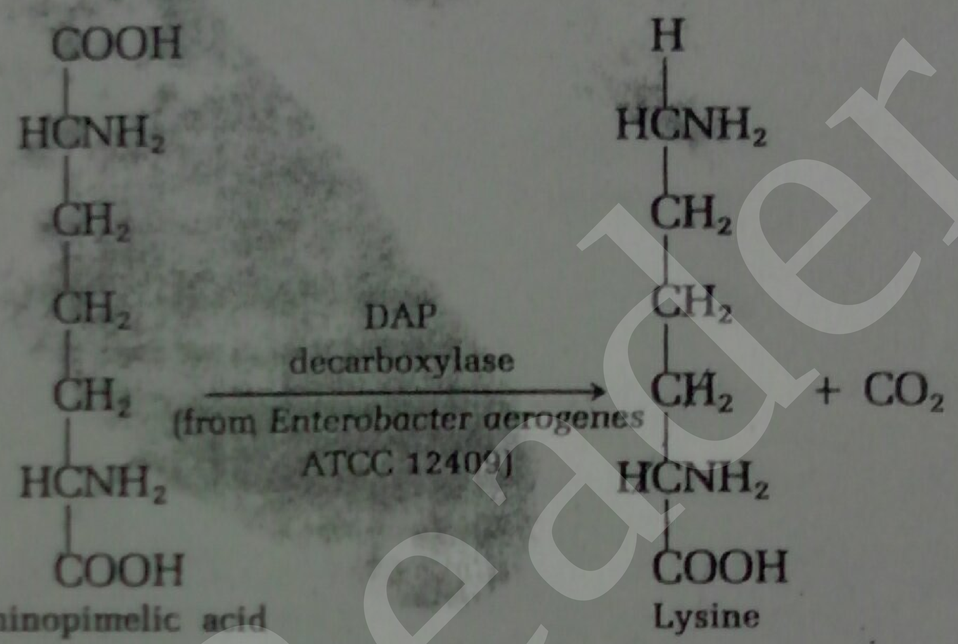


Figure 29-4. Frings vinegar generator. A dilute solution of alcohol percolates through wood shavings that are covered with a growth of acetobacters. The bacteria oxidize the alcohol to acetic acid.

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

L-Lysine Production

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



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

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

L-Glutamic Acid Production

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

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

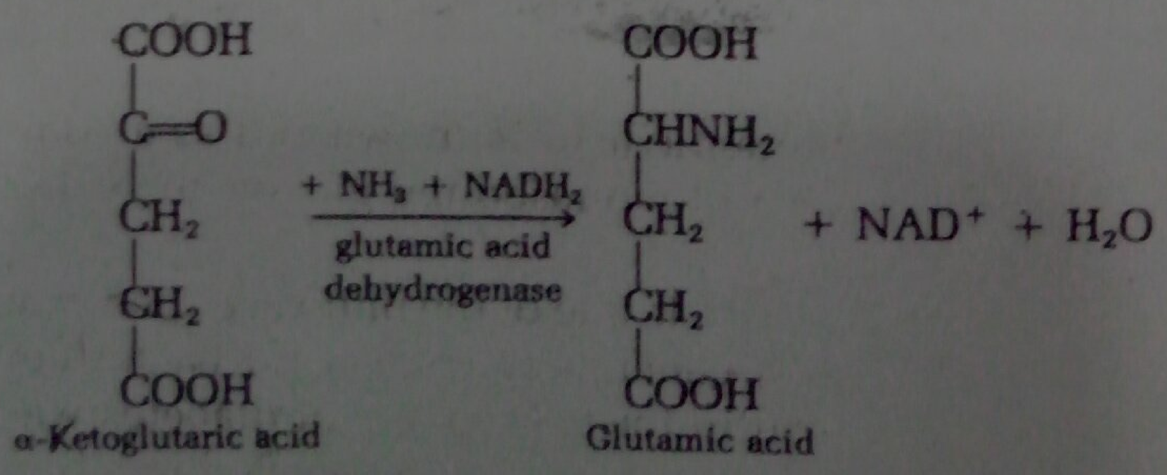


Figure 29-5. (A) Transmission electron micrograph of *E. coli* (X35,000) containing insulin A chain chimeric protein. Arrows indicate concentrations of this chimeric protein in the cells. (B) The first crystals ever obtained of human insulin made by recombinant DNA technology. *(Courtesy of Eli Lilly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

