ive or enzymatic conversion of chemically syn-
esized intermediates. The procedures have in-
lved primarily the use of immobilized cells or
mobilized enzymes. To optimize the process,
combinant DNA technology has been used.
e disadvantage of the enzymatic process is the
gh cost of the starting materials, indole, serine,
ruvate, or anthranilic acid. Because of this, ex-
sive work has been carried out to develop a
ect fermentation of L-tryptophan. Because of
complex regulatory mechanism of trypto-
an biosynthesis, these studies have not led to
rs sufficiently high so that L-tryptophan could
used in the food industry, although direct fer-
ntation can be used to produce this amino acid
medical uses.

le 9.8 Production of tryptophan by transformation
precursors

anism	Precursor	Amount used (g/l)	Yield (g/l)
senula nomala	Anthranilic acid	4.2	5.7
illus subtilis Anthranilate⁻)	Anthranilic acid	5.0	5.5
dida utilis	Anthranilic acid	4.2	6.4
piceps purpurea	Indole	1.3	1.5
herichia coli	Indole +	3.0	
	DL-Serine	6.0	5.2
illus subtilis FT⁻AG⁻)	Anthranilic acid	15.75 (feeding)	15.6

T, 5-fluorotryptophane; AG, 8-azaguanine)
n Kinoshita and Nakayama (1978), with additions.

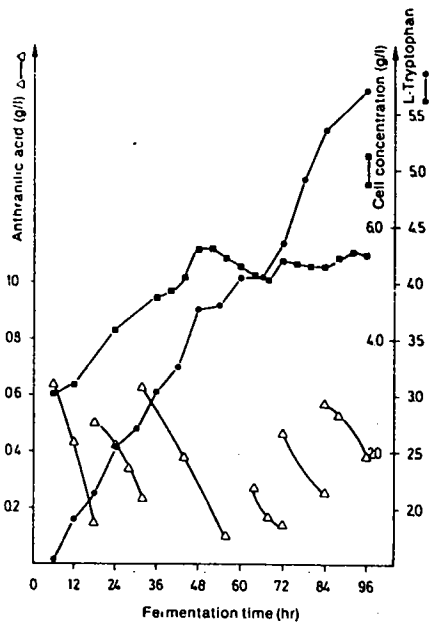


Figure 9.12 Production of L-tryptophan from anthranilic acid by *Hansenula anomala*. Cultured on the following medium (%): glucose 5; NH_4NO_3 0.3; KH_2PO_4 0.05; $Na_2HPO_4 \cdot 12\,H_2O$ 0.2; $NaH_2PO_4 \cdot 2\,H_2O$ 0.1; $MgSO_4 \cdot 7\,H_2O$ 0.05; NaCl 0.01; $FeSO_4 \cdot 7\,H_2O$ 0.001; yeast extract 0.1; anthranilic acid 0.2; $CaCO_3$ 2.0 (From Terui, 1972)

le 9.9 Enzymatic processes for the production of L-tryptophan

yme	Organism	Reaction
ryptophan hydantoin racemase yptophan hydantoin hydrolase arbamoyl-L-tryptophan ydrolase	*Flavobacterium aminogenes*	DL-Tryptophan hydantoin + H_2O→ L-Tryptophan + CO_2 + NH_3
tophanase	*Proteus rettgeri* *Achromobacter liquidum* *E. coli*	Indole + Pyruvate + NH_3→L-Tryptophan + H_2O Indole + L-Serine→L-Tryptophan + H_2O
tophan synthetase	*E. coli*	Indole + L-Serine→L-Tryptophan + H_2O

Fermentative conversion of intermediates stages

The most important fermentation processes are listed in Table 9.8. Figure 9.12 shows a typical fermentation sequence with *Hansenula anomala*. Starch hydrolysate or molasses is used as a carbon source and the feeding of sugar/NH_4NO_3-solution and anthranilate begins after the culture is grown.

Enzymatic process for tryptophan

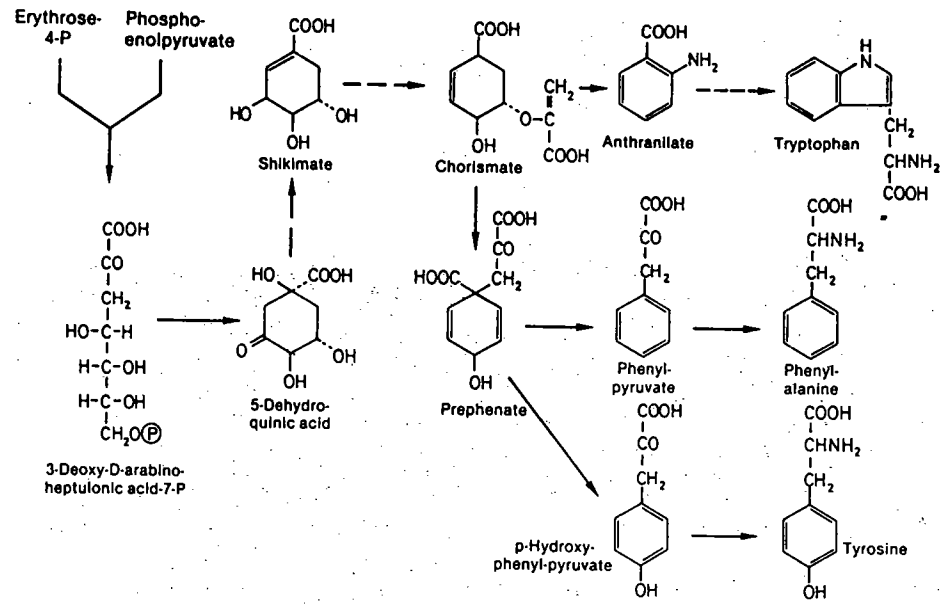
Table 9.9 lists enzymatic processes for L-tryptophan production. The methods include either the stereoselective hydrolysis of hydantoin compounds derived by chemical synthesis or the bioconversion of intermediates.

In the **hydantoinase technique**, an enzyme

Table 9.10 Mutants for the production of tryptophan by direct fermentation with glucose

Organism	Genetic characteristics	Yield (g/l)
Bacillus subtilis	5FT⁺, Arg⁻ or Leu⁻	6
*Enterobacter cloacae*TA 599	5MT⁺THA⁺Str⁺Tyr⁻	10
Corynebacterium glutamicum Px-115-97	Phe⁻ Tyr⁻ 5MT⁺ TrpHx⁺ 6FT⁺ 4MT⁺ PFP⁺ PAP⁺ TyrHx⁺ PheHx⁺	12
*Brevibacterium flavum*S-225	5FT⁺AZ⁺SG⁺PFP⁺Tyr⁻	19

Resistance: 5FT 5-fluorotryptophan; PFP p-fluorophenylalanine; 5MT 5-methyltryptophan; TrpHx Tryptophan hydroxamate; 6FT 6-fluorotryptophan; 4MT 4-methyltryptophan; PAP p-aminophenylalanine; TyrHx Tyrosine hydroxamate; PheHx Phenylalanine hydroxamate; AZ azide; SG sulfaguanidine; THA thienylalanine; Str streptomycin.
Auxotrophy: Arg arginine; Leu leucine; Phe phenylalanine; tyr tyrosine.



F ure 9.13 Pathways for the biosynthesis of tryptophan, phenylalanine and tyrosine

from *Flavobacterium aminogenes* which hydrolyzes D,L-tryptophan hydantoin is used. This strain has a reduced tryptophan-degrading ability and the enzyme is induced by the addition of D,L-tryptophan hydantoin to the medium. A 100% conversion of the hydantoin to L-tryptophan is obtained (starting material, 5% D,L-tryptophan hydantoin; 40°C; 100 hours), the tryptophan formed being removed from the reaction mixture by complex formation with inosine.

The most widely used commercial process is the **tryptophanase technique**, which involves the conversion of indole using *Proteus rettgeri*. The reaction mixture contains, per liter culture solution, 60 g indole (dissolved in 100 ml methanol), 80 g sodium pyruvate, 80 g ammonium acetate, 0.01 g pyridoxal phosphate, and 1 g sodium sulfate. After incubation at 34°C for 48 hours, 75 g of L-tryptophan is produced. By precipitation of the tryptophan with inosine as it is formed, the yield can be even higher (83.3 g/l), resulting in a molar conversion efficiency of 96%. In a related process, an enzyme reactor containing tryptophanase from *E. coli* has been used to convert indole and L-serine, resulting in a tryptophan concentration of 200 g/l and an efficiency of 95%.

Another process involves the use of the enzyme **tryptophan synthetase** which transforms indole and L-serine to L-tryptophan. In a mixed culture of *E. coli* and *Pseudomonas putida*, using D,L-serine as the starting material, the racemization of the D-form of tryptophan leads to yields of 23 g/l (81% efficiency).

Direct fermentation processes

The possibilities for a direct synthesis of L-tryptophan starting with inexpensive carbon sources are outlined in Table 9.10.

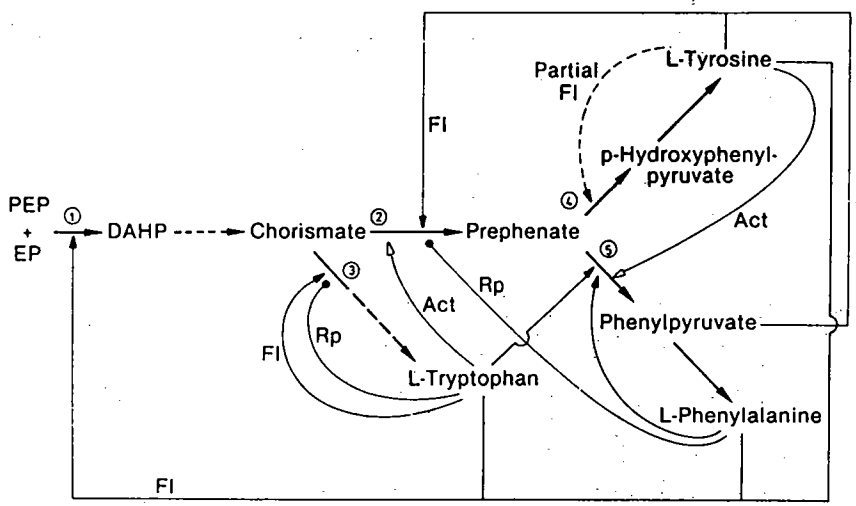


Figure 9.14 Regulation of aromatic amino acid biosynthesis in *Corynebacterium glutamicum*. The main sites of regulation are numbered, and the lines and symbols indicate the type of regulation. 1, DAHP synthetase; 2, Chorismate mutase; 3, Anthranilate synthase; 4, Prephenate dehydrogenase; 5, Prephenate dehydratase.
Fl, Feedback inhibition; Rp, Repression; Act, Activation.
EP, Erythrose-4-P; PEP, Phosphoenolpyruvate; DAHP, 3-Deoxy-D-arabinoheptulonic acid-7-P.
(From Nakayama et al., 1976)

The tryptophan biosynthetic pathway illustrated in Figure 9.13 has been found in many microorganisms, but there are considerable differences in the regulation of the individual organisms. In *Corynebacterium glutamicum*, for instance, tryptophan biosynthesis is regulated by the activity of DAHP synthetase and anthranilate synthase (Figure 9.14). DAHP synthetase, which catalyzes the condensation of erythrose-4-phosphate and phosphoenol pyruvate, shows concerted feedback inhibition by phenylalanine and tyrosine; tryptophan increases the inhibition so that the activity of DAHP synthetase is inhibited up to 90% in the presence of all three amino acids. Anthranilate synthase, the first enzyme of tryptophan biosynthesis after the branch point, shows feedback inhibition and repression due to L-tryptophan. The DAHP synthetase of *C. glutamicum* Px-115-97 is completely sensitive to regulation by phenylalanine and tyrosine. The maximum yield is determined not only by the effect of regulatory mechanisms but also by the specific activities of the individual enzymes of the biosynthetic chain. More yield increases are to be expected from the application of recombinant DNA technology to the development of derepressed strains of tryptophan producers, following the methods that have already been perfected with *E. coli*. The genes for the biosynthetic pathway from anthranilic acid to tryptophan are present in a single cluster. A tryptophanase-negative strain with a deletion of the *trp* operon has been transformed with a plasmid containing the *trp* operon in which the gene for anthranilate synthase was resistant to feedback inhibition by tryptophan. The *E. coli* strain Tna (pSC101 *trp* I15–14), whose plasmid contains five copies per genome, forms 4–5 g/l tryptophan when using glucose as carbon source. By the addition of a multicopy plasmid with the *trp* operon, it has also been possible to obtain yields of 23.5 g/l with *E. coli* strain JP 4114.

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10

Nucleosides, nucleotides, and related compounds

10.1 INTRODUCTION

Microbially produced nucleosides and nucleotides have found wide use as flavor-enhancers for food. The use of synthetic flavor-enhancers developed first in the Orient and has spread throughout the world. Since the beginning of the seventeenth century, the Japanese have used different substances (fungi, dried fish, seaweed, etc.) to enhance the flavor of foods. L-Glutamic acid (see Chapter 9) and the histidine salt of inosinic acid were identified in 1908 and 1913 as flavor-enhancing components of these substances. Further studies showed that a number of other substances, all purine ribonucleoside-5′-monophosphates, also improve flavor. In descending order of effectiveness, these are: guanylic acid (5′-GMP), inosinic acid (5′-IMP) and xanthylic acid (5′-XMP). Substances which have no effect are 5′-AMP, and the 2′ and 3′ isomers, as well as the 5′-deoxyribonucleotides, nucleosides and pyrimidine nucleotides.

The process of producing 5′-IMP and 5′-GMP through enzymatic hydrolysis of yeast RNA was developed in Japan in 1959 and was commercialized in 1961. The development of direct fermentation processes for nucleotide production was accomplished at about the same time, also in Japan. The discovery of synergism between the flavor-enhancing properties of sodium glutamate and the sodium salts of 5′-IMP and 5′-GMP was another research advance. IMP·Na_2, and GMP·Na_2, as well as mixtures of sodium and calcium salts of both nucleotides, are approved food additives and are frequently used in combination with sodium glutamate in prepared food products. To enhance the flavor of soups and sauces, for example, as little as 0.005–0.01% 5′-ribonucleotide additives is sufficient; moreover, addition of these additives can help eliminate undesirable properties, such as the metallic taste of cans. The amount of seasoning mixture consumed per meal is 0.1–0.2 g; the mixture contains 8–12% 5′-IMP and 1.5–2.0% 5′-GMP

added in combination with sodium glutamate (based on the Na glutamate content). Both hydrolytic RNA-splitting and direct fermentation processes are about equally used in Japanese commercial production, for an annual production of 3000 tons.

In addition to their use in the food industry, nucleotides, nucleosides and related compounds are being studied for **therapeutic purposes**. The antibiotic and cytostatic effects of purine analogs (8-azaguanine, 6-mercaptopurine) are being tested, particularly for cancer chemotherapy. 9-β-D-arabinofuranosyladenine is effective against herpes simplex and herpes zoster in humans.

The substances which are microbially produced in Japan and their applications are given in Table 10.1. The following Japanese companies are producers of 5′-ribonucleotides and nucleosides: Ajinomoto Co., Asahi Chemical Ind., Kyowa Hakko Kogyo Co., Takeda Chemical Ind. Ltd. and Yamasa Shoyu Co. Ltd.

Table 10.1 Nucleotides, nucleosides, and related compounds manufactured in Japan

Substance	Amount manufactured (tons/year)	Application
5′-IMP	2000	Flavor enhancer
5′-GMP	1000	Flavor enhancer
Inosine	25	Heart ailments
ATP	6	Muscular dystrophy
CDP-Choline	2	Stroke
FAD	Small amounts	Liver or kidney diseases
NAD	Small amounts	Liver or kidney diseases
Adenine	11	Leukopenia
Adenosine	5	Coronary deficiencies, angina pectoris, arteriosclerosis, high blood pressure
5′-AMP	?	Circulation problems, rheumatism
cAMP	?	Diabetes, asthma, cancer therapy, biochemical reagent
Orotic acid	20	Liver diseases
5-Azauridine	?	Cancer chemotherapy
Ara A (9-β-D-arabino-uranosyladenine)	?	Antiviral

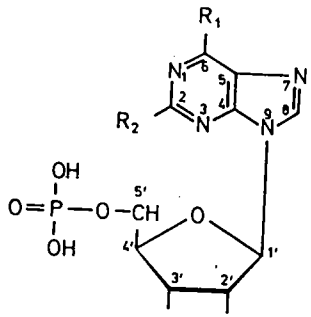
(Yamada, 1977, with additions)

10.2 STRUCTURE AND BIOSYNTHESIS

The structures of the economically relevant compounds are shown in Figure 10.1.

As seen in Figure 10.2, the biosynthesis of purine-5′-ribonucleotides proceeds from 5′-phosphoribosylpyrophosphate (PRPP), which is formed from ribose-5-phosphate and ATP. The first complete purine derivative, inosinic acid (5′-IMP), is produced after several reaction steps and 5′-IMP is then the precursor of 5′-AMP, 5′-XMP and 5′-GMP. AMP biosynthesis involves the action of adenyl succinate lyase (step M, Figure 10.2), a bifunctional enzyme which also catalyzes reaction H to AICARP.

In GMP biosynthesis, IMP is oxidized (reaction N) to XMP and aminated (reaction O) to GMP.



Nucleotide	R_1	R_2
5′-AMP	$-NH_2$	-H
5′-GMP	-OH	$-NH_2$
5′-IMP	-OH	-H
5′-XMP	-OH	-OH

Figure 10.1 Structure of the purine nucleotides

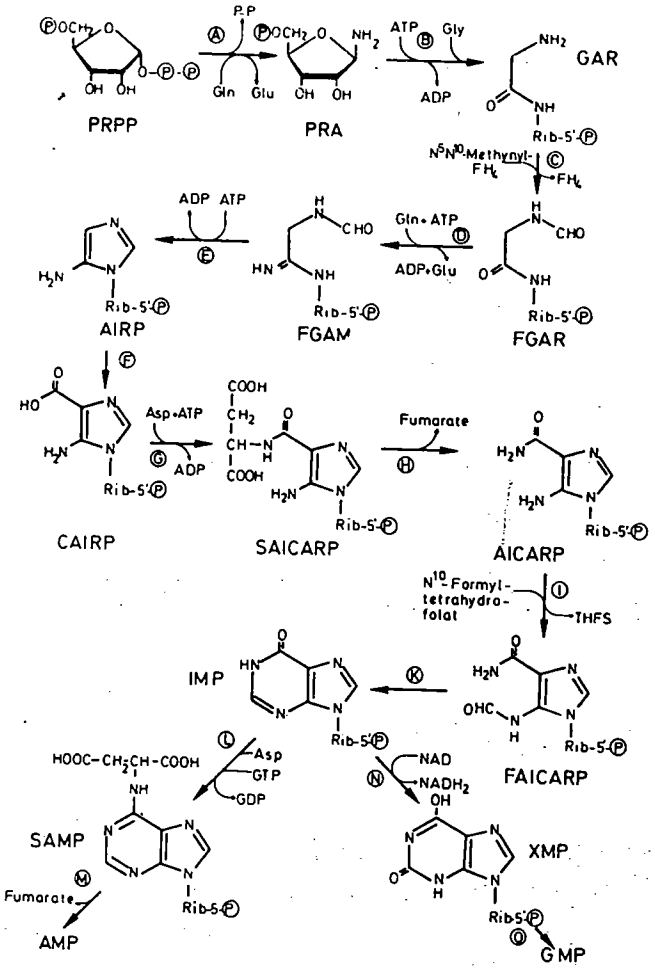


Figure 10.2 Biosynthesis of AMP and GMP.

PRPP = 5-Phospho-α-D-ribosylpyrophosphate;
PRA = 5-Phospho-β-D-ribosylamine;
GAR = 5′ Phosphoribosylglycinamide;
FGAR = 5′-Phosphoribosyl N′-Formylglycinamide;
FGAM = 5′-Phosphoribosyl-N′ formylglycinamidine;
AIRP = 1-(5′-Phosphoribosyl)-5 aminoimidazole;
CAIRP = 1-(5′-Phosphoribosyl)-5 aminoimidazole-4-carboxylate;
SAICARP = 1-(5′-Phosphoribosyl)-4-(N-Succinocarboxamide)-5-aminoimidazole;
AICARP = 5-Amino-1-(5′-phosphoribosyl)-imidazole-4-carboxamide;
FAICARP = 5-Formamido-1-(5′-phosphoribosyl)-imidazole-4 carboxamide;
SAMP = Adenylsuccinate

an excess of AMP or GMP is formed, GMP
e transformed through GMP reductase into
and AMP through AMP deaminase into
or through several steps into AICARP.

REGULATION

verproduction of purine nucleotides in wild
bacteria is prevented by feedback regula-
For commercial production, the regulatory
anisms must be partially eliminated. One
ach is to isolate auxotrophic mutants and
he required end product in growth-limiting
ntration, so that feedback inhibition and
sion are prevented. Another alternative is
late mutants with resistance to purine an-

e regulation of GMP and AMP synthesis
illus subtilis (Figure 10.3) has several pe-
ities. IMP, the precursor of AMP and GMP,
sformed mainly into GMP. IMP dehydro-
e, the first enzyme of this biosynthetic
ay, has a 10- to 30-fold greater specific ac-
han adenyl succinate synthetase. Feedback
tion and repression of IMP dehydrogenase
sed by GMP and XMP; the last enzyme of
ain, GMP synthetase, is only slightly af-
by GMP. Under these conditions the pro-
n of AMP is increased. AMP synthesis is
regulated by feedback inhibition of the
mmon enzyme, PRPP amidotransferase.
is **asymmetric control** by AMP, but the
tory effect of GMP is considerably lower:
f the inhibition of PRPP amidotransferase
mplished by 0.2 mM AMP or 2 mM GMP.
nzymes of AMP biosynthesis, adenyl suc-
synthetase and adenyl succinate lyase, are
ted by AMP.

RODUCTION

nt methods are used to produce nucleo-

ymatic or chemical breakdown of nu-
acids through

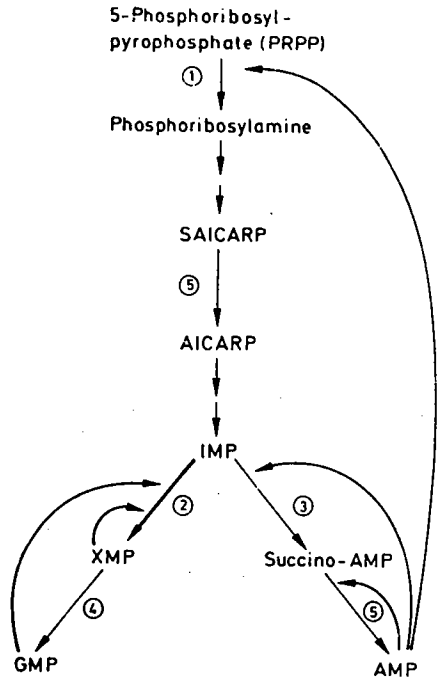


Figure 10.3 Regulation of purine nucleotide biosynthesis in *Bacillus subtilis*. 1, PRPP amidotransferase; 2, IMP dehydrogenase; 3, Adenylsuccinate synthetase; 4, GMP synthetase; 5, Adenylsuccinate lyase (From Shiio, 1979)

a. hydrolysis of yeast RNA by microbial enzymes
b. breakdown of cellular RNA by endogenous cell enzymes, leading to excretion of 5'-mononucleotides
c. chemical hydrolysis of yeast RNA into nucleosides with subsequent chemical phosphorylation.

2. **Direct fermentation** using mutants with a block in nucleotide biosynthesis, in which endproduct regulation is eliminated (as discussed above).
a. manufacture of nucleosides by fermentation and their chemical phosphorylation
b. direct manufacture of 5'-nucleotides through fermentation
c. microbial conversion of bases of nucleosides to 5'-nucleotides.

Methods 1a, 1c, 2a and 2b are used for industrial production of 5'-IMP and 5'-GMP.

Production of 5'-IMP and 5'-GMP by enzymatic hydrolysis of RNA

The breakdown of RNA was the first method used to produce 5'-nucleotides commercially. As late as 1975, 50% of the 5'-nucleotides in Japan were still obtained using this method. The multistage process consists of the following steps: 1) growth of yeast cells containing a high RNA content; 2) extraction of RNA from cell material; 3) production of enzymes used in RNA hydrolysis; 4) enzymatic hydrolysis of RNA; and 5) isolation and purification of 5'-IMP and 5'-GMP.

Obtaining RNA The nucleic acid content of different microorganisms varies considerably:

Microorganisms	DNA content (%)	RNA content (%)
Bacteria	0.37–4.5	5 –25
Yeast	0.03–0.52	2.5–15
Fungi	0.15–3.3	0.7–28

The RNA of the cell consists of 5% messenger RNA, 10–15% transfer RNA and 75–80% ribosomal RNA. Because of their relatively low DNA content, yeasts are the best sources of RNA. Moreover, there are well-developed fermentation and recovery techniques for yeast. The yeast *Candida utilis* is most commonly used, followed by *Saccharomyces cerevisiae*. The RNA content of yeast cells is very dependent on culture conditions. A high proportion of RNA is found in the early logarithmic phase, particularly in media with a low carbon/nitrogen ratio. The RNA content of cells growing on molasses or glucose can be further increased by adding zinc ions (0.25 ppm) and phosphate (0.15%). The yeast fermentation is generally done in continuous culture in an airlift fermenter (aeration rate, $K_a > 20 \times 10^{-4}$ g·mole·O_2/atm·min·ml), using either sulfite waste liquor or molasses. In Japan, a large production plant may produce 10,000–20,000 tons of yeast cells per year.

The cell mass produced in this process is 35 g/l, with an RNA content of 10% (sulfite waste liquor) or 15% (molasses). The cell material is separated and dried by means of yeast separators, and the RNA is then extracted with hot alkaline saline (5–20% NaCl, 8 h, 100°C). After separating the cell residue, the RNA is precipitated by adding HCl or ethanol and is dried. The crude preparation, which has an RNA content of 70–90% with a molecular weight distribution of 10,000–150,000, can be used directly for enzymatic hydrolysis.

Another process for the production of nucleotides makes use of acidophilic, methanol-utilizing bacteria such as *Methylobacter acidophilus*.

Enzymatic hydrolysis As Figure 10.4 shows, several distinct enzymatic reactions can be used for the hydrolysis of RNA. For optimal production of 5'-IMP and 5'-GMP, organisms must be used which have higher activities for the enzymes which catalyze steps a–d (Figure 10.4) than for steps e–g, in order to avoid the production of undesirable substances. Two organisms, *Penicillium citrinum* and *Streptomyces aureus*, have been found to contain enzymes which meet these requirements, and enzymatic RNA hydrolysis on an industrial scale is carried out exclusively with these two organisms.

One process makes use of hydrolysis with nuclease P_1 from *Penicillium citrinum*. A pigment-free mutant from *P. citrinum* is cultivated on wheat bran for 5 days at 30°C. The aqueous extract contains the thermostabile nuclease P_1, which quantitatively splits the 3'–5'-phosphodiester linkage of polynucleotides and the 3'-phosphomonoester linkage of mono- and oligonucleotides. The undesired phosphomonoesterase activity, which produces 3'-nucleotides, can be almost completely inactivated by heat treatment. To eliminate effects of the residual phosphatase activity (optimal temperature

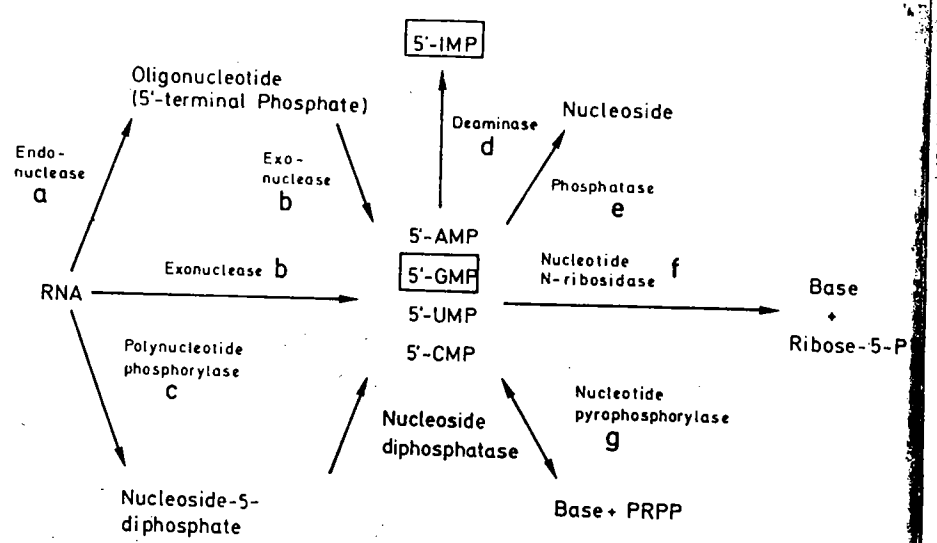


Figure 10.4 Enzymes which are involved in the production of GMP and IMP via RNA hydrolysis (see text for explanation)

45°C), hydrolysis of a solution of 2% RNA is performed in 4 hours at pH 5.0 at the optimal temperature of nuclease P_1 (65°C). In this reaction, 5'-GMP, 5'-AMP, 5'-CMP, and 5'-UMP are produced. The 5'-AMP is deaminated by adenyl deaminase from *Aspergillus oryzae* to 5'-IMP.

A process with immobilized nuclease P_1 has also been described.

Separation of the 5'-nucleotides formed through this enzymatic hydrolysis is performed by means of anion exchange chromatography.

Another process involves hydrolysis with nucleases from *Streptomyces aureus*. *S. aureus* forms a mixture of endo- and exonucleases; any 5'-AMP is thus deaminated by AMP deaminase to 5'-IMP. By isolating appropriate mutants, researchers have attempted to reduce the amounts of a 5'-nucleotidase and an unspecific alkaline phosphatase which occur in the enzyme mixture produced by *S. aureus*. Table 10.2 shows the enzyme activities of different mutants compared with the wild type. Mutant A-5 is the best mutant because of its clearly reduced phosphatase activity. The production of the enzymes takes place during a 30-hour incubation in a submerged culture ($K_d = 2.72 \times 10^{-3}$ g·Mol·O_2/atm·min·ml) at 28°C on a medium containing starch hydrolysate, soy meal, and corn steep liquor. Typical progress of a 6 m³ fermentation of *Streptomyces aureus* is given in Figure 10.5. Because of the high 5'-nucleotidase activity of the *S. aureus* enzymes, RNA hydrolysis is carried out at a different temperature and pH. A 0.5–1.0% RNA solution is hydrolyzed for 10 hours at 42–65°C in a pH range between 7.0–8.0.

The purification of 5'-GMP and 5'-IMP is done by means of a preliminary adsorption with activated carbon, then ion exchange chromatography, followed by a fractional precipitation with methanol.

Table 10.2 Enzyme production by *Streptomyces aureus* mutants relative to the wild type

Strain	Endonuclease	Exonuclease	AMP deaminase	5'-Nucleotidase	Alkaline phosphatase
Wild type	100	100	100	100	100
K-1	310	180	110	160	20
A-5	400	210	150	180	10
S-8	100	80	100	60	10

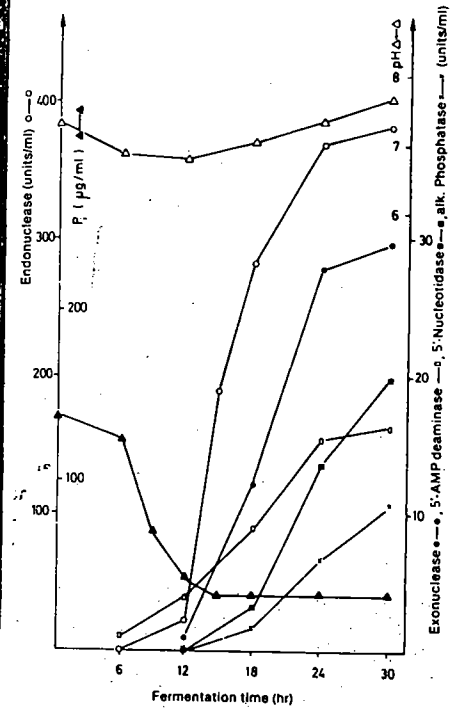


Figure 10.5 Fermentation process for nuclease production with *Streptomyces aureus*

Production of 5'-IMP and 5'-GMP through chemical hydrolysis of RNA

In the chemical hydrolysis of RNA under alkaline conditions, 5'-nucleotides are not produced directly, but a mixture of 2'- and 3'-nucleotides is produced via an intermediate 2',3'-cyclic phosphodiester. However, nucleosides can be produced quantitatively from RNA by heating to 130°C for 3–4 hours in calcium hydroxide. The chemical phosphorylation of these nucleosides to 5'-IMP and 5'-GMP then can be done, after the deamination of adenosine to inosine.

Production of 5'-IMP by direct fermentation

5'-IMP can be produced fermentatively by various methods:

- Through microbial production of inosine and subsequent chemical phosphorylation to 5'-IMP
- Through direct fermentation to 5'-IMP
- Through fermentative production of adenosine or 5'-AMP and subsequent chemical or enzymatic conversion into 5'-IMP
- Through microbial conversion of chemically synthesized hypoxanthine into 5'-IMP.

For economic reasons only, the first two processes are used commercially.

Fermentative production of inosine The cell membrane is not permeable to nucleotides but is permeable to the dephosphorylated derivatives, the nucleosides. When a cell synthesizes 5'-IMP, it cannot be excreted through the cytoplasmic membrane; however, it can be dephosphorylated by the cell and then can be excreted, so that it accumulates in the medium as inosine. Because of the regulatory effect of AMP and ADP on PRPP amidotransferase in *Bacillus subtilis*, the adenine nucleotide content of the cells must be

kept at a low level to produce IMP. This is accomplished through a block in the AMP biosynthesis (absence of SAMP-synthetase). Reduced nucleosidase activity, absence of IMP-dehydrogenase and/or an increase in 5'-nucleotidase activity also favor the accumulation of inosine. Inosine-producing microorganisms were first found among adenine auxotrophs of different strains of the genera *Bacillus, Brevibacterium, Corynebacterium, Streptomyces,* and *Saccharomyces. Corynebacterium petrophilum* (Ade⁻) accumulates 1.6 g/l inosine on a medium with n-alkanes (C_{12}–C_{16}). Various strains of *Bacillus subtilis, Brevibacterium ammoniagenes,* and *Microbacterium* have been developed for commercial production, as shown in Table 10.3.

In a typical fermentation process with *Bacillus*, starch hydrolysate is most commonly used as the carbon source, but glucose can also be used. Dry yeast or crude RNA serves as the source of adenine. Ammonia is added both to regulate the pH and as a nitrogen source. The optimal pH range is 6.0–6.2, the optimal temperature 30–34°C. Maximal inosine accumulation is obtained with an oxygen transfer coefficient of $K_d = 5.8–7.0 \times 10^{-6}$ g·Mol·O_2/atm·min·ml. At the same time, the CO_2 content of the medium must be low. The progress of an inosine fermentation with *Brevibacterium ammoniagenes* is illustrated in Figure 10.6.

The final stage is the phosphorylation of inosine. At pH 11, inosine is precipitated from a culture filtrate and crystallized. The chemical phosphorylation of inosine is carried out using trialkyl phosphate with PCl_3. The amount of undesired 2'(3')5'-phosphodiester can be reduced to < 10%; under these conditions the 5'-monoester content is 90%.