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

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

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

Table 29-2. Some Commercial Products of Yeast

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

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

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

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

INDUSTRIAL USES OF YEASTS

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

Alcohol Fermentations

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

The Substrate

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

The Organism

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

The Reaction

The biochemical change accomplished by the yeast is as follows:

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

Bakers' Yeast

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

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

Food Yeasts

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

INDUSTRIAL USES OF MOLDS

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

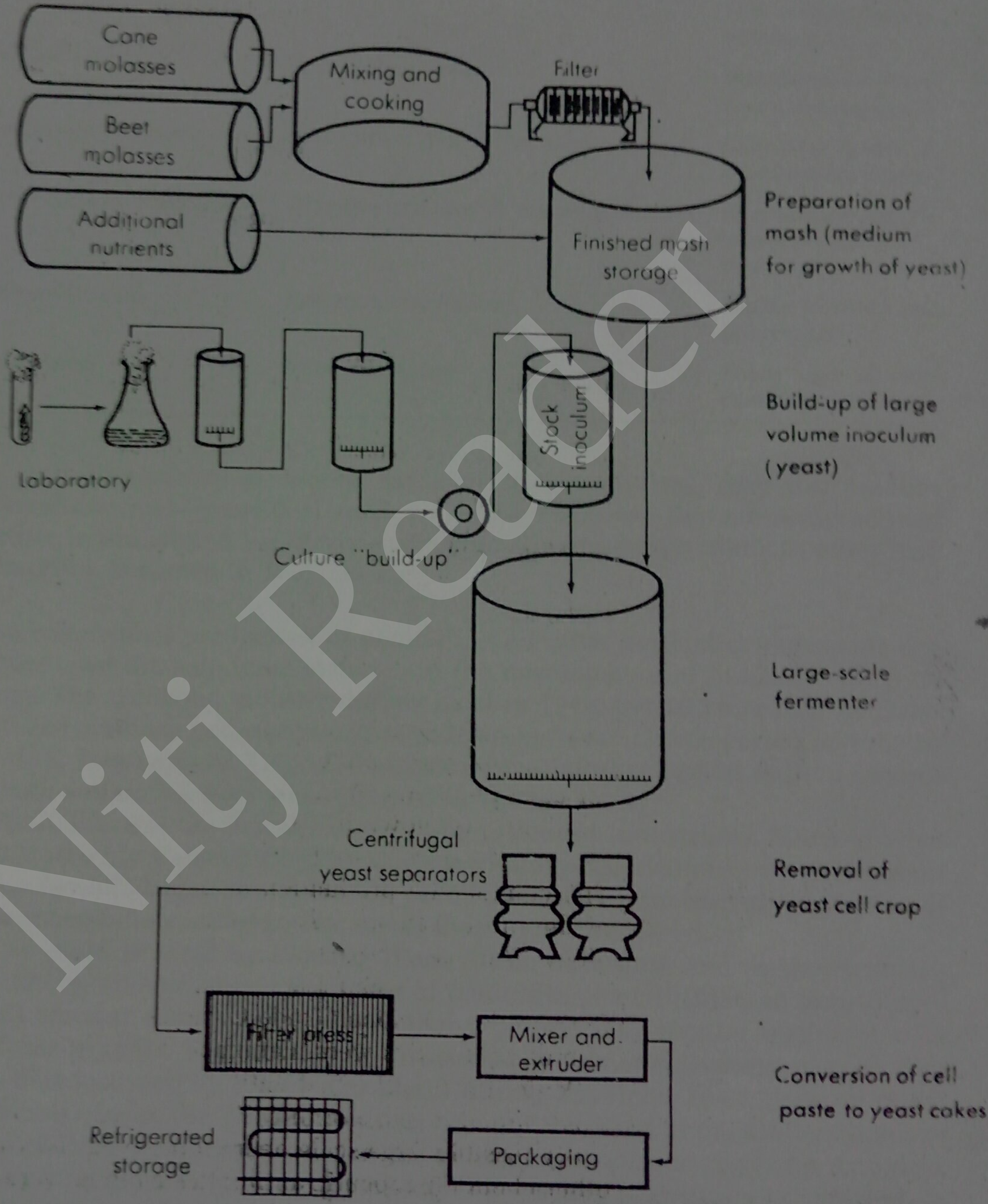


Figure 29-6. Steps in the commercial production of bakers' yeast.