Direct fermentation of 5'-IMP Mutants for the direct fermentation of 5'-IMP should have the following properties:

Table 10.3 Mutants used for the inosine fermentation

Mutants	Genetic characteristics	Inosine production (g/l)
Bacillus subtilis		
strain		
No. 2	Ade⁻	0.21
B-4	Ade⁻His⁻	4.46
C-30	Ade⁻His⁻Tyr⁻	10.5
RDA-16	Ade⁻ Trp⁻ Red⁻ Dea⁻ 8AG^r	18.0
Brevibacterium ammoniagenes		
KY 13714	Ade⁻ 6MG^r Gua⁻	13.6
KY 13761	Ade⁻ 6MG^r 6MTP^r Gua⁻	30.0
Microbacterium sp.		
No. 250	Bio⁻ 6MP^r 8AG^r MSO^r 6TG^r	35.0

Auxotrophy: Ade Adenine; Bio Biotin; Gua Guanine; His Histidine; Tyr Tyrosine; Trp Tryptophan; Red⁻ GMP reductase is absent; Dea⁻ AMP deaminase is absent. Resistance: 8AG 8-Azaguanine; 6MG 6-Mercaptoguanine; 6MTP 6-Methylthiopurine; MSO Methionine sulfoxide; 6TG 6-Thioguanine; 6MP 6-Mercaptopurine
(Furuya, in Ogata et al., 1976)

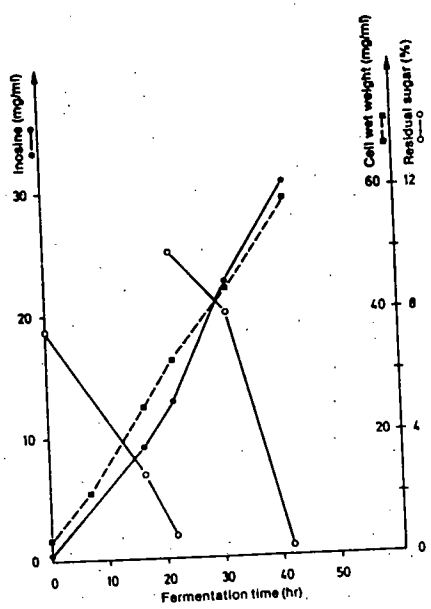


Figure 10.6 Inosine fermentation with *Brevibacterium ammoniagenes* KY 13761 (From Kotani et al., 1978)

- Absence of SAMP synthetase to eliminate AMP regulation at the level of PRPP amidotransferase,
- Low activity of the enzymes which break down 5'-IMP,
- Increased permeability of the cytoplasmic membrane to bring about 5'-IMP excretion.

Mutants with reduced nucleotidase were first isolated among inosine-producing *B. subtilis* strains. Mutants able to grow on adenine but not on AMP showed a lower incidence of nucleotide breakdown. A further inhibition of nucleotidase activity was obtained in mutant A-1-25, which is listed in Table 10.4 along with the other 5'-IMP producers. Industrial production of 5'-IMP is carried out with *Brevibacterium ammoniagenes.* The isolation of permeability mutants whose IMP-accumulation is not affected by Mn^{2+} was an important advance. In the mutants KY 7208 and KY 13102, which are sensitive to Mn^{2+}, the optimal Mn^{2+} content of the medium is 0.01–0.02 mg/l; at higher levels, IMP production decreases. Figure 10.7 illustrates the relationship of the IMP accumulation of both mutants to the Mn^{2+} concentration.

In the fermentation by strain KY 13102 (Figure 10.8), the production of hypoxanthine continues during the first 2–3 days. Then it decreases

Table 10.4 Microorganisms producing 5'-IMP

Mutants	Genetic characteristics	5'-IMP Yield (g/l)
B. subtilis A-1-25	Ade⁻ Nuc⁻	0.6
Corynebacterium glutamicum	Ade⁻ 6MP^r	2.0
Brevibacterium ammoniagenes		
KY 7208	Ade⁻	5.0
KY 13102	Ade⁻	12.8
KY 13105	Ade⁻ Mn^{2+} insensitive	19
KY 13369	Ade⁻ Mn^{2+} insensitive Gua⁻	20–27

Ade⁻ Adenine-requiring; Nuc⁻ Nucleotidase-negative; 6MP^r 6-Mercaptopurine-resistant.

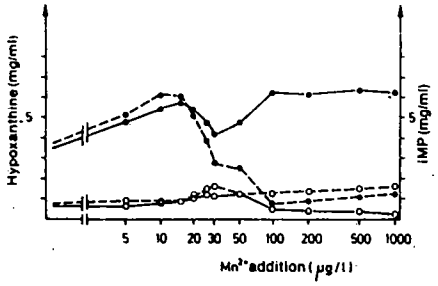


Figure 10.7 Effect of Mn^{2+} on IMP and hypoxanthine production. Mutant KY 13102 (Mn^{2+} sensitive): • - - - • IMP; O - - - O Hypoxanthine. Mutant KY 13105 (Mn^{2+} resistant): • — • IMP; O — O Hypoxanthine

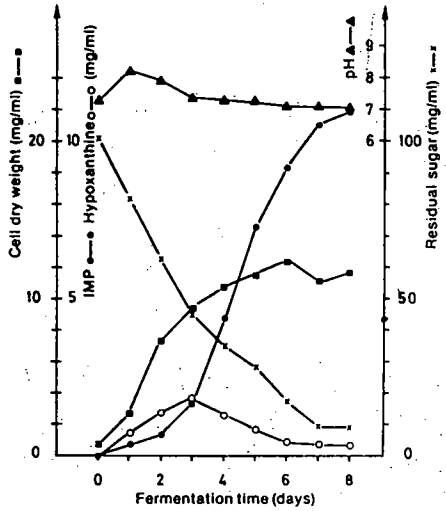


Figure 10.8 5'-IMP fermentation with *B. ammoniagenes* KY 13102 (From Furuya et al., 1968)

as IMP accumulation begins. With Mn^{2+} as a limiting factor, abnormal cells with reduced permeability are formed. They excrete hypoxanthine, which is extracellularly phosphorylated to 5'-IMP, as shown in Figure 10.9. Moreover, 5'-IMP is synthesized *de novo* in the subsequent fer-

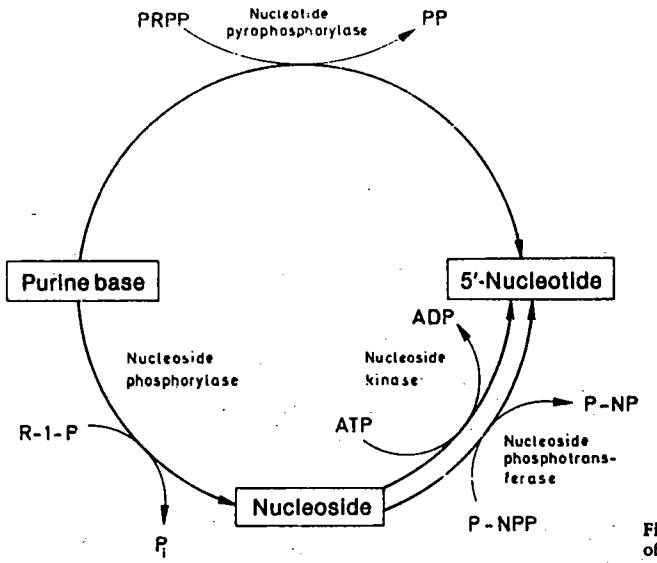


Figure 10.9 Extracellular conversio of purine bases to purine nucleotide

mentation phase and is excreted directly into the culture medium.

For fermentations with *B. ammoniagenes*, it is important that the phosphate and $MgSO_4$ concentrations be kept at 2 and 1%, respectively. The typical composition of the culture medium for Mn^{2+}-insensitive *B. ammoniagenes* is as follows (measured in g/l): KH_2PO_4 10.0; K_2HPO_4 10.0; $MgSO_4 \cdot 7\ H_2O$ 10.0; $CaCl_2 \cdot 2\ H_2O$ 0.1; $FeSO_4 \cdot 7\ H_2O$ 0.01; urea 6.0; cysteine 0.02; thiamine 0.05; nicotinic acid 0.05; Ca pantothenate 0.01; biotin 3×10^{-5}; $MnCl_2 \cdot 4\ H_2O$ 0.001; adenine 0.04; glucose 100.0; pH 8.3.

The Ajinomoto Company has developed a process for IMP production that uses a nitrosoguanidine-induced mutant of *Brevibacterium ammoniagenes*. Inosine (50–2000 mg/l) or hypoxanthine (25–1000 mg/l) is added during the fermentation. This process greatly increases the amount of 5'-IMP production from sugars such as glucose, fructose, sucrose, or starch hydrolysate.

Fermentative production of 5'-GMP

The excretion of sizable amounts of 5'-GMP i wild type strains is rare, due to regulatory effec primarily at the levels of PRPP amidotransferas IMP dehydrogenase, and GMP synthetase Moreover, 5'-GMP can be broken down by 5 nucleotidases or nucleosidases or can be con verted to IMP by GMP reductase. Various mean of production have therefore been developed.

- Fermentation from AICAR (5-amino-4-im idazole carboxamide riboside, Figure 10.2 which is chemically converted to 5'-GMP.
- Microbial production of guanosine with sub sequent chemical phosphorylation to 5 GMP.
- Fermentative production of xanthine or 5 XMP and enzymatic conversion into 5'-GMP
- Direct fermentation of 5'-GMP.

Only the first two processes are used for in dustrial production of 5'-GMP. In mutants use in production, intracellular accumulation is a

GMP, but the nucleoside guanosine is actually excreted.

Production from AICAR AICAR is commercially produced as the starting material for production of 5'-GMP and as an intermediate for the enzymatic production of 5-amino-4-imidazole carboxamide (AICA), which is used as the starting material for the chemical synthesis of purine derivatives.

Escherichia coli, B. subtilis, B. megaterium, and *Brevibacterium flavum* have been examined as AICAR producers. Purine auxotrophs exhibit especially significant AICAR production, and the purine auxotroph *B. megaterium* No. 366 (ATCC 15117) and its mutants have been used in industrial production. Under optimal fermentation conditions, this strain produces 16 g/l AICAR from 80 g/l glucose. The genetic properties that make this strain suitable for AICAR accumulation are: 1. The strain is a purine auxotroph and has a block in the AICARP formyltransferase reaction, which causes AICARP to be converted into FAICARP (see Figure 10.2). 2. The strain has no enzyme activities causing the hydrolysis of AICA-riboside. 3. The enzymes which catalyze AICAR biosynthesis, especially PRPP amidotransferase, are insensitive to regulation by intracellular purine nucleotides.

Starch hydrolysate is the carbon source used with mutant *B. megaterium* No. 366. Fifty percent of the total AICAR synthesis occurs after the metabolism of glucose is complete. As an intermediate step in glucose breakdown, gluconic acid accumulates and is then subsequently metabolized during AICAR synthesis. For maximal AICAR production, the medium must contain a purine source, such as dry yeast or yeast RNA, in suboptimal concentration. Soy protein hydrolysate and NH_4Cl are used as nitrogen sources and mineral salts ($MgSO_4 \cdot 7\ H_2O$, $FeSO_4 \cdot 7\ H_2O$, $MnSO_4 \cdot H_2O$) are also used. Gaseous ammonia is used both as a nitrogen source and pH regulator.

The AICAR yield during the fermentation is directly affected by the sporulation process; sporulating cultures have a strongly reduced AICAR production. Sporulation can be suppressed by i hibitors such as butyric acid, or by reducing t O_2 supply. If good aeration is provided durir the period about 8–12 hours after the start of t fermentation, sporulation occurs and AICA production is reduced. However, if O_2 supply suboptimal, especially during this period, spo ulation is suppressed and AICAR production increased.

The final step is the chemical conversion AICAR to 5'-GMP. The AICARP formed is e creted into the fermentation medium as the d phosphorylated compound, AICAR. The isol tion of AICAR can be accomplished with a 90 yield through several purification stages. For th transformation into 5'-GMP, AICAR is first con verted to guanosine in several chemical steps an then phosphorylated.

Production of guanosine by direct fermentatio Another cost-effective process for the productio of 5'-GMP is the production of guanosine by fe mentation, followed by chemical phosphoryla tion. Guanosine-excreting strains should hav the following properties: 1. SAMP synthetase negative, 2. GMP reductase-negative, 3. reduce nucleosidase activity, and 4. enzymes of GMI biosynthesis unregulated, particularly PRPP ami dotransferase, IMP dehydrogenase, and GMI synthetase.

Microorganisms which possess the ability t excrete guanosine include *Bacillus subtilis, B pumilus, B. licheniformis, Corynebacterium petro philum, C. guanofaciens,* and *Streptomyces griseus* Mutants of *B. subtilis*, also an inosine producer are chiefly used for commercial production (Ta ble 10.5).

IMP dehydrogenase is regulated by 5'-GMP *B. subtilis* AJ 1993 is a mutant which is resistan to the purine analog 8-azaguanine. It shows IMP dehydrogenase activity three times greater than the initial strain, as well as reduced GMP reductase activity. Mutation to resistance to methionine sulfoxide (a glutamine analog) in mutant MG-1 causes another increase in the IMP dehydrogenase activity. In mutants which are re-

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Table 10.5 Guanosine-excreting *B. subtilis* mutants

Mutants	Genetic characteristics	Guanosine production (g/l)
B. subtilis AJ 1993	Ade⁻ Red⁻ 8AG⁺	4.3
No. 30-12	Ade⁻ Trp⁻ Red⁻ 8AX⁺	5
MG-1	Ade⁻ His⁻ Red⁻ MSO⁺ Psi⁺ Dec⁺	16

For the mutant designations, see Table 10.3; Resistance: 8AX 8-azaxanthine; Psi Psicofuranine; Dec Decoyinine (Psicofuranine and Decoyinine are inhibitors of GMP synthetase)

sistant to nucleoside antibiotics such as psicofuranine and decoyinine (which inhibit production of GMP), the GMP synthetase activity is clearly increased, while regulation by GMP is completely eliminated. The high guanosine excretion of mutant MG-1 is thus due to several factors: adenine auxotrophy, the absence of GMP reductase, the elimination of the feedback regulation by GMP, and the increased activity of IMP dehydrogenase and GMP synthetase.

The production medium for *B. subtilis* AJ 1993 has the following composition (g/l): glucose 70; adenine 0.3; soy protein hydrolysate 0.48; NH_4Cl 15; KH_2PO_4 1; $MgSO_4 \cdot 7\,H_2O$ 0.4; $FeSO_4 \cdot 7\,H_2O$ 0.01; $MnSO_4 \cdot H_2O$ 0.01; $CaCO_3$ 25. The fermentation temperature is 30°C. Under these conditions, 4.3 g/l guanosine and 3.1 g/l inosine are produced.

Production of adenosine and adenine nucleotides

The production of adenosine and adenine nucleotides has gained significance due to the use of these substances as starting materials for the synthesis of medically important adenine derivatives.

For adenosine production, the *Bacillus subtilis* mutant P 53-18 (His⁻ Thr⁻ Xan⁻ 8AX⁺) was isolated. This mutant has a block in adenosine deaminase, GMP reductase, and IMP dehydrogenase. An adenosine accumulation of 16 g/l was obtained under optimal fermentation conditions. Progress of a typical fermentation is given in Figure 10.10.

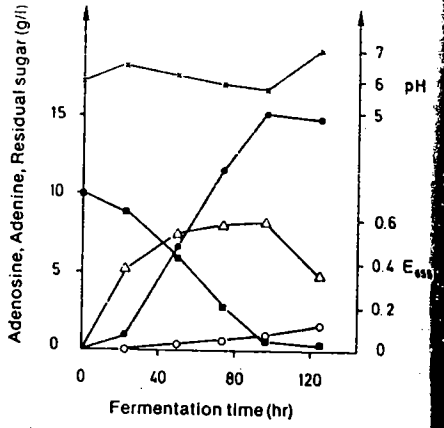

Figure 10.10 Adenosine fermentation with *Bacillus* sp. strain P 53-18. • — • Adenosine; ○ — ○ Adenine; ■ — ■ Residual sugar; △ — △ Growth (Absorbance at 655 nm); crosses, pH (From Haneda et al., 1971)

Another organism, a xanthine auxotroph of *Brevibacterium ammoniagenes*, carries out a direct synthesis of 5'-AMP to a level of 2.1 g/l.

Adenosine triphosphate, ATP, can be obtained through phosphorylation of adenine, adenosine, and ATP by various microorganisms in the presence of glucose or methanol plus phosphate. Protoplasts of the yeast *Candida boidinii* produce ATP at a yield of 100 g/l (77.4% efficiency) from a mixture of adenine and methanol.

Cyclic adenosine-3',5'-monophosphate, cAMP, can be produced either chemically or microbially. cAMP production has been shown in a variety of bacteria, including *E. coli*, *Corynebacterium murisepticum*, *Microbacterium* sp., and *Brevibacterium liquefaciens*. A mutant of *Microbacterium* sp. resistant to 8-azaguanine, azaserine, methionine sulfoxide, and 6-mercaptoguanine has been shown to synthesize cAMP *de novo*, producing as much as 16.6 g/l in a medium containing glucose and inorganic salts. If 3 g/l of adenine or hypoxanthine is added, the production is increased to 20.5 or 21.3 g/l respectively.

Studies on cloning the gene for adenyl cyclase in *E. coli* and *Salmonella typhimurium* have been undertaken. Although the activity of the enzyme in *S. typhimurium* increased by a factor of 20, there was only a 60% increase in cAMP formation.

Fermentative production of other substances related to nucleotides

Other substances related to nucleotides are produced by fermentation in Japan for **medical purposes**, although the market for such products is limited (see also Table 10.1). Production strains and yields of these processes are listed in Table 10.6.

An enzyme/membrane reactor has been developed for the production of **coenzymes NADP/NADPH**, substances that find considerable value in analytical biochemistry.

A group of pharmacologically interesting compounds are the **purine arabinosides.** Substances which possess antibiotic, antiviral, or antitumor activity include guanosine derivatives and adenosine analogs such as arabinosyladenine. A major site of action of these compounds is the enzyme S-adenosylhomocysteine hydrolase, a key enzyme in biochemical systems for biological transmethylation. S-adenosylmethione and S-adenosylhomocysteine, intermediates in the transmethylation pathway, have been studied clinically as sedatives and for the treatment of mental illness. Both microbiological and enzymatic processes for the production of these substances are under study.

Over 100 **antibiotics** have been described which are derived from nucleosides. Several representative examples are discussed in Section 13.7.

10.6 Production by fermentation of substances related to nucleic acids

ct	Organism	Yield (g/l)
	Sarcina lutea	1
	Brevibacterium ammoniagenes	1.9
yme A	*Brevibacterium ammoniagenes* IFO 12071	2
acid	*Arthrobacter paraffineus*	20
choline	*Saccharomyces carlsbergensis*	17

et al., 1976; Hirose et al., 1979)

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11

Enzymes

11.1 INTRODUCTION

The first enzyme produced industrially was the fungal amylase takadiastase, employed as a pharmaceutical agent (for digestive disorders) in the United States as early as 1894. Otto Roehm's patented "laundry process for any and all clothing via tryptic enzyme additives" was announced in 1915. By 1969, 80% of all laundry detergents contained enzymes, chiefly proteases. Along with these, additional enzymes such as lipases, amylases, pectinases, and oxidoreductases were used experimentally in the detergent industry.

Due to the occurrence of allergies among production workers and consumers, the use of proteases in detergents was drastically reduced in 1971 and the world sales fell from $150 million to one-third this amount. Only when special processing techniques, such as microencapsulation, were developed could dustless protease preparations be produced that were risk-free to workers and consumers. Today 95% of laundry detergents in Europe contain proteases, although such detergents are much less widely used in the United States.

The development of **amylases** and **amyloglucosidases** for the production of glucose from starch has led to a new industrial application of enzymes. Also, the use of glucose isomerase for the production of fructose has become widespread since 1967.

Microbial rennin is next in order of significant enzymes. It has been used instead of calf's rennin in cheese production since 1965.

Gross world sales for enzymes in 1987 was $450 million. The following enzymes are currently produced commercially:

- Enzymes used in industry, such as amylases, proteases, catalases, isomerases, and penicillin acylases;
- Enzymes used for analytical purposes, such as glucose oxidase, galactose oxidase, alcohol dehydrogenase, hexokinase, muramidase, and cholesterol oxidase;
- Enzymes used in medicine, such as asparaginase, proteases, lipases, and streptokinase.

These three areas of application require varying levels of quality and quantity (Table 11.1). On a tonnage basis, the most important are the industrial enzymes (about 1200 tons of pure protein/year) (Table 11.2). The United States and Western Europe produce about 40–45% of the world market, and nine firms account for 90% of the total market. Industrial enzymes are produced in 120 m³ fermenters, while enzymes for analytical or medical purposes can frequently be produced in pilot-plant-sized fermenters.

The low concentration of enzymes which are normally produced by wild strains is a considerable hindrance for enzyme production. Although in 1985 about 2500 enzymes were known, only 250 were marketable, mostly in amounts around 10 g, some even in mg amounts. Figure 11.1 shows the overall picture of commercial enzyme production.

The prospects for enzyme application have improved due to developments in the following areas:

- Microbial genetics. High yields can be obtained by genetic manipulation. For instance, the yeast *Hansenula polymorpha* has been genetically modified so that 35% of its total protein consists of the enzyme alcohol oxidase.
- Optimization of fermentation conditions via induction of enzyme production, use of low cost nutrients, optimal utilization of components in nutrient solution, and introduction of fed-batch fermentations.
- Release of enzymes from cells by means of new cell-breaking methods.

Table 11.2 Production of industrial enzymes

Enzyme	Enzyme preparation tons/year	Sales, percent of total
Bacillus proteases	6200	45
Glucoamylases	4500	13
Bacillus amylases	4200	5
Glucose isomerases	1900	6
Rennin (microbial)	1500	10
Amylases (fungal)	800	4
Pectinases	100	3
Proteases (fungal)	100	2
Others	–	12

(Aunstrup et al., 1979)

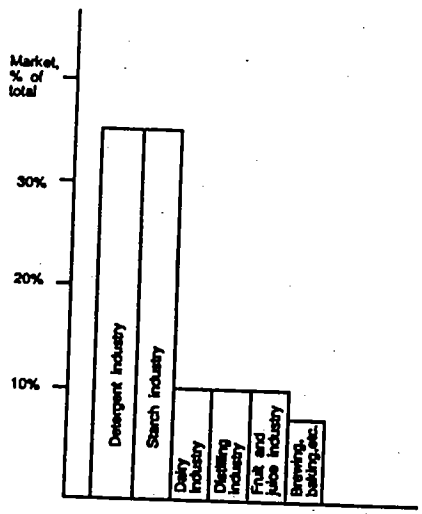

Figure 11.1 Percentage of industrial enzymes used in various markets

Table 11.1 Differences in enzyme qualities

	Industrial enzyme	Analytical enzyme	Clinical enzyme
Scale of application	Tons	Milligrams–grams	Milligrams–grams
Degree of purification	Crude	Pure crystalline	Pure crystalline
Secondary activities	Present	Usually none. If yes, then defined	Only isoenzymes
Origin	Microbial, usually extracellular	Microbial, animal, plant, usually intracellular	Microbial, animal, plant, usually intracellular
Multiple applications	Possible in part	Possible in part	Not yet possible
Production costs	Low	Middle–high	High

- Modern purification processes such as countercurrent distribution, ion-exchange chromatography, molecular-sieve chromatography, affinity chromatography, and precipitation.
- Development of processes for the immobilization of enzymes and for their recycling. The amount of enzyme needed per amount of substrate converted is thus considerably decreased. The proportion of enzyme cost in some processes becomes only a few percent (Figure 11.2).
- Continuous enzyme production in special reactors. The investment cost for a new system can be minimized in a continuous operation, since smaller-sized equipment is used.

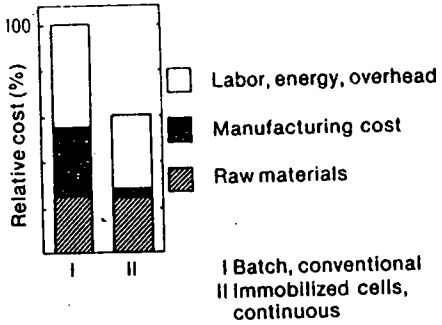

Figure 11.2 Reduction in the cost of L-aspartic acid production through use of cell immobilization techniques (From Chibata and Tosa, 1977)

In this chapter, we will discuss those reactions which involve more or less purified enzymes (either intracellular or extracellular). Additional enzymatic processes involving living or dead cells will be discussed in Chapter 15 (Transformation).

11.2 AMYLASES

Starch, a glucose polymer, is one of the most widely available plant polysaccharides. Amylases are enzymes which hydrolyze starch. One of the main uses of amylases is in the production of sweeteners for the food industry. The hydrolysis of starch with amylase results first in the production of short-chain polymers called dextrins, then the disaccharide maltose, and finally glucose. Maltose syrup (>80% maltose), which is produced primarily in Japan, is of low viscosity, is weakly hygroscopic, not crystallizable, only slightly sweet, but has good heat stability and does not undergo browning reactions. Glucose is not nearly as sweet as its isomer fructose, so the next step is the conversion of glucose to fructose using the enzyme glucose isomerase (discussed in Section 11.3). Commercial sweeteners based on fructose have some economic and manufacturing advantages over the more widely used sweetener sucrose. The most important enzymes in the starch-saccharification process are α-amylases, β-amylases, glucoamylases, glucose isomerases, pullulanases, and isoamylases. Figure 11.3 shows the manner of action of these enzymes on starch.

α-Amylases

α-Amylases (1,4-α-glucan-glucanohydrolases) are extracellular enzymes which hydrolyze α-1,4-glycosidic bonds. These enzymes are **endoenzymes**, splitting the substrate in the interior of the molecule. Their action is not inhibited by α-1,6-glycosidic bonds although such bonds are not split.

α-Amylases are formed by many bacteria and fungi. They are classified according to their starch-liquefying and/or saccharogenic effect, pH optimum, temperature range, and stability. Saccharogenic amylases produce free sugars, whereas starch-liquefying amylases break down the starch polymer but do not produce free sugars. Many organisms produce several α-amylases.

Bacteria which produce α-amylases are: *Bacillus subtilis, B. cereus, B. amyloliquefaciens, B.*

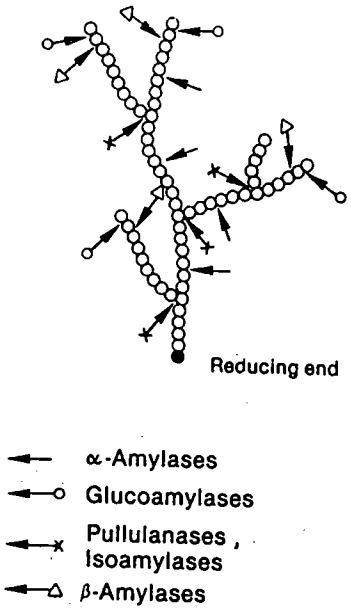

Figure 11.3 Mechanism of starch hydrolysis by amylases

coagulans, B. polymyxa, B. stearothermophilus, B. caldolyticus, B. acidocaldarius, B. subtilis var. *amylosaccharaticus, B. licheniformis, Lactobacillus, Micrococcus, Pseudomonas, Arthrobacter, Escherichia, Proteus, Thermomonospora,* and *Serratia.*

Three very similar strains producing saccharogenic α-amylases are *B. subtilis* Marburg, *B. subtilis* var. *amylosaccharaticus,* and *B. natto.* The strain *B. amyloliquefaciens* differs from these in that it produces a liquefying α-amylase. The substrate concentration determines the extent to which the enzymes act in a liquefying or saccharogenic manner.

Some α-amylase-producing fungi are from the genera *Aspergillus, Penicillium, Cephalosporium, Mucor, Candida, Neurospora,* and *Rhizopus.*

The molecular weights of various α-amylases do not differ considerably (Table 11.3). They all contain a large proportion of tyrosine and tryptophan in the enzyme protein and most require calcium as a stabilizer.

Table 11.3 Molecular weight of some α-amylases from different microorganisms

Organism	Molecular weight $\times 10^3$
Aspergillus oryzae	51– 52
A. niger	58– 61
Bacillus acidocaldarius	68
B. amyloliquefaciens	49
B. subtilis	24–100
Thermomonospora curvata	62

(Fogarty, 1983)

The most important α-amylases are produced by *Bacillus amyloliquefaciens, Bacillus licheniformis,* and *Aspergillus oryzae. Bacillus* amylases are used much more extensively than those of *Aspergillus.* The most important areas of application for these two enzymes are shown in Table 11.4.

Production of bacterial α-amylases Bacterial amylase production involves the function of the normal cell machinery for protein synthesis. This is shown by experiments involving the addition of antibiotics. If the specific antibiotics are used to inhibit protein synthesis in *B. subtilis* during the production of α-amylases, both growth and production of amylase cease (Figure 11.4). Compared to the mRNA of intracellular enzymes (stability about 2 minutes for *Escherichia coli*), the mRNA for the production of extracellular hydrolases has an extremely long lifetime. When actinomycin D is added to the amylase-producing culture to inhibit RNA synthesis, both RNA synthesis and growth cease after 30 minutes but the production of amylase continues (Figure 11.5).

For industrial production, α-amylases are produced either in batch or in fed-batch fermentation. The enzyme formation rate is very low during exponential growth in many production strains, but just before the growth rate decreases and spore formation begins, amylase production increases (Figure 11.6).

The production of α-amylases is regulated by several genes, which have been only partially

Table 11.4 Important applications of α-amylases

Industry	Source		Application
	Bacillus	*Aspergillus*	
Starch industry	+		Liquefaction of starch for production of glucose, fructose, maltose
Milling		+	Modification of α-amylase-deficient flour
Alcohol	+	+	Liquefaction of starch before the addition of malt for saccharification
Baked goods		+	Increase in the proportion of fermentable carbohydrates
Brewing	+		Barley preparation, liquefaction of additives
		+	Improved fermentability of grains, modification of beer characteristics
Paper	+		Liquefaction of starch without sugar production for sizing of paper
Textiles	+		Continuous desizing at high temperatures
Feed industry	+		Improvement of utilization of enzymatically treated barley in poultry and calf raising
Sugar	+		Improvement of filterability of cane sugar juice via breakdown of starch in juice
Laundry and detergent	+		Increase in cleansing power for laundry soiled with starch, additive in dishwasher detergents

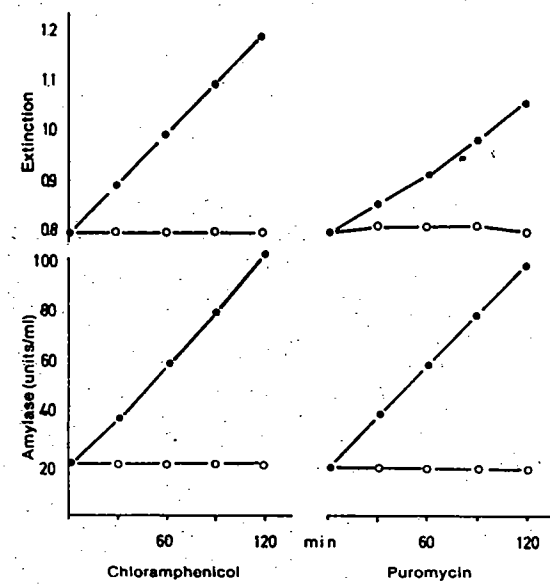

Figure 11.4 Effect of chloramphenicol and puromycin on α-amylase production in *B. subtilis.* ○ — ○ antibiotic added (10 μg/ml chloramphenicol, 100 μg/ml puromycin); • — • control. (From Terui, 1973)

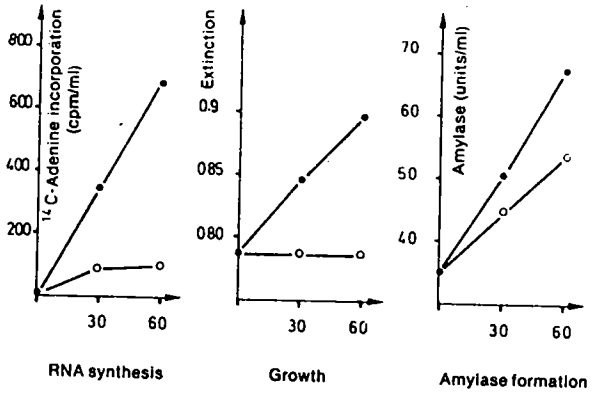

Figure 11.5 Effect of actinomycin D on growth, RNA synthesis, and α-amylase production. O — O actinomycin D (0.6 μg/ml); • — • control. (From Terui, 1973)

characterized. Whereas single-step mutations increase yields by a factor of 2–7, mutants have been selected after 5 steps which produce yields 250 times greater than the wild strain.

Since starch is a macromolecule, it cannot be taken up by the cells and hence cannot act as an inducer of α-amylase synthesis. It is assumed that a small amount of enzyme is produced constitutively and excreted into the medium where its action on starch leads to the production of low-molecular-weight inducers. **Catabolite repression** is significant in the production of most extracellular enzymes. Glucose promotes the best growth compared to other substrates, but is the least favorable in terms of amylase production. In continuous fermentation with carbohydrate as a limiting factor, growth is inversely proportional to the α-amylase production. When nitrogen is used as a limiting factor with excess glucose, only traces of amylase are produced.

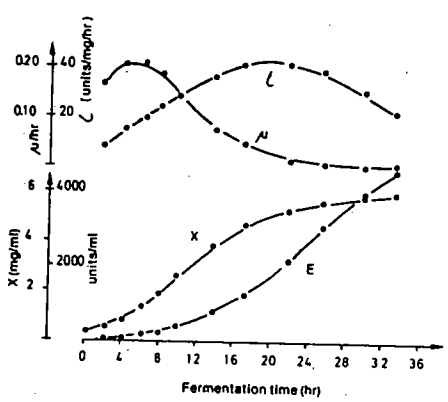

Figure 11.6 Amylase production in *Bacillus amylosolvens*. μ specific growth rate, ξ specific enzyme production rate, X cell concentration, E enzyme concentration (From Terui, 1973)

It seems unlikely that amylase is excreted by pure diffusion, since the molecule is quite large and is hydrophilic. There is good evidence that the biosynthesis as well as the steps leading to excretion of extracellular proteins such as amylases and proteases takes place at the outer membrane of the cell. As it is formed, the protein contains an additional peptide, the *signal peptide*, which participates in the transfer of the nascent protein from the ribosome through the membrane. Only after the protein has passed through the membrane does it fold into its tertiary configuration and the signal peptide is removed.

A **medium** for the production of α-amylases in a 100 m³ fermenter with *B. subtilis* consists of: 5% starch, 0.56% NH_4NO_3, 0.28% sodium citrate, 0.13% KH_2PO_4, 0.05% $MgSO_4 \cdot 7\ H_2O$, 0.01% $CaCl_2 \cdot 2\ H_2O$, 0.5% peptone, 0.2% yeast extract; pH 6.8.

The effect of temperature on enzyme production and growth rate is given in Figure 11.7. At 45°C, the maximal specific enzyme formation rate is reached with the test strain after 18 hours; however, the maximal amount of enzyme produced (up to 3000 units/ml) is obtained at considerably lower temperatures (27 to 30°C). Thermophilic strains are used in newer processes. *Thermomonospora*, isolated from compost, has a temperature optimum for growth and amylase production at 53°C. Figure 11.8 shows the narrow pH optimum for amylase production by this strain.

α-Amylase production from fungi The production of fungal amylases is constitutive, but as with other enzymes, it is repressed by regulators. For amylase production using *Aspergillus oryzae*, the following nutrient solution can be used: 8% starch, 1.2% $NaNO_3$, 0.1% K_2HPO_4, 0.1% $MgSO_4$, 0.05% KCl, 0.003% $FeSO_4$, 0.08% $Mg(NO_3)_2$, 0.05% $Mg(H_2PO_4)_2$, 2.0% malt extract. The medium composition must be optimized, because as seen in Table 11.5, a marked shift in enzymatic activities is observed when different carbohydrates are used.

The optimal temperature lies in a narrow range between 28–30°C, and the duration of the fermentation process is 3–4 days.

β-Amylases

β-Amylases (α-1,4-glucan-maltohydrolases) are usually of plant origin, but some microbial producers are also known: *Bacillus polymyxa*, *B. cereus*, *B. megaterium*, *Streptomyces* sp., *Pseudomonas* sp., and *Rhizopus japanicus*. Although yields in wild strains are usually low, mutants have been discovered which produce 200 times more enzyme than the wild type.

Bacterial β-amylases have greater heat resistance (>70°C) than plant β-amylases and the pH optimum is also higher (about pH 7.0). In contrast to α-amylase, calcium is not necessary for stabilization and activation of bacterial β-amylase.

In the future, it is likely that β-amylases will be used for the production of maltose syrup.

Glucoamylases

Glucoamylases (α-1,4-glucan-glucohydrolases) act on starch by splitting glucose units from the nonreducing end. Maltose is broken down only slowly, while 1,6-bonds in the branched polysaccharides are hardly attacked. Thus glucose, maltose and limit dextrins are the end products of glucoamylase action.