29-3. Some Industrial ...ics (Other than Anti-...ics) Derived from Molds

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

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

Penicillin Production

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

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

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

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

The major steps in the commercial production of penicillin are

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

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

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

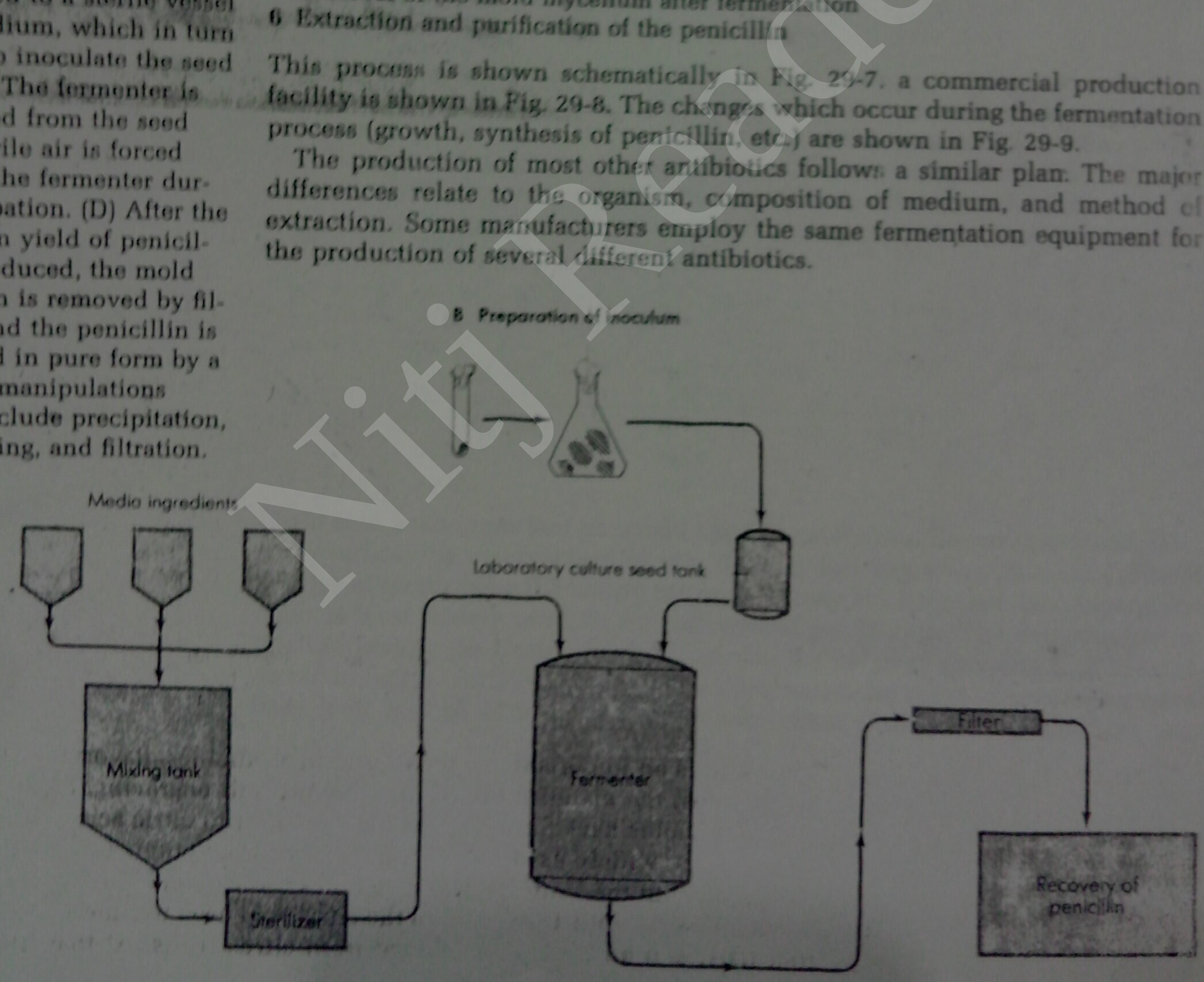


Figure 29-7. Manufacture of penicillin shown schematically. (A) A medium of corn-steep liquor, lactose, salts, and other ingredients is mixed, sterilized, cooled, and pumped into the fermenter. (B) The mold *Penicillium chrysogenum* is transferred from slant cultures to bran, and spore suspensions from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

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

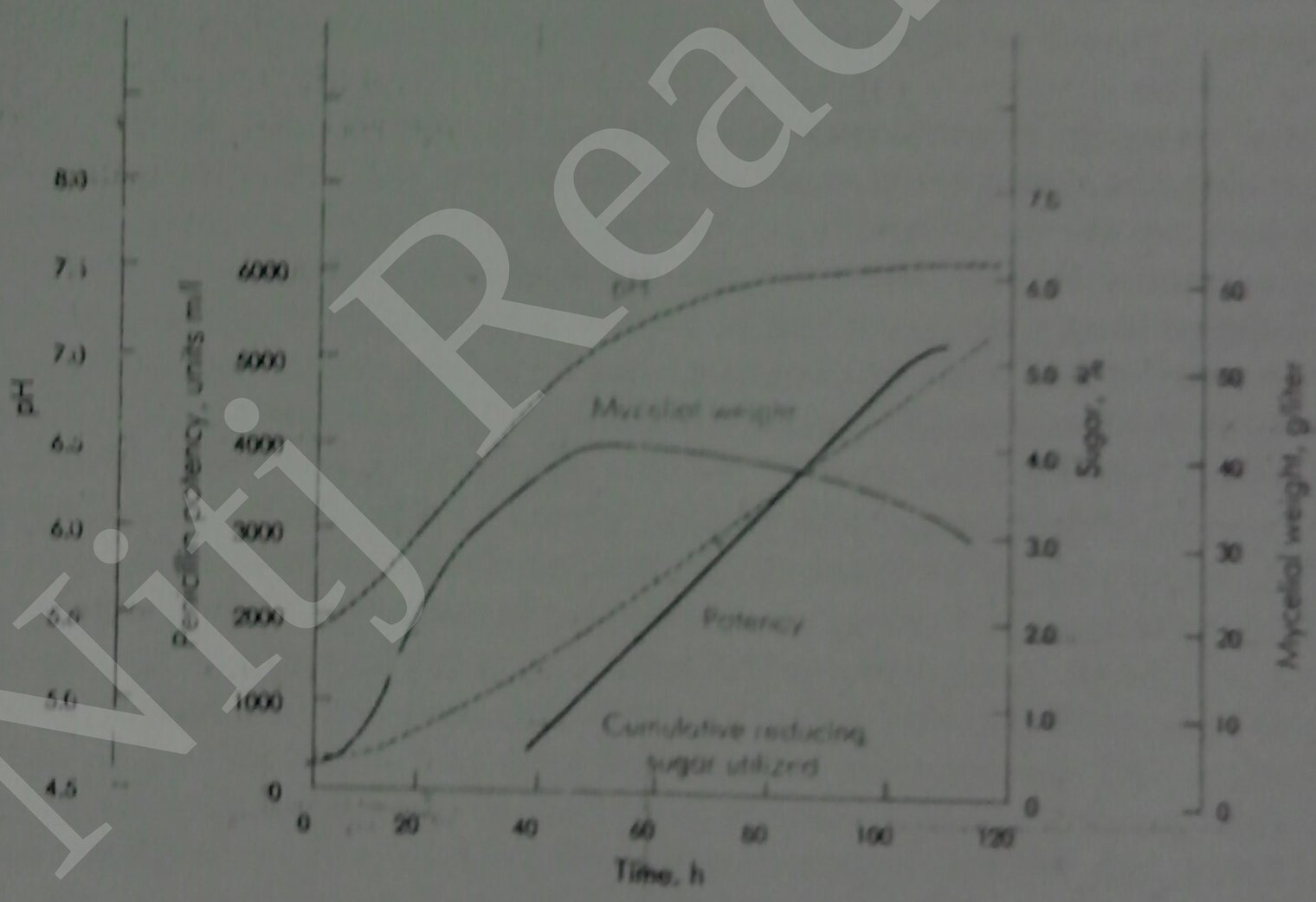


Figure 29-9. Biochemical changes that occur in the fermenter during production of penicillin by *Penicillium chrysogenum*. *(Courtesy of R. Donovick, Appl Microbiol, 8:117, 1960.)*

Citric Acid

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

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

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

Enzyme Production

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

Immobilized Enzyme Technology

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

29-4. Net Distribution
Selected Biologics
United States)

Product Description	Net Doses Distributed, January–June, 1983
Influenza vaccine	
Trivalent	45,630
Bivalent	
Diphtheria toxoid and tetanus toxoid (pediatric)	447,213
Diphtheria and tetanus toxoids with pertussis vaccine	8,897,380
Tetanus and diphtheria toxoid (adult)	4,733,327
Tetanus toxoid	3,818,263
Poliomyelitis vaccine, inactivated	17,320
Poliomyelitis vaccine, live, oral, trivalent	9,362,070
Measles virus vaccine, live, attenuated*	2,993,000
Rubella virus vaccine, live*	3,015,030
Mumps virus vaccine, live*	2,605,329
Smallpox vaccine	2,246,507
Immune serum globulin, human (reported in cc)	1,010,602
Tetanus immune globulin, human (reported in cc)	353,380

* All products containing this antigen.

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

HYBRIDOMAS AND MONOCLONAL ANTIBODIES

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

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

BIOLOGICS FOR IMMUNIZATION

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

PETROLEUM MICROBIOLOGY

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

Petroleum Formation

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

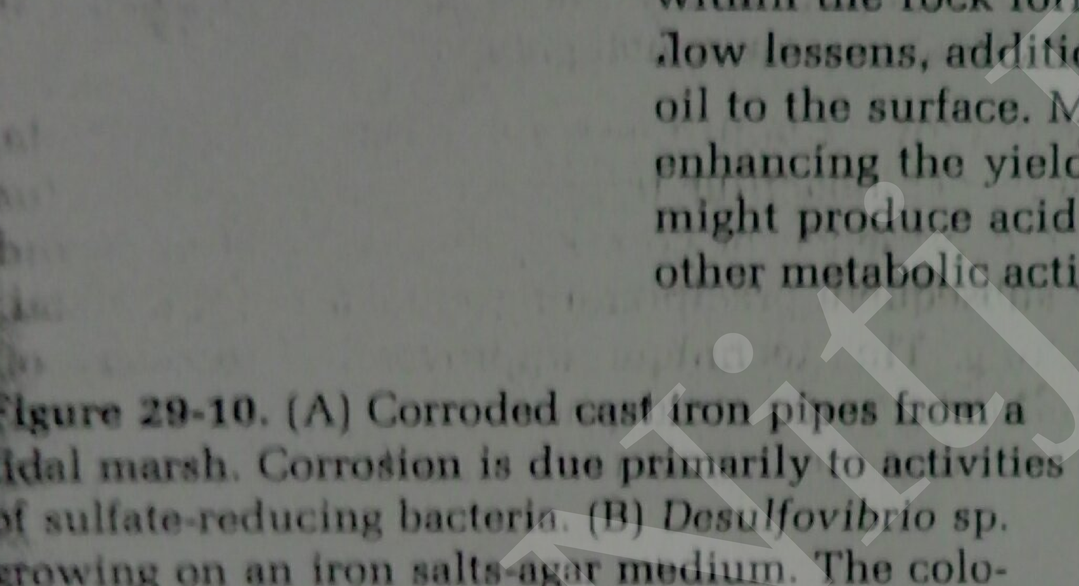
Petroleum Exploration

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

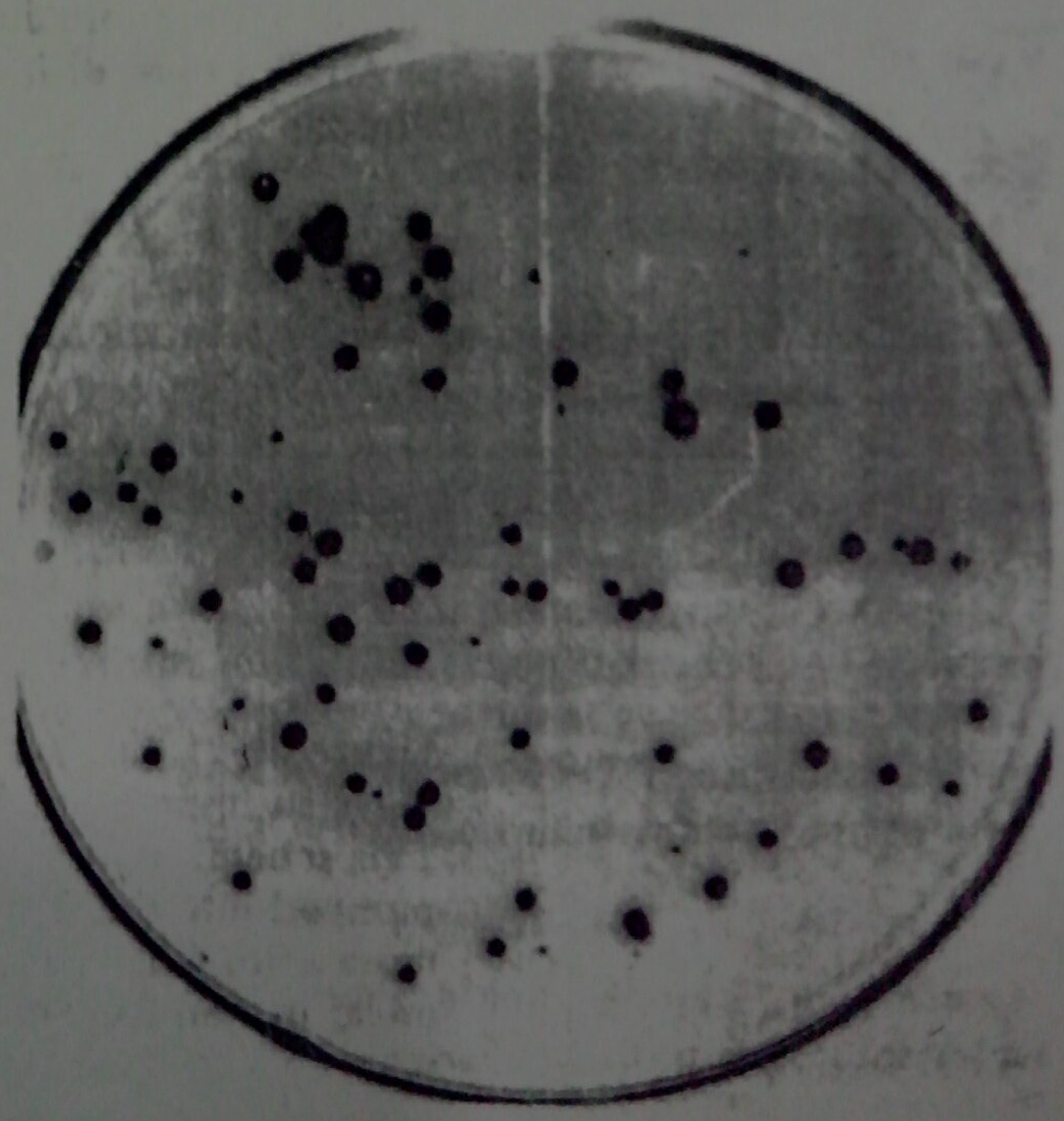
Petroleum Recovery