Microorganisms used to produce glucoamylases are *Aspergillus niger*, *A. oryzae*, *A. awamori*, *Rhizopus niveus*, *R. delemar*, *R. formosaensis*, and *R. javanicus*. Production strains in western Europe and in the United States are mutants of *A. niger* and *R. niveus*. Today, industrial fermentation of glucoamylase is carried out almost exclusively in submerged fermenters up to 150 m³ in volume. Due to the increasing demand for glucoamylase in fructose-syrup production, smaller systems which involve growth on the surface of liquid or solids are no longer economical. However, in genetic studies, strains are frequently isolated which produce higher yields in surface culture than in submerged culture (Table 11.6). Experiments with batch-fed fermentations have been successfully carried out over periods as long as 320 hours.

Frequently, several glucoamylase isoenzymes are produced by one strain; in addition, the enzymes may be modified during the fermentation process. *A. awamori* var. *kawachi* produces three glucoamylases, only one of which hydrolyzes crude corn starch. Due to the action of proteases and glycosidases, which are also present under various experimental conditions, one of the other two enzymes may be formed instead of the glucoamylase which hydrolyzes crude corn starch. These other enzymes can only act on corn starch which has been swollen by heat or chemical treatment. Therefore the changes in glucoamylase activity must be carefully examined

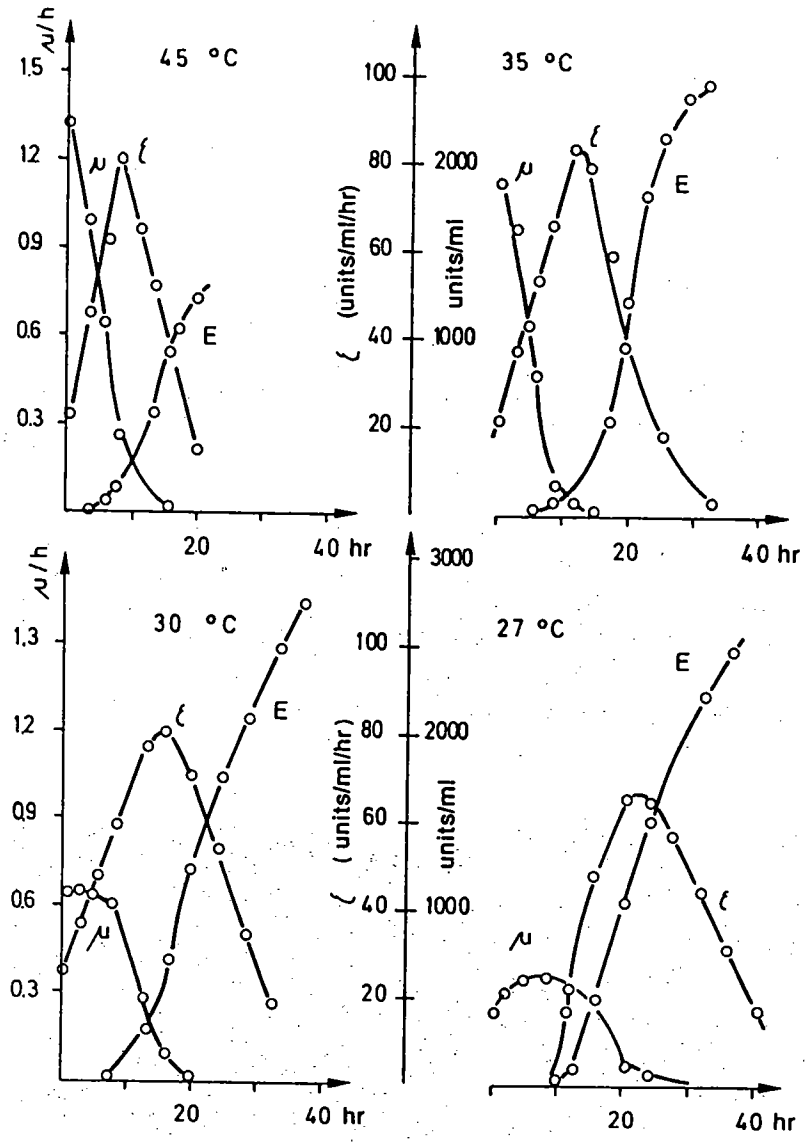

Figure 11.7 Relationship of growth and α-amylase production to temperature. See Figure 11.6 for symbols. (Modified from Terui, 1973)

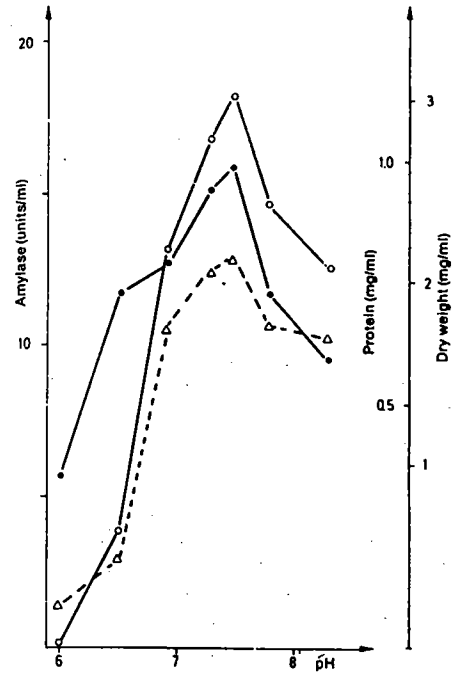

Figure 11.8 Effect of pH on α-amylase production, extracellular protein and growth. O — O α-amylase; • — • protein; △ — △ cell dry weight (From Glymph and Stutzenberger, 1977)

Table 11.5 Effect of carbon source on amylase and protease production in *Trichoderma viride*

Carbon source	Amylase units/ml	Protease units/ml
Corn starch	235	351
Maltose	179	175
Glucose	52	243
Sucrose	17	350
Lactose	3	175
Control	0	324

(Upton and Fogarty, 1977)

when high-yielding strains are isolated and when fermentation conditions are being optimized.

Table 11.6 Glucoamylase production in some mutan of *Rhizopus formosaensis*

Strain	Submerged culture units/100 ml	Surface culture (liquid) units/100 ml	Surface culture (solid) units/g
R 13-5	295	2745	858
R 13-59	491	3067	1322
R 13-591	1595	3788	2160
R 13-59136	4432	7425	5120

(Lin, 1972)

Either starch or dextrins induce enzyme pr duction; nutrient solutions for enzyme produ tion are generally based on starch. Glucose, gl tamic acid, and lactose cause catabolit repression. Fermentation time varies from 3- days, depending on the organism, and the fe mentation temperature can range from 28–35°(In addition to glucoamylase, α-amylase may al be produced in the same fermentation.

The quality of the spores used as inoculu has an effect on the enzyme yield in gluc amylase production with *A. niger*. When spor are produced on a rich agar medium, such as or containing a supplement of malt extract, th glucoamylase formation rate of later cultures d creases with each transfer, although the spo formation rate does not diminish. After 6 tran fers only 50% of the original enzyme activity produced. However, if the inoculum spores a produced on a minimal medium (Czapek Do the enzyme formation rate remains constant, b the spore formation rate decreases.

1,6-Glycoside-splitting enzymes

Enzymes which can split the side chains of am lopectin via hydrolysis of α-1,6-bonds are im portant in commercial processes. These enzym can be subdivided into two groups: a) thos which directly affect amylopectin, such as pu lulanases and isoamylases, and b) those whic have an indirect effect after enzymatic modif cation of the substrate. Pullulanases and iso mylases hydrolyze amylopectin. Moreover, pu

lulan, the α-glucan from the fungus *Pullularia pullulans*, is split by means of pullulanases. This latter reaction cannot be carried out with isoamylases. Table 11.7 shows the effect of pullulanase on starch hydrolysis by β-amylase.

Late in the log phase, *Aerobacter aerogenes* ATCC 15050 excretes pullulanase into the medium. With inducible strains, either maltose or pullulan can be used as a carbohydrate source for enzyme induction, but constitutive mutants are now generally used instead. Both batch fermentations and continuous processes are in use. Several other pullulanase producers besides *Aerobacter aerogenes* have been described: *Bacillus polymyxa, Pseudomonas saccharophila, Streptococcus* sp., and *Streptomyces* sp. Among the isoamylase-producers, *Pseudomonas amyloderamosa* is the most important strain. In addition, there are other strains: *Agrobacterium, Erwinia, Staphylococcus, Serratia, Nocardia, Bacillus, Pediococcus, Lactobacillus*, and *Leuconostoc*.

Starch hydrolysis

In the food industry, starch hydrolysates are used as additives in the manufacture of candies, baked goods, canned goods, and frozen foods. Syrups used can contain maltose or glucose (after saccharification), or fructose (after isomerization, Section 11.3). Starch hydrolysates affect the taste, sweetness, and texture of the food products.

To produce a hydrolysate, starch must first be converted to paste at 65–90°C. During this process, the viscosity of a 20% solution increases significantly to 3000 centipoise. Acid treatment at 120–140°C (pH 1.8–2.0) or treatment with α-amylases cause starch liquefaction in the next step; an alternative method is a combined acid/enzyme liquefaction. The disadvantages of acid treatments are generally low glucose yields, higher purification costs, risk of deterioration and corrosion of the system used.

Enzymatic liquefaction has therefore generally displaced acid treatment. Liquefaction is carried out at 110°C with amylases from *B. subtilis* which are added to the solution before conversion to paste. When α-amylases from *A. niger* or *A. oryzae* are used, the process is carried out at 55°C and the hydrolysis continues to the point of a maltose/maltotriose mixture without the addition of other enzymes.

Starch solution which has been liquefied with α-amylases can be saccharified with glucoamylases at a pH of 4.0–5.0 and a temperature of 55–60°C without an intermediate purification stage. Pullulanases or isoamylases may also be added at this stage to bring about splitting of bonds other than the 1–4 linkages. The specificity of enzymes used and the conditions of conversion determine the composition of the end product.

Figure 11.9 shows a diagram of the glucose syrup production process.

Table 11.7 Conversion of 10% potato starch (pretreated with α-amylase) via pullulanase and β-amylase

Type of process	Maltose %	Glucose %	Maltotriose %	Dextrin %
25 units β-Amylase/g starch; 16 hr 45°C	65.5	1.1	3.5	29.9
10 units Pullulanase + 25 units β-amylase/g starch; 16 hr 45°C	90.5	0.4	1.2	7.9

(Hayashibara, 1970)

11.3 GLUCOSE ISOMERASES

Glucose isomerase (D-glucose ketoisomerase) causes the isomerization of glucose to fructose. The reaction is reversible and a mixture of glucose and fructose is produced, the ratio of the components depending on the enzyme used and the reaction conditions, such as temperature. The enzyme has become of commercial value because the price of sugar has increased compared to that of starch. In the Western world, the current sucrose consumption rate is 50 kg per person per year. In the United States in 1985, 4×10^6 tons of isosyrup (glucose/fructose mixture) were produced. As an example of European usage, the

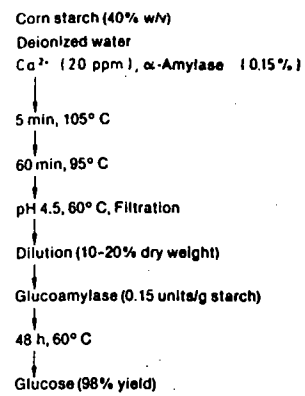

Figure 11.9 Production of glucose solution from starch (Novo Industries, 1977)

size of the German fructose production is estimated at 10,000–20,000 tons per year.

Starch can be converted to glucose by either acid or enzyme hydrolysis (see Section 11.2). The advantage of starch hydrolysis over direct sugar production is that the initial materials used, such as wheat, corn, cassava, and to some extent potatoes, are nonperishable, but sugar beets (the only direct source of sugar in temperate climates) are only available about 100 days per year.

Glucose has 70–75% the sweetening strength of beet sugar (sucrose) but β-D-fructopyranose (fructose), the sweetest monosaccharide, has twice the sweetening strength of sucrose. Thus, processes for the manufacture of fructose are of considerable value. Fructose can be produced biochemically from sucrose or starch by way of glucose production, as shown in Figure 11.10. It can also be produced chemically from glucose at high temperatures under alkaline conditions, but byproducts are formed such as psicose which cannot be metabolized in the body (Figure 11.11). Thus, chemical production of fructose is not acceptable to the food industry.

The glucose isomerases in use today are actually D-xylose-isomerases which have additional activity with D-glucose and D-ribose. The

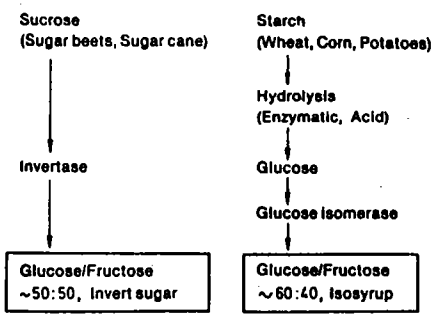

Figure 11.10 Use of biochemical methods for the production of high-fructose syrup

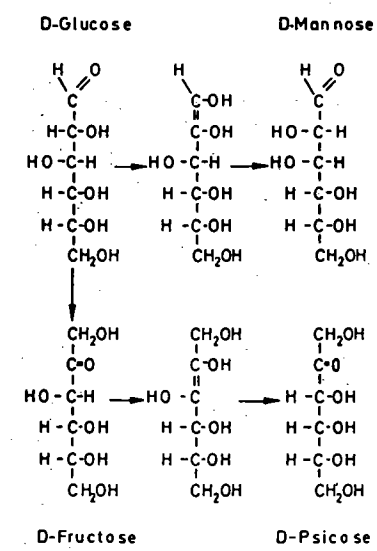

Figure 11.11 Chemical isomerization of glucose to fructose

glucose isomerase used must fulfill the following criteria: low pH optimum to avoid side reactions, high specific activity, high temperature optimum.

A large group of bacteria and some yeasts are known which carry out the direct isomerization

(Table 11.8). There are other bacteria (*Escherichia intermedia*, *E. freudii*, and *Aerobacter aerogenes*) which have a somewhat different glucose isomerase activity. The enzyme produced by these bacteria is actually a glucose phosphate isomerase. These organisms are not usable industrially, however, since their enzymes require arsenic for maximal activity, and arsenic cannot be used in food production.

The most important glucose isomerase producers are *Bacillus coagulans* (Sweetzyme®, Novo Industri), *Streptomyces rubiginosus* (Optisweet®, Miles-Kali), *Actinoplanes missouriensis* (Ketozyme®, Universal Oil Products; Maxazyme®, Gist Brocades), *Flavobacterium arborescens* (Taka-Sweet®, Miles Laboratories).

Glucose isomerase from *Bacillus*

Novo Industries has developed glucose isomerase from *B. coagulans* for commercial use. This **immobilized enzyme process** is used today in many plants in the United States, Europe, Japan and Korea. Since this glucose isomerase is primarily a xylose isomerase, xylose must be added for induction of the enzyme with all wild strains. Xylose is quite expensive, however, and may be replaced by xylan or wheat bran, which also contain xylose. Constitutive mutants have also been isolated.

Catabolite repression regulates the production of glucose isomerase. Glucose acts as a repressor in both batch process and continuous fermentation. In batch process, *B. coagulans* does not form the enzyme during the log phase. As soon as the glucose content in the nutrient solution approaches zero, growth ceases. In a typical diauxy, additional carbon sources present in the medium are then metabolized, and enzyme production begins. Maximal enzyme activity is obtained after 24 hours' incubation.

Cobalt and magnesium are prerequisites for maximal enzyme production in wild strains, but mutants have been isolated which produce optimal enzyme titers in the absence of cobalt. Either yeast extract or corn steep liquor can be used as the nitrogen source. As in the case of many other fermentations, the choice of a nitrogen source is vital for the yield and the concentration must be optimized for each individual process.

The difficulties that arise when attempting to optimize the nitrogen source for glucose isomerase production are illustrated in Table 11.9 by results for *Actinoplanes missouriensis*. As seen, there is wide variation in yield using various complex nitrogenous substrates. Because it is so difficult to standardize the nitrogen source, the fermentation yield may vary considerably from batch to batch.

Table 11.8 Glucose isomerase producers

Bacillus coagulans
Streptomyces phaeochromogenes
Streptomyces rubiginosus
Streptomyces olivochromogenes
Arthrobacter sp.
Actinoplanes missouriensis
Microbispora rosea
Micromonospora coerula
Microellobospora flavea
Nocardia asteroides
Nocardia dassonvillei
Brevibacterium imperiale
Flavobacterium arborescens

Table 11.9 Effect of organic nitrogen sources on glucose isomerase production in *Actinoplanes missouriensis*

Nitrogen source	Manufacturer	Activity	
		GI/ml	%
Corn steep liquor	Anheuser-Busch	17.8	100
O. M. peptone	Amber Lab.	13.4	75
Casein hydrolysate (Amber EHC)	Amber Lab.	13.0	73
Bacto-soytone	Difco	11.8	66
Yeast extract (BYF-100)	Amber Lab.	10.3	58
Distiller's solubles	National Distillers	7.9	44
Yeast extract (BYF-300)	Amber Lab.	5.8	33
Bacto-peptone	Difco Lab.	5.2	29
Yeast extract (BYF 50 X)	Amber Lab.	4.3	24
Malt extract	Difco Lab.	2.5	14
Atlantic Menhaden peptone (fish)	Haynie Products	1.1	6

(Activity with corn steep liquor = 100%)

In a continuous fermentation process, the growth-limiting substrate must be glucose. For optimal enzyme yields, oxygen limitation is also necessary since microaerophillic conditions inside the cells stabilize the system.

Glucose isomerase from *Streptomyces*

Glucose isomerases from *Streptomyces* strains are produced only in batch processes today. There are mutants which require neither xylose nor cobalt. Production is begun with a spore inoculum of the working strain, which is cultivated for 24–48 hours at 30°C in shaken culture. The culture medium used contains soy meal, yeast extract, glucose, starch, phosphate, and deionized water. A number of such shake flasks are used to inoculate (5% inoculum) the production fermenter (which is 80–150 m³ in volume). *Streptomyces olivaceus* NRRL 3588, produces glucose isomerase at pH 8.5 and between 60–70°C. A typical fermentation with *Streptomyces albus* is shown in Figure 11.12.

Figure 11.13 shows the pH and temperature optima of the glucose isomerase produced in this process.