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

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



A



B

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

Oil Spills

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

MICROBIOLOGY AND MINING

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

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

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

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

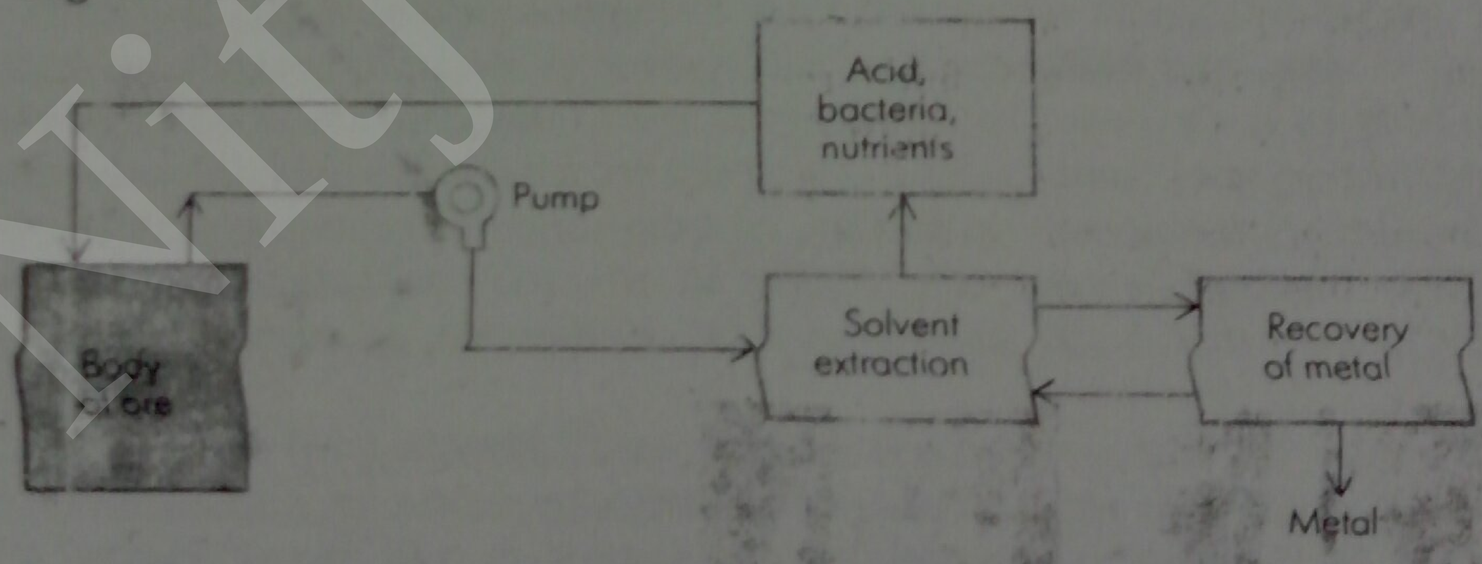


Figure 29-11. Leaching of low-grade ores using bacteria. *Thiobacillus ferrooxidans* plays an important role in the extraction (leaching) of metal from low-grade ores. This scheme shows an arrangement whereby the bacteria, nutrients, and acid are pumped into the ore bed. Continued growth of *Thiobacillus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