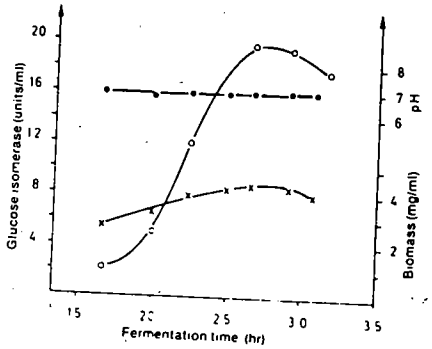

Figure 11.12 Industrial production of glucose isomerase with *Streptomyces albus*. 22 m³ fermenter, temperature 30°C, impeller speed 150 rpm, aeration rate 0.33 vvm. •—• pH; X — X cell growth; O — O glucose isomerase (From Takasaki et al., 1969)

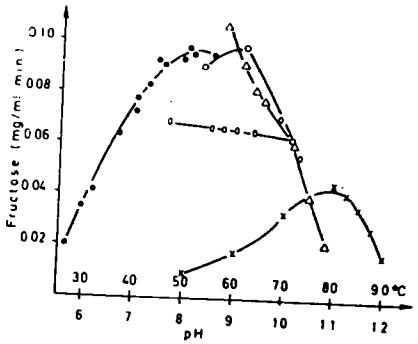

Figure 11.13 Effect of temperature and pH on gluco isomerase activity. The enzyme was derived from *Stre tomyces albus*. •—• Na_2HPO_4-KH_2PO_4 buffer; O — Na_2HPO_4-NaOH buffer; □—□ NaOH-glycine buffe △—△ Na_2CO_3-$NaHCO_3$ buffer; X — X temperatu experiment with phosphate buffer 0.05 M (From Takasal et al., 1969)

Immobilization of glucose isomerase

The commercial process for production of fructose from glucose became feasible only when procedures for immobilization of the enzyme were developed (see Section 11.11), since this permitted the same batch of enzyme to be used many times. Since glucose isomerase is formed intracellularly in most strains, many commercial processes are carried out with immobilized cells or by addition of partly broken cells.

The Novo process uses glutaraldehyde to cross-link cell suspensions of *B. coagulans*. The cross-linked material is processed into 0.3–1.0 mm diameter pellets and used either in a batch reaction or continuously with several bioreactors connected in series. Figure 11.14 shows a system which produces 100 tons of fructose per day with 2 parallel rows, each with three 2.2 m³ columns.

The starting material is glucose syrup with a dry weight of 40–45% containing 93–96% glucose. The solution must first be purified by filtration, carbon treatment, and ion exchange to prevent loss of enzyme activity during isomerization. Magnesium is added as an activator and

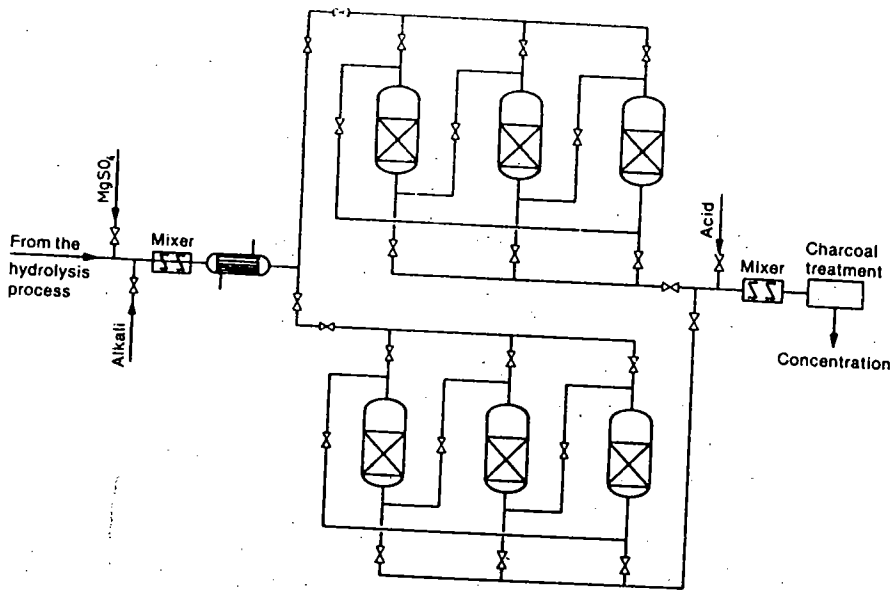

Figure 11.14 Flow chart of a process for fructose syrup production (100 tons/day)

the reaction is run at pH 8.5 and a temperature of 60–65°C. The time required for isomerization is 0.8–4 hours.

When compared to the batch process, at the same conversion temperature, a considerably better product is obtained in a continuous system in 20% of the reaction time and with only 4% of the reactor volume (Table 11.10).

In the recovery process, the converted solution is first acidified and then passed over activated carbon and ion exchange resins to remove salts and colored materials. Fructose syrup is then concentrated to about a 70% solution (dry weight basis), stored, and used in this form in the food industry. Since di- and trisaccharides are not converted by glucose isomerase, a typical product has the following composition:

Fructose	42	%	Dry weight
Glucose	53	%	Dry weight
Oligosaccharide	5	%	Dry weight
Psicose	0.1	%	Dry weight
Ash	0.05–0.1%		Dry weight
Dye value	0.003		
pH	4–5		

Table 11.10 Comparison of batch and continuous methods for production of fructose

Parameters	Batch process	Continuous conversion
Bioreactor volume	750 m³	30 m³
Enzyme consumption	17–20 tons	10 tons
$MgSO_4 \cdot 7\,H_2O$-consumption	4.3 tons	2.2 tons
$CoSO_4 \cdot 7\,H_2O$-consumption	2.2 tons	0
Color formation OD_{420nm}	0.05–0.10	0–0.02
Psicose formation	0.1%	0.1%
Purification	Carbon treatment, cation and anion exchangers	Carbon treatment

(Zittan et al., 1975)

11.4 L-ASPARAGINASES

L-Asparaginases (L-asparagine amidohydrolases) are used as **antitumor agents** in the treatment of some leukemias and lymphomas. The principle behind the use of this enzyme as an antitumor agent is as follows: The metabolism of these tumor cells requires L-asparagine, and since asparagine is split into aspartic acid and ammonium ions by asparaginase, these malignant cells can be destroyed.

The enzyme is produced by several bacteria, but for commercial production (80 m³ fermenters) only mutants of *Escherichia coli* ATCC 9637, *Serratia marcescens* ATCC 60, and *Erwinia carotovora* are used. An appropriate medium is 3% corn steep liquor, 0.6% sodium acetate, and 0.2% ammonium sulfate at pH 7.0, used in reactors up to 80 m³ in volume. Oxygen supply is critical during the 16- to 40-hour fermentation. Table 11.11 shows that the prerequisite for a high enzyme titer is a low pO_2.

At the end of the fermentation process, the cells are broken open and the enzyme is crystallized.

11.5 PROTEASES

After amylases, the second most important industrial enzymes currently are the proteases. About 500 tons of these enzymes (based on the pure protein) are produced per year. Proteases are used primarily in the detergent industry and in the dairy industry (rennin, see Section 11.6). Other areas in which proteases are used include the pharmaceutical industry, the leather industry, the manufacture of protein hydrolysates, the food industry, the film industry, and waste processing.

Proteases are produced commercially both from bacteria and fungi. An important distinguishing feature is the pH optimum of the protease. The proteases on the market include **alkaline, neutral,** and **acid proteases.**

Akaline proteases

Many bacteria and fungi excrete alkaline proteases. The **most important producers** are *Bacillus* strains such as *B. licheniformis, B. amyloliquefaciens, B. firmus, B. megaterium,* and *B. pumilis; Streptomyces* strains such as *S. fradiae, S. griseus,* and *S. rectus;* and the fungi *Aspergillus niger, A. sojae, A. oryzae,* and *A. flavus.* Enzymes used in detergents are mainly proteases from *Bacillus* strains (Bacillopeptidases). The best known proteases are Subtilisin Carlsberg from *B. licheniformis,* and Subtilisin BPN and Subtilisin Novo from *B. amyloliquefaciens.* These enzymes contain serine at the active site of the molecule and are not inhibited by EDTA (ethylene diamine tetraacetic acid), but are inhibited by DFP (diisopropyl fluorophosphate).

The proteases of this type have many features of value for use as detergent enzymes:

- stability at high temperature
- stability in the alkaline range (pH 9–11)
- stability in association with chelating agents and perborates.

Table 11.11 L-asparaginase formation with *Erwinia aroideae* at 28°C in a small fermenter

Aeration rate vvm	Baffles	Impeller speed rpm	Cell dry weight g/10 l	L-Asparaginase Units/g
0.5	–	300	14	960
1.0	+	300	16	580
1.5	+	300	18	340
2.0	+	300	18	230

(Peterson and Ciegler, 1969)

However, their stability in the presence of surface-active agents is low, thus limiting their shelf life.

Screening Because the enzymes must be stable under alkaline conditions, screening for better producers is done using strongly basic media. Single colonies have been tested on protein-agar plates at pH 10.0. Figure 11.15 shows the results of screening with a large number of strains. As seen, strains of *B. licheniformis* and *B. subtilis* showed optimal growth in the pH range of 6–7, but some new strains had maximal growth rates at pH 8–9 and grew somewhat at pH 11.

Since the enzyme yields of wild-type strains are insufficient for industrial utilization, extensive genetic studies have been carried out to increase the yield. The genes for the formation of several proteases have been cloned. Protein engineering has been used to develop modified *Bacillus* subtilopeptidases with altered amino acid sequences and corresponding changes in enzymatic properties such as substrate specificity, pH optimum, and stability to bleaching agents.

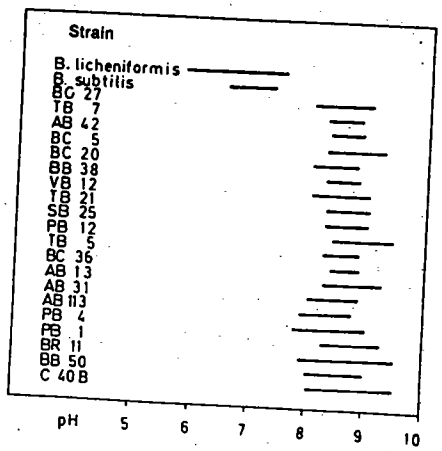

Figure 11.15 Screening for alkaline proteases. Within the indicated pH range, at least 90% of the maximal growth rate is attained. (From Aunstrup et al., 1972)

Fermentation process Sterility is mandatory for protease production, as with other enzyme fermentations. Cultures are stored in the lyophilized state or under liquid nitrogen. Initial growth is carried out in shaken flasks and small fermenters at 30–37°C. For production, 40–100 m³ fermenters are used. Production of extracellular proteases is chiefly regulated by the medium composition.

The fed-batch process is generally used in order to keep down the concentration of ammonium ions and amino acids, since these nitrogenous materials repress protease production. Although continuous processes have been described, they are not used commercially.

High oxygen partial pressure is generally necessary for optimal protease titers. Aeration rates are 1 vvm and the time span of the fermentation is 48–72 hours, depending on the organism.

Proteases must be converted into particulate form before they are added to detergents, since if dry enzyme powder is inhaled by production workers or users, allergic reactions may result. Enzyme concentrates are marketed in a microencapsulated form. To make a suitable encapsulated product, a wet paste of enzyme is melted at 50–70°C with a hydrophobic substance such as polyethylene glycol and then converted into tiny particles. These solidified spherical particles are not hazardous when added directly to the detergent. A further development is the immobilization of enzymes in fibrous polymers and in granules.

Neutral proteases

Neutral proteases are excreted by both bacteria and fungi. Producing organisms include: *Bacillus subtilis, B. cereus, B. megaterium, Pseudomonas aeruginosa, Streptomyces griseus, Aspergillus oryzae, A. sojae,* and *Pericularia oryzae.*

Neutral proteases are relatively unstable and calcium, sodium, and chloride must be added for maximal stability. The pH range of activity is fairly narrow and these enzymes are not very stable to increased temperatures (Figure 11.16).

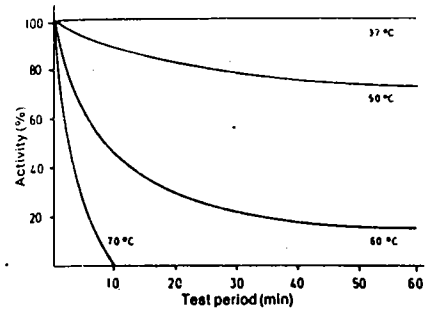

Figure 11.16 Temperature stability of a neutral protease (Neutrase®, Novo, 1978)

The neutral proteases are also quickly inactivated by alkaline proteases. Because of these limitations, they have restricted industrial application, but do find some uses in the leather industry and in the food industry for the manufacture of crackers, bread, and rolls.

Acid proteases

In this category are rennin-like proteases from fungi which are chiefly used in cheese production (see Section 11.6). In addition, other acid proteases which are similar to mammalian pepsin are also on the market. These enzymes have a pH optimum of 2–4. Acid proteases are used in medicine, in the digestion of soy proteiln for soy sauce production, and to break down wheat gluten in the baking industry.

11.6 RENNIN

In earlier centuries, curds for cheese production were produced by the action on milk casein of microorganisms which were naturally present in milk. These organisms produced organic acids, primarily lactic acid, which lowered the pH to the isoelectric point of casein, leading to a natural precipitation of casein in the form of curds which could easily be removed from the whey. Another possibility for curd production, salting out, rarely used. A third possible method is heat p cipitation but this method is used for only a f processes. A fourth method, the addition of milk-coagulating enzyme rennin, has be known for a long time and is used in most ca today (alone or in combination with pH prec itation).

In modern processes, **starter cultures** added which convert lactose to lactic acid a lower the pH. The most commonly used stra include *Streptococcus lactis, S. cremoris, S. th mophilus, Lactobacillus helveticus,*and *L. bulga cus.* These organisms are cultured industria and the culture selected depends upon the sired curd structure and flavor, a minimal production during fermentation, a low sensitiv to bacteriophages, and the extent of acid p duction.

Rennet has been used for generations a milk-coagulating enzyme. It is an extract fr the fourth stomach of 3- to 4-week-old cal which have been raised on milk. The purifi enzyme is called *rennin, chymase,* or *chymos* The main binding site of rennin on κ-casein the phenylalanine residue at location 105 and t methionine at location 106. The kinetics of m coagulation with microbial rennins has been tensively studied.

Due to the increasing production of chee (in 1974, six million tons were manufactur worldwide) and a decline in the number slaughtered calves, intensive research has be underway since 1960 to develop rennin produ of microbial origin.

Enzyme systems must meet the following quirements:

- Good coagulation of casein without hydr ysis
- Good odor and structure of the cheese
- No unpleasant odor of its own
- Nontoxic
- Low proteolysis in order to prevent the d velopment of bitterness in the ripening p cess

- Low lipase activity, to avoid the development of rancidity in the cheese.

A variety of fungi and bacteria have been isolated as producers and have been examined for the above characteristics.

The following are the most important genera of bacteria: *Alcaligenes, Bacillus, Corynebacterium, Lactobacillus, Pseudomonas, Serratia, Streptococcus,* and *Streptomyces.* The *Bacillus* strains (*B. subtilis, B. polymyxa,* and *B. mesentericus*) have been marketed but have not proved commercially successful.

There are several genera of fungi which produce milk-coagulating enzymes: *Aspergillus, Candida, Coriolus, Endothia, Enthomophthora, Irpex, Mucor, Penicillium, Rhizopus, Sclerotium,* and *Torulopsis.*

Only three strains of fungi are used worldwide in production. They are subdivided into 2 groups:

Type I	*Mucor pusillus* var. Lindt	Solid surface culture
Type II	*Endothia parasitica*	Submerged culture
	Mucor miehei	Submerged culture

It has not yet been possible to cultivate *Mucor pusillus* successfully by the submerged culture process; in a stirred bioreactor, only about half as much rennin activity is formed as in the surface process. Moreover, there are substantial side products in submerged fermentation, such as proteases and lipases, which prohibit the direct use of enzyme preparations without extensive purification. The enzyme, which is produced at 30°C in 70 hours, is sold commercially as a liquid preparation. The rennin produced by *Endothia parasitica* was the first rennin marketed commercially, placed on the market in 1967. The enzyme is an acid protease, stable at pH 4.0–5.5, with a molecular weight of 34,000–37,500. Within the designated pH range at 50°C, only 30% of the activity is lost within 30 minutes. A nutrient solution for enzyme production contains 3% soy meal, 1% glucose, 1% skim milk, 0.3% $NaNO_3$, 0.05% K_2HPO_4, 0.025% $MgSO_4 \cdot 7\,H_2O$; pH 6.8. The fermentation takes 48 hours at 28°C. At the end of the fermentation, after removal of the mycelium, the extracellular enzyme is concentrated and precipitated in an evaporation process.

Several *Mucor miehei* strains produce usable rennin. The enzyme is stable around pH 4.5 and the molecular weight is 38,000–41,800. A typical medium for production is 4% potato starch, 3% soy meal, 10% ground barley, 0.5% $CaCO_3$. The fermentation takes 5–6 days at 40°C.

After the curd has formed, most of the enzyme remains in the whey and is therefore lost. Because of this, studies have been carried out to immobilize the enzyme, but so far these studies have been unsuccessful. Microbial rennins are actually too temperature stable, remaining active in the curd after precipitation and subsequently causing harmful proteolysis. Research is thus being done to clone the gene for calf rennin into microorganisms. Complementary DNA (cDNA) from calf prorennin has been successfully expressed in *Escherichia coli,* making possible the first commercial production by a microorganism of the calf rennin enzyme.

11.7 PECTINASES

Pectinase preparations contain at least six enzymes, splitting pectins at different sites of the molecule. The basic structure of pectin is α-1,4-linked galacturonic acid, with up to 95% of its carboxyl groups esterified with methanol. Pectinases are classified according to their point of attack on the pectin molecule (Figure 11.17). The methyl ester is split by pectinesterase and the glycosidic bonds of the pectin chain are split by hydrolysis with endo-polygalacturonases or exo-polygalacturonases. Another method of splitting is transelimination by means of bacterial endo-pectate lyases, or fungal exo- and endo-pectin lyase (Figure 11.18).

A number of commercial firms produce fungal pectinases using *Aspergillus niger* or *A. wentii* and one fermentation plant uses *Rhizopus.* Both surface and submerged processes are used. Pectinases have been discovered in other fungi and also in bacteria, protozoa, insects and higher plants.