DETERIORATION OF MATERIALS

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

Paper

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

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

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

Textiles and Cordage

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

Painted Surfaces

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

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

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

Prevention of Microbial Deterioration

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

ANALYTICAL MICROBIOLOGY

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

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

Lactobacillus arabinosus requires the vitamin niacin for growth. When this

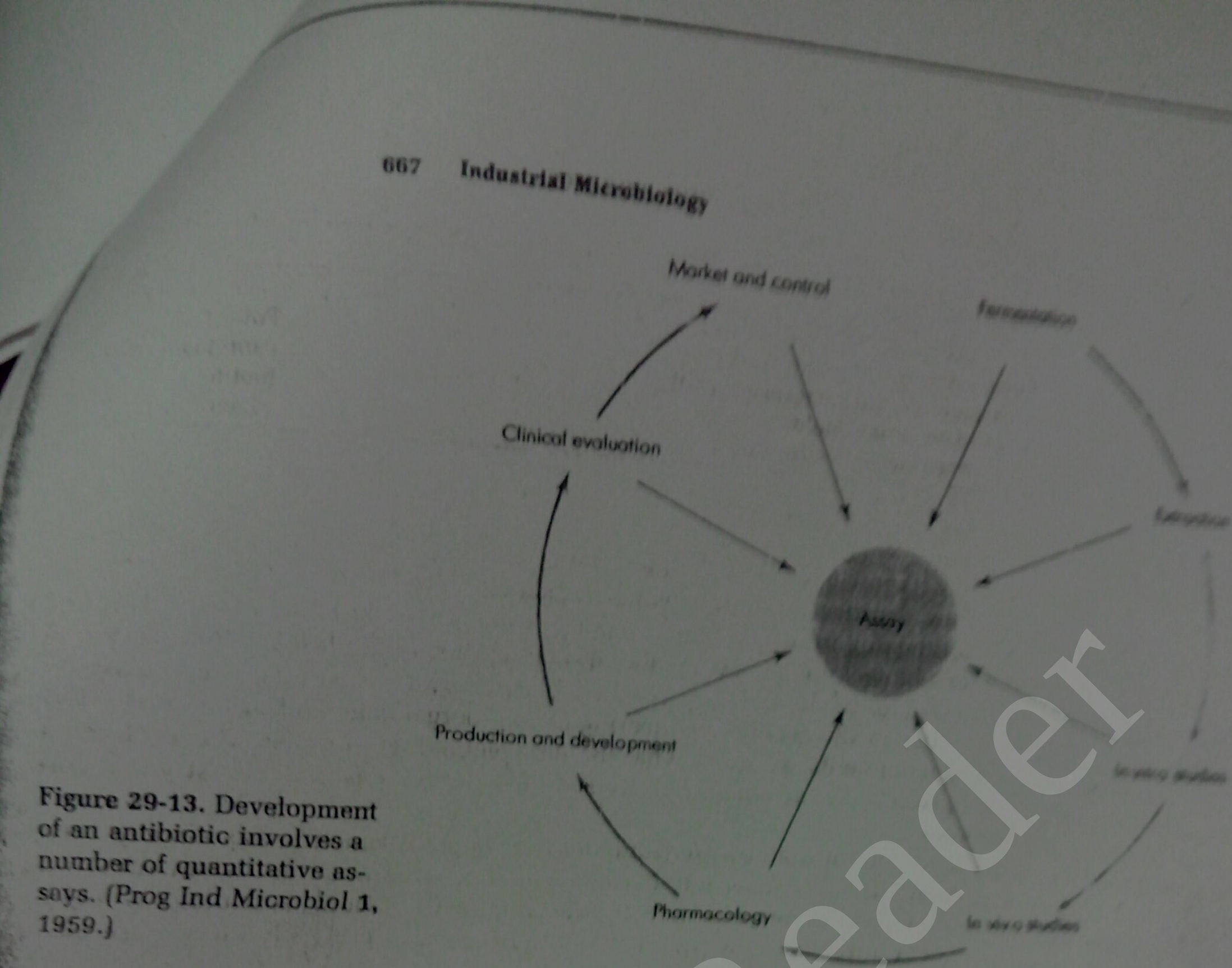


Figure 29-13. Development of an antibiotic involves a number of quantitative assays. (*Prog Ind Microbiol* **1**, 1959.)

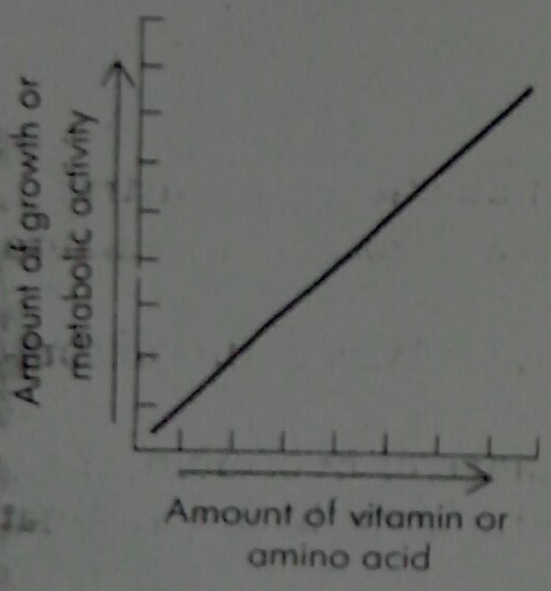


Figure 29-14. Principle of microbiologic assays as used for the measurement of vitamins and amino acids. Within limits of concentration of the substance being assayed, the amount of growth of the organism is proportionate to the amount of substance present.

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

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

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

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