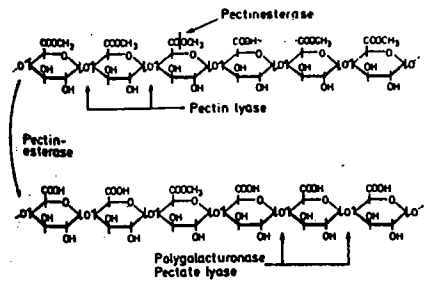

Figure 11.17 Pectin hydrolysis with various individual pectinases

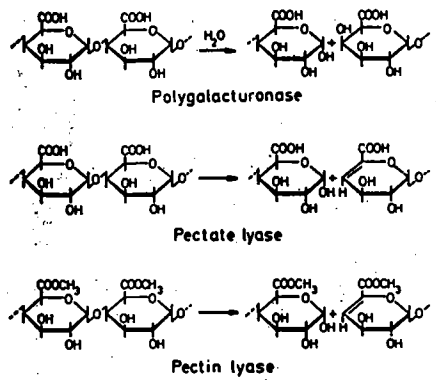

Figure 11.18 Splitting of glycosidic bonds in pectin through hydrolysis with polygalacturonase and through transelimination with pectate lyase and pectin lyase

The fermentation with *Aspergillus niger* runs for 60–80 hours in fed-batch cultures at pH 3–4 and 37°C, using 2% sucrose and 2% pectin. The purification of the enzyme is simple: the biomass is removed by filtration or centrifugation, stabilizing agents are added, the enzyme is precipitated with organic solvents, and the crude protein dried.

Pectinases are used primarily to clarify fruit juices and grape must, for the maceration of vegetables and fruits, and for the extraction of olive oil. Pectin-like substances are present at concentrations ranging from 0.2–1% in grapes to 30% in sugar beet pulp. By treatment with pectinase (1–2 hours at 54°C or 6–8 hours at 18°C) the yield of fruit juice during pressing is considerably increased.

11.8 LIPASES

Lipases (glycerol ester hydrolases) split fats (glycerol esters) into di- or monoglycerides and fatty acids (Figure 11.19). They are usually extracellular enzymes. Fungi producing lipases include *Aspergillus, Mucor, Rhizopus, Penicillium, Geotrichum,* and the yeasts *Torulopsis* and *Candida.* Bacteria producing lipases include *Pseudomonas, Achromobacter,* and *Staphylococcus.* Of these organisms, only *Aspergillus, Mucor, Rhizopus,* and *Candida* are used commercially.

In most cases, enzyme production must be induced by adding oils and fats. However, there are some cases in which fats have no effect on lipase production, and with *Penicillium roqueforti,* they actually repress enzyme production. Glycerol, a product of lipase action, represses lipase formation. This is also true for glucose, so that lipase production in *Geotrichum candidum* is first induced at the end of the log phase (around 15 hours). Lipases are generally bound to the cells and hence inhibit an overproduction, but addition of a cation such as magnesium ion liberates the lipase and leads to a higher enzyme titer in the production process. Isoenzymes with

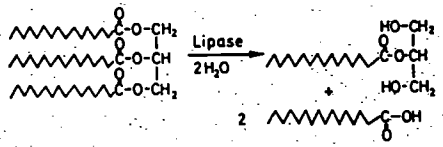

Figure 11.19 Splitting of fats into monoglycerides and fatty acids with lipases

Table 11.12 Temperature and pH optima of several microbial lipases

Organism	Optimal pH	Optimal temperature
Penicillium chrysogenum	6.2–6.8	37
Pseudomonas fragi	7.0–7.2	32
Rhizopus delemar	5.6	35
Aspergillus niger	5.6	35
Penicillium roqueforti	8.0	37
Staphylococcus aureus	8.5	45
Geotrichum candidum	8.2	37
Achromobacter lipolyticum	7.0	37

(Shahani, 1975)

varying pH optima, temperature optima, or substrate specificities are frequently produced.

Temperature and pH optima for lipases are given in Table 11.12.

The commercial use of lipases has been limited. They are primarily marketed for therapeutic purposes as digestive enzymes to supplement pancreatic lipases. The enzymes also find some use in the dairy industry. Since free fatty acids determine the odor and taste of cheese, and the cheese ripening process is affected by lipases, microbial affects during the aging process can be due to lipase action. For instance, in the production of roquefort cheese (blue cheese) spores of *Penicillium roqueforti* are added and serve as a sort of lipase preparation. In the soap industry, the lipase from *Candida cylindraceae* is used to hydrolyze oils. A potentially important future use of lipases is for the synthesis of esters from acids and alcohols in nonaqueous media. Another potential use is improvement of fat quality by the exchange of one fatty acid by another.

11.9 PENICILLIN ACYLASES

As we will describe in Chapter 13, many penicillin antibiotics used in medicine are produced **semisynthetically** by chemical modification of the basic penicillin ring structure. The starting material for chemical modification is the nonacylated thiazolidine-β-lactam ring system of penicillins, 6-aminopenicillanic acid (6-APA, Figure 11.20). 6-APA is excreted in low concentrations by *Penicillium chrysogenum* when the organism is grown in a medium without addition of phenylacetic acid as a precursor. This process for production of the basic ring system cannot currently be commercially utilized due to its high cost. 6-APA can also be produced by the splitting-off of the acyl side chain of microbially produced penicillin G with the enzyme penicillin acylase (also called penicillin amidase or benzylpenicillin amidohydrolase). It is essential that with the enzyme used the β-lactam ring not open (Figure 11.20). Penicillin acylases thus have commercial use in the production of semisynthetic penicillins.

Classification of penicillin acylases

Penicillin acylases are produced by yeasts, fungi, and bacteria. They can be subdivided into 2 types. Type I acylases, also called the fungal type, split phenoxymethyl penicillin (penicillin V) but attack benzyl penicillin (penicillin G) much less efficiently. Known producers of these acylases are *Penicillium* sp., *P. chrysogenum, Aspergillus ochraceus, Trichophyton mentagrophytes, Epidermophyton floccosum, Cephalosporium* sp., and *Fusarium semitectum*. Aside from fungi, the bacterium *Streptomyces lavendulae* also produces this

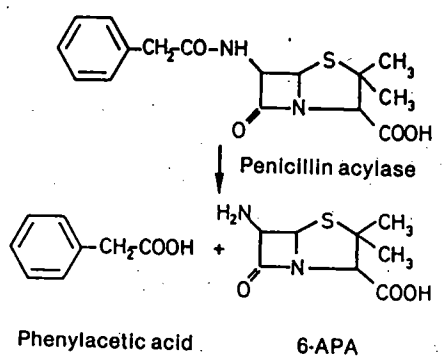

Figure 11.20 Splitting of penicillin G into 6-aminopenicillanic acid and phenylacetic acid

type of acylase. In addition to penicillin V, penicillin K (heptyl penicillin), dihydro penicillin F (pentyl penicillin), and a number of synthetic penicillins are split. These acylases, which are usually extracellular, have a pH optimum of 10 and a temperature optimum of 50°C.

Type II acylases, also designated as the bacterial type, are produced by *Aerobacter, Alcaligenes, Bordetella, Cellulomonas, Corynebacterium, Erwinia, Escherichia, Flavobacterium, Micrococcus, Nocardia, Proteus, Pseudomonas, Salmonella, Sarcina,* and *Xanthomonas*. The temperature optimum is 40°C and the pH optimum is pH 8, which is lower than that of the Type I acylases. Phenylacetic acid acts as a competitive inhibitor and 6-APA as a noncompetitive inhibitor.

The extracellular and intracellular acylases of various microorganisms differ in substrate specificities. The substrate specificity is determined by the nature of the acyl moiety and not by the 6-APA.

Penicillin acylase from *Escherichia coli*

Penicillin acylases from *Escherichia coli* are used almost exclusively for 6-APA production. The production strains are mutants of *Escherichia coli* ATCC 11105 and *Escherichia coli* ATCC 9637. Intracellular acylase production is induced in wild-type strains by addition of phenylacetic acid. Glucose represses enzyme production and must therefore be present in the culture medium only in low concentrations. The production rate is also strongly affected by the O_2 partial pressure. Although *E. coli* can grow under anaerobic conditions, enzyme yields are low; however, enzyme production is also suppressed by high aeration rates.

A suitable nutrient medium consists of 2% corn steep water at pH 7.0. The inoculum consists of 0.25% of an 18-hour preculture. To induce enzyme formation, after 8 hours of fermentation at 24°C, 0.1% of a sterile ammonium phenylacetate solution is added at hourly intervals for the next 13 hours.

The cells are concentrated 20-fold on a s arator and the enzyme is then released by us a pressure disintegrator (500 kg/cm² pressu The crude enzyme preparation so obtaine subsequently purified by conventional bioch ical procedures.

The enzyme yield in commercial product has been substantially improved by optimi the culture medium and by use of classical netic techniques. Recombinant DNA techniq promise to increase the yields even more. instance, by cloning the penicillin acylase g on a multicopy plasmid, strains have been tained having a 28-fold increase in enzyme mation rate in noninduced fermentation an further 6-fold increase in induced fermenta (Table 11.13).

There are 3 methods used commercially producing 6-APA from penicillin G. A stri chemical method involves converting penic G in a series of steps to N-iminomethoxyp cillin ester, which is hydrolyzed with aque ammonia to 6-APA. Two microbial methods used for the enzymatic conversion, one usir bacterial slurry and the other using carrier-bo penicillin acylase.

Enzymatic splitting with whole bacteria The o inal process uses cell-bound penicillin acyl *Escherichia coli* slurry is added as a crude enz solution directly to a penicillin G solution batch process which has been run at pH 8.0 37°C. At the end of the reaction, the cells removed by filtration and discarded. The filt is adjusted to pH 2.0 and the 6-APA separ

Table 11.13 Penicillin G-acylase formation in hybri strains

Escherichia coli Strain	Specific activity	
	Not induced	Induced
ATCC 11105	0.02	0.12
5 K pHM6	0.56	0.70
5 K pHM7	—	0.44
5 K pHM8	—	0.36
5 K pHM11	—	0.28

(Mayer et al., 1979)

from phenylacetic acid and nonconverted penicillin by extraction with methylisobutyl ketone. 6-APA is then precipitated at its isoelectric point (pH 4.3) and the crystals are washed and dried.

Carrier-bound penicillin acylase Several hundred tons of 6-APA are produced each year using this widely applied process. Its advantages are: conservation of the penicillin acylase by recycling, greater purity of the 6-APA, and fewer losses due to side reactions. There are processes for the immobilization of whole cells, in which acrylamide monomers are polymerized with cells. However, methods in which the purified penicillin acylase is immobilized are more widely used.

In one method, penicillin acylase from *Escherichia coli* is rendered insoluble by formation of a covalent bond with a polymer. Penicillin (100,000 units/ml) can be quantitatively split at 38°C and pH 7.8 in 6 hours when substrate and enzyme are used in a proportion of 5×10^4 units of substrate (1670 International Units = 1 mg penicillin G) per unit of penicillin acylase. One unit of enzyme is defined as the activity which hydrolyzes 1 μMole penicillin G in one minute to 6-APA and phenylacetic acid at 37°C. With this method, 6-APA can be isolated with a recovery of 87% of the theoretical yield and at 97% purity.

Cephalosporin acylases

Cephalosporins are antibiotics with a β-lactam ring similar to but different from that found in the penicillins. Semisynthetic cephalosporins are also produced in a manner analogous to the penicillins. In the past, the side chain L-α-aminoadipic acid was chemically split to produce the ring structure needed for the synthetic process, since microbiological methods resulted only in low yields. Today, however, there is a process which allows the microbiological splitting of cephalosporin C into the 7-acyl side chain and 7-aminocephalosporanic acid (7-ACA). A solution of 35% cephalosporin C can be split in 7 hours using an enzyme from *Pseudomonas* sp. (BN-188), which is closely related to *Pseudomonas putida*.

11.10 LACTASES

Lactase, also called β-galactosidase, splits lactose into glucose and galactose. The enzyme is intracellular in bacteria and yeast, but it is excreted by many fungi. The genetics of some bacterial β-galactosidase systems have been intensively studied, but the enzymes produced commercially are obtained from fungi or yeasts and are less well known. Lactases of commercial interest include those from the fungi *Aspergillus oryzae* and *A. niger*, and those from the yeasts *Kluyveromyces lactis*, *K. fragilis*, and *Torula cremoris* and *Bacillus* sp. (in immobilized form). The enzymes differ mainly in the following:

	Fungi	Yeasts
pH optimum	2.5–4.5	6.0–7.0
Temperature optimum	55°C	35°C

The yeasts are cultured in a submerged process, but the aspergilli must be cultivated on the surface of wheat bran at 30°C. Lactose is generally added to induce enzyme formation.

Lactases are used as digestive enzymes in cases of lactose-intolerance (usually due to lactase deficiency) and as a feed additive to increase the nutritive value of some animal feeds. In the dairy industry, lactases are used to break down lactose in milk. For example, in Europe lactose-low milk is produced by pasteurization and then treating with yeast lactase (4 hours at 35°C, which results in 70–80% hydrolysis of the lactose). The resulting product is then sterilized by the ultra-high temperature process and marketed. Lactases are also used commercially to process whey.

11.11 STABILIZATION OF ENZYMES AND CELLS

Purified enzyme preparations generally cannot be stored for long periods without losing their effectiveness. Native enzymes are subject to inactivation by chemical, physical, and biological factors, and the inactivation can occur either in storage or during use. There is thus a need to stabilize enzymes because of the high cost of enzyme production. Ideally, enzymes should be stable for months with as much activity as possible under varied conditions. Moreover, it should be possible to stabilize enzymes in commercial processes in such a way that they can be used over and over again and so that conversion of substrate to product can be carried out continuously.

If a cofactor such as NAD or an energy-rich substrate such as ATP is needed in the process, it is essential that regeneration be possible. Under such conditions, it is more cost-effective to use immobilized cells rather than immobilized enzymes. The technique for immobilizing cells is actually very analogous to that for immobilizing enzymes.

There are four basic methods of achieving enzyme stability:

1. **Stabilization of soluble enzymes.** Stabilization of soluble enzymes is accomplished by additives or by chemical modification. This improves the stability of enzymes against physical and chemical agents without decreasing their solubility. Such stabilized enzymes can be successfully stored but since they are still soluble they cannot be recycled after use.
2. **Stabilization by cross-linkage (immobilization) of enzyme molecules or cells.** Cross-linked enzyme molecules are linked to each other in such a way that their activity is not affected. They are no longer soluble and can be repeatedly or continuously used.
3. **Bonding to carriers.** In these procedures, the enzyme molecules are not bound to each other, but to a carrier. Immobilization is also possible within the original microbial cell. The fixation is done in such a way that it does not affect the enzyme activity, and the preparation can be used repeatedly or continuously as a carrier-bound enzyme.
4. **Incorporation within semipermeable membranes.** Encapsulated enzymes are separated from the surrounding substrate and product by a semipermeable membrane. Cells with enzymatic activity can also be encapsulated. The activity of the enzyme is not affected as a result of the encapsulation process and the preparation can be used repeatedly or continuously.

Stabilization of soluble enzymes

In some cases, the enzyme cannot be immobilized but must be used in soluble form. Examples include the enzymes used in liquid detergents, as diagnostic reagents, and as food additives. In such cases, some procedure for stabilization of the enzyme is necessary in order to prolong the shelf life of soluble enzymes. Following are some methods of stabilization:

Substrate stabilization The active site of an enzyme is responsible for its specific activity and this site can be stabilized by adding the substrate. For instance, α-amylase is stabilized by adding starch and glucose isomerase is stabilized against heat damage by addition of glucose. Figure 11.21

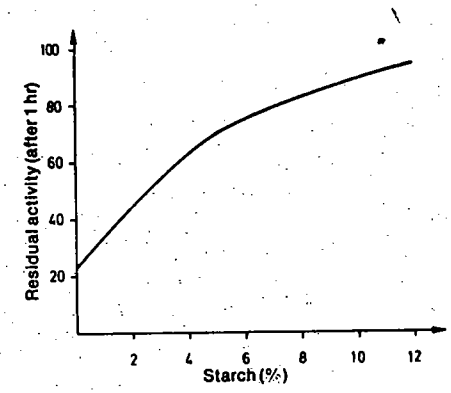

Figure 11.21 Stabilization of α-amylase by its substrate starch (Novo, 1970)

shows the stabilization of a bacterial amylase at pH 7.0 and a temperature of 80°C using starch.

On the other hand, there are enzymes such as pyruvate dehydrogenase whose activity is decreased when substrate is added.

Solvent stabilization Enzymes can also be stabilized by adding solvents; many of these can cause denaturation at high concentrations but afford considerable stabilization at low concentrations. Figure 11.22 shows several examples of the stabilizing effect of solvents on benzylalcohol dehydrogenase.

Stabilization by means of salts Cations such as Ca, Cu, Fe, Mn, Mo, and Zn have an effect on the stability and activity of metalloenzymes. For example, calcium helps to stabilize the tertiary structure of α-amylases of *Bacillus caldolyticus* and proteases.

Stabilization by means of polymer additives Natural or synthetic polymers such as gelatin, albumin, fatty alcohol ethylene oxide adducts, or polyethylene glycols can increase the heat stability of enzymes.

Stabilization by chemical means In addition to enzyme-bonding to soluble carriers, stabilization can be accomplished by chemical modification without a loss of solubility. One method is the formation of enzymes with polyamino side chains, such as the production of polytyrosyl or polyglycyl enzymes. Another method is acylation with acetyl, formyl, propionyl, or succinyl groups. Asparaginase in blood can be considerably protected by acylation. In other enzymes, such as α-amylases, the temperature sensitivity and sensitivity to proteases can be reduced.

By means of bifunctional or multifunctional additives, enzymes can also be polymerized in such a way that they remain soluble.

Stabilization by means of immobilization

The bonding of an enzyme to another enzyme or to a carrier must take place without changing the three-dimensional structure at the active site of the molecule. Neither the substrate specificity nor the specificity of the reaction must be lost as a result of the immobilization process.

Functional groups on the enzyme molecule that are suitable for use in the immobilization process are free α-, β-, or γ-carboxyl groups, α- or β-amino groups, and phenyl, hydroxyl, sulfhydryl, or imidazole groups of the appropriate amino acids. The groups used must not be critical for the activity of the enzyme.

The three major approaches to enzyme immobilization are illustrated in Figure 11.23 and are summarized below.

Cross-linked enzymes Several examples of this method for immobilizing enzymes can be given. Glutaraldehyde is commonly used as a polymerizing agent, and Figure 11.24 shows how this

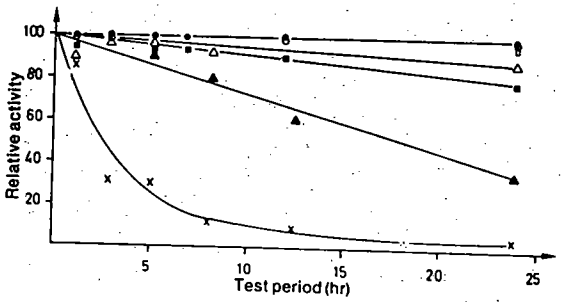

Figure 11.22 Stability of benzyl alcohol dehydrogenase in organic solvents. × — × without solvent; △ — △ 2% acetone; □ — □ 5% acetone; ○ — ○ 10% acetone; ▲ — ▲ 2% ethanol; ■ — ■ 5% ethanol; ● — ● 10% ethanol (From Katagiri et al., 1967)

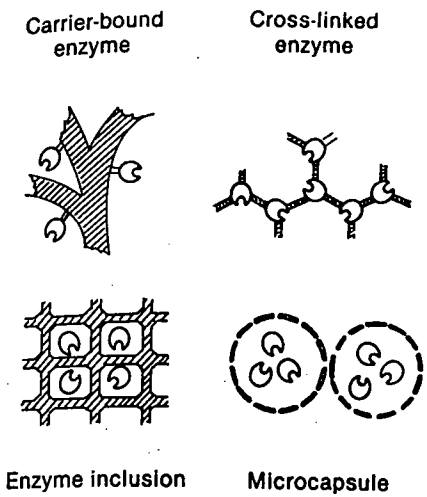

Figure 11.23 Procedures for the immobilization of enzymes (From Chibata, 1978)

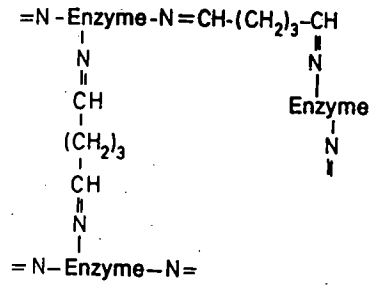

Figure 11.24 Cross linkage of enzymes with glutaraldehyde

compound reacts with amino groups of the enzymes (for example, amylases and alcohol dehydrogenase) in the formation of Schiff bases. Immobilization of cells is also frequently accomplished with glutaraldehyde. Another cross-linking agent is hexamethylene diisocyanate, which reacts with enzymes by peptide bond formation (Figure 11.25).

Carrier-bound enzymes The bonding of an enzyme to a carrier through adsorption, ionic bonding, or covalent bonding is the oldest method of enzyme immobilization, and it is the most frequently used commercial method. The structure of the carrier, the type of functional bonding sites, the ratio of hydrophilic to hydrophobic groups, the particle size, and the surface/volume ratio must be suitable for the individual enzyme and for its eventual commercial use.

Bonding by means of adsorption causes the least damage or conformation alteration of the protein. Fixation is easily accomplished and the method is applicable to a wide variety of enzymes. In addition, the carrier can be regenerated in most cases. However, the bonding strength is low, and this is a disadvantage of adsorption procedures; any temperature changes in the bioreactor cause elution of enzyme from the carrier and hence activity loss. Both organic and inorganic carriers can be used for the adsorption process and several types are listed in Table 11.14.

Ion exchangers can be used as enzyme carriers, the enzyme being held to the carrier by ionic bonds. This method overlaps in part with the adsorption method. Ionic bonding is an easily accomplished method with low activity loss, al-

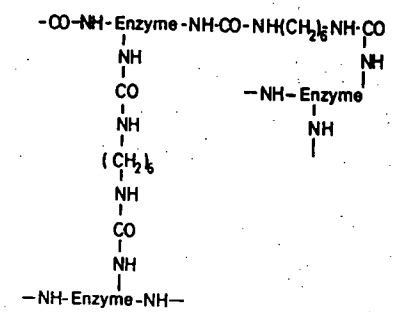

Figure 11.25 Cross linkage of enzymes with hexamethylene diisocyanate

Table 11.14 Adsorption carriers for immobilization of enzymes

Type of carrier	Carrier	Enzyme used
Inorganic	Aluminium oxide	Glucoamylase
	Bentonite	Invertase
	Glass	Lipase
	Ca phosphate gel	Aspartase
Organic	Activated carbon	Glucose oxidase
		α-Amylase
		β-Amylase
		Glucoamylase
		Invertase
	Starch	α-Amylase
	Tannin aminohexyl cellulose	Glucose isomerase
		Aminoacylase

though changes in pH or ionic strength can result in enzyme loss from the carriers. A variety of commercially available ion exchangers can be used as carriers.

Immobilization by covalent bonding has the advantage of strong bonding forces. In this procedure, the side chain of one or more amino acids of the protein is modified so that it forms a covalent link with the carrier. The preparation of a covalently bonded enzyme is expensive and difficult to carry out because amino acids of the active center may also become involved in the reaction. Covalent bonding is the most widely used commercial process. The kinds of covalent bonds used include peptide bonds and diazo bonds, as well as alkylation and isourea bond formation with BrCN-activated carbohydrates.

For enzyme bonding to occur, the functional groups of the carrier must be activated. For the production of peptide bonds with primary amino groups of the enzyme protein, the carboxyl groups of the carriers can be activated through the production of azide or anhydride derivatives. An example of covalent linking is that shown with carboxymethyl cellulose (CM cellulose) in Figure 11.26. Carboxymethyl cellulose is first converted to the methylester and subsequently treated with hydrazine. The resulting hydrazide then reacts with sodium nitrite and the corresponding azide derivative is produced which reacts with the amino groups of the enzyme.

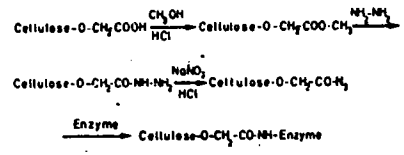

Figure 11.26 Immobilization of enzymes with carboxymethylcellulose

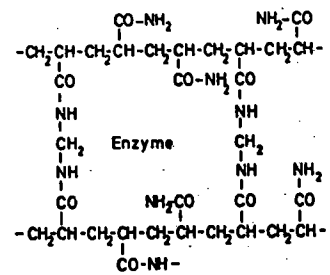

Figure 11.27 Enzyme incorporation into a polyacrylamide gel

Encapsulation of enzymes

When enzymes are **physically enclosed** in gels, microcapsules, or fibrous polymers, there must be pores which are so small that the enzyme molecules cannot be washed out, yet which are still large enough to permit the unimpeded diffusion of low-molecular-weight substrates and products across the barrier. Unfortunately, there may be considerable yield loss in the production of an encapsulated enzyme, but the finished products are very stable.

Gel enclosure Cross-linked, water-insoluble polymers such as polyacrylamide, polyvinyl alcohol or starch can be used to surround the enzyme. The method most frequently used involves incorporation into a polyacrylamide gel (Figure 11.27). Acrylamide and the enzyme are polymerized within minutes and at room temperature into a gel by means of a cross-linking compound (e.g. NN'-methylene bisacrylamide), a starter (sodium persulfate), and an accelerator (β-dimethylamino propionitrile). The gel can then be cut up and used in a batch or column process.

Microcapsules The advantage of immobilization of enzymes using microcapsule preparations is that free enzyme or free cells can be surrounded by a semipermeable polymer membrane. Production takes place by means of a polymerization reaction at the surface of aqueous enzyme droplets which are suspended in a non-water-soluble organic phase. Microcapsulated enzymes have not yet found commercial application, but they appear promising for future clinical and analytical uses.

Immobilization in fibrous polymers This method of immobilization is similar to the method using microcapsules. An emulsion of aqueous enzyme solution and organic water-immiscible solvent is extruded into a precipitation bath in which the fibrous polymer forms and encloses the enzyme. Cellulose triacetate and other cellulose derivatives serve as suitable polymers.

Industrial application of immobilized enzymes and cells

The first large-scale applications of immobilized cells were optimized empirically and have been in use for many years. The best example is the use of the *trickling filter* for sewage treatment (see Chapter 17). A modern application of this procedure is the immobilization of cultures of anaerobic bacteria on sintered glass particles, which makes it possible to increase the rate of anaerobic sewage treatment. For instance, in the effluent from a cellulose processing plant, 84% of the chemical oxygen demand (COD) was removed in a 12 hour holding time with a loading of 88 kg COD/m³/day. Another well-known example of the use of immobilized cells is in the trickling generator used for vinegar production.

A number of processes have also been introduced in more recent times. Among these are the use of immobilized cells containing **glucose isomerase** for the production of high-fructose syrup (see Section 11.3). The half-life of the immobilized isomerase in large-scale operation ranges from 70–120 days. World-wide production with this method amounts to 7.5×10^6 tons per year, of which about half is in the United States. **Glucose amylase** for the production of glucose from starch is now frequently used in an immobilized state. The enzyme **β-galactosidase** from yeast is now being immobilized in some countries (for instance, Italy) for the splitting of lactose to glucose plus galactose. Some studies are under way to use this process to produce a sweet syrup from whey. A large-scale process for the immobilization of **invertase** has been developed for the splitting of sucrose to glucose plus fructose. The enzyme **α-galactosidase** splits the trisaccharide raffinose to galactose plus sucrose. Raffinose is present in sugar beet juice at a concentration of around 0.1% and reduces the yield of sucrose during sugar beet production because of its effect on the crystallization process. A large-scale process is under study to immobilize the α-galactosidase from the fungus *Absidia* sp. for use in the sugar beet industry. A number of processes for **amino acid production** have been developed using immobilized enzymes or cells (see Section 9.3).

In addition to the food industry, immobilized systems have also been developed for fine-chemical production. The process using penicillin acylase has already been discussed in Section 11.9. In another example, malic acid is being produced from fumaric acid using the fumarase of *Brevibacterium ammoniagenes*. In this process, an enzyme/membrane reactor has been developed which carries out the following reaction:

$$HOOC{-}CH{=}CH{-}COOH + H_2O \rightarrow HOOC{-}CHOH{-}CH_2COOH$$

In this process, a 1 m³ column reactor has been

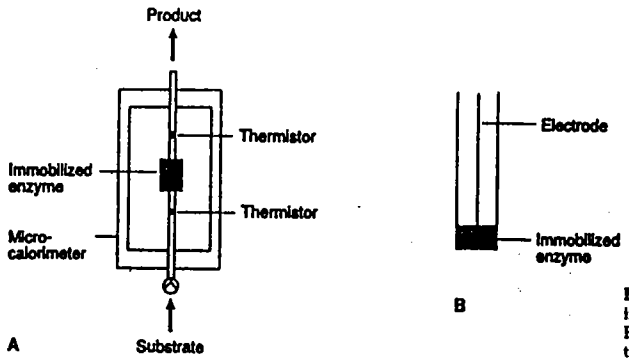

Figure 11.28 Biochemical assays using immobilized enzymes or cells. A. Enzyme/thermistor. B. Enzyme electrode

Table 11.15 Examples of biochemical assays carried out by means of the enzyme/thermistor method

Substrate	Enzyme	Concentration (mMol/l)
Ascorbic acid	Ascorbic acid oxidase	0.05–0.6
ATP	Hexokinase	1–8
Cholesterol	Cholesterol oxidase	0.03–0.15
Cephalosporin	Cephalosporinase	0.005–10
Ethanol	Alcohol oxidase	0.01–1
Galactose	Galactose oxidase	0.01–1
Glucose	Hexokinase	0.5–25
Lactose	Lactase/Glucose oxidase/Catalase	0.05–10

Table 11.16 Electrode sensors employing immobilized enzymes or cells

Substrate	Strain/enzyme	Measurement principle
Sucrose	Invertase/Mutarotase Glucose oxidase	O_2 uptake
Monoamine	Monoamine oxidase	O_2 uptake
Cholesterol	Cholesterol oxidase	O_2 uptake
Acetic acid	*Trichosporon brassica*	O_2 uptake
Ethanol	*Trichosporon brassica*	O_2 uptake
Nystatin	Yeast	O_2 uptake (as a result of cell death)
Cephalosporin	*Citrobacter* with cephalosporinase	pH measurement

used, with a flow rate of 0.3 h^{-1} at pH 7.5 and 37°C. The half-life of the enzyme in this process was about 160 days.

In Japan, the large-scale production of ethanol has been carried out using growing, immobilized cells of the yeast *Saccharomyces*. The cells are immobilized by photochemical reaction with a cross-linked gel. The bioreactor, which produces more than 1.5 mol/l·h ethanol, has been operated successfully over long periods of time. A process for the production of glycerol has been developed using immobilized *Saccharomyces cerevisiae*, but is still at the laboratory stage.

Special bioreactors for use in immobilized processes were discussed in Section 5.3.

Uses of immobilized cells and enzymes for analytical biochemistry

An immobilized system can be used in the development of a precise and sensitive biochemical assay. The basic principle is that the enzyme acts on the substrate and a decrease in substrate concentration, increase in product concentration, or change in cofactor concentration can be followed, using one of many assay methods. The reaction can be followed spectrophotometrically, polarimeterically, photometrically, or by mass spectroscopy. Additionally, as shown in Figure 11.28 A, the reaction can be followed calorimetrically by measuring heat production with a thermistor, or as shown in Figure 11.28 B, by measuring potential difference with an electrode.

Table 11.15 gives several examples of substances that can be assayed calorimetrically and Table 11.16 gives some examples in which electrodes are used.

In some cases, a coupled series of reactions can be employed. As an example, the measurement of sucrose can be linked to the measurement of oxygen uptake via the following reaction chain:

$$\text{Sucrose} + H_2O \rightarrow \alpha\text{-D-Glucose} + \text{D-Fructose}$$
$$\alpha\text{-D-Glucose} \rightarrow \beta\text{-D-Glucose}$$
$$\beta\text{-D-Glucose} + O_2 + H_2O \rightarrow \text{D-Glucose-}\delta\text{-lactone} + H_2O_2$$

This particular enzyme electrode, which involves invertase, mutarotase, and glucose oxidase, respectively, functioned for over 85 separate determinations over a 10 day period.

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12

Vitamins

12.1 INTRODUCTION

Microorganisms can be used for the commercial production of certain vitamins, such as thiamine, riboflavin, folic acid, pantothenic acid, pyridoxal, vitamin B_{12}, and biotin. Also, microorganisms (including algae) synthesize β-carotene, which is provitamin A. Ergosterol (provitamin D_2), produced with a yeast fermentation, can occur in certain *Saccharomyces* strains at concentrations as high as 0.1–10% of the cell weight. In addition to direct fermentation, certain vitamins can also be produced by combined chemical/microbiological means, the microorganism carrying out specific reaction steps (so-called *biotransformation*, see Chapter 15). Biotransformation plays an important role in the production of ascorbic acid and tocopherol. In Japan, a wide variety of vitamins are produced by microbial fermentation. However, on a worldwide basis only the production of vitamin B_{12}, riboflavin, and ascorbic acid have any major economic significance. The microbial production of β-carotene, although possible, is not economic under large-scale conditions.

12.2 VITAMIN B_{12}

Occurrence and economic significance

In 1926, G. R. Minot and W. B. Murphy determined that liver extracts cure human pernicious anemia, a discovery for which they were awarded the Nobel Prize in medicine in 1934. Independently, E. L. Ricke and L. Smith isolated crystalline vitamin B_{12} from liver extracts in 1948. Vitamin B_{12} (cyanocobalamin) is a vitamin that is synthesized in nature exclusively by microorganisms; because it is required by animals it is present in every animal tissue in very low concentrations (e.g. 1 ppm in the liver). Although the substance isolated from tissues is cyanocobalamin, in the cell only the coenzyme form (adenosyl- or methylcobalamin) is present, cyanocobalamin not appearing until the product recovery stage. The vitamin B_{12} needs of animals

are covered by food intake or by absorption of vitamin B_{12} produced by intestinal microorganisms. However, humans obtain vitamin B_{12} only from food, since the B_{12} synthesized by microorganisms in the large intestinal tract cannot be assimilated.

The concentrations of vitamin B_{12} which are present in animal tissues are too low for use in commercial production. Activated sludge from sewage treatment contains 4–10 mg B_{12}/kg, but isolation from this source is expensive due to the problem of separating the various B_{12} analogs. Chemical synthesis is also impractical, since it requires 70 reaction steps. Vitamin B_{12} was first obtained commercially as a byproduct of streptomycete fermentations for the production of the antibiotics streptomycin, chloramphenicol, or neomycin, with a yield of about 1 mg/l. As the demand for vitamin B_{12} increased, fermentation processes were developed with higher-yielding strains. Commercial production is currently carried out entirely by fermentation. The most important manufacturers of vitamin B_{12} are: Farmitalia S.p.A. (Italy); Glaxo Lab., Ltd. (England); Merck & Co., Inc. (United States); Rhône Poulenc S.A. (France); Roussel UCLAF (France); G. Richter Pharmaceutical Co. and Chinoin (both Hungary). The current annual world production of vitamin B_{12} is estimated at about 12,000 kg. Approximately 3500 kg cyanocobalamin, 2000 kg hydroxocobalamin, 1000 kg coenzyme B_{12} and a small amount of methylcobalamin is supplied to the pharmaceutical industry; the remainder goes to the animal feed industry. For swine and poultry feeds, 10–15 mg vitamin B_{12} is added per ton of feed, since animal protein can be replaced with less-expensive vegetable protein if the vegetable protein is fortified with vitamin B_{12}.

Structure

Corrin, the base structure of vitamin B_{12}, consists of a tetrapyrrole ring which differs from the porphyrin ring system in that the methene bridge between rings A and D is missing. The C-1 of ribose from cobamide (see structural formula, Figure 12.1) is linked to the heterocyclic base 5,6-dimethylbenzimidazole, which in turn occupies another coordination site of the cobalt next to CN in the final cyanocobalamin product. Vitamin B_{12} analogs having other heterocyclic bases (purines or substituted benzimidazoles) are either spontaneously produced by microorganisms or are produced after the addition of these substances to the culture medium. However, they are less biologically effective in vertebrates than the corresponding 5,6-dimethylbenzimidazole compounds. It is noteworthy that vitamin B_{12} derivatives with a purine base are ineffective in humans.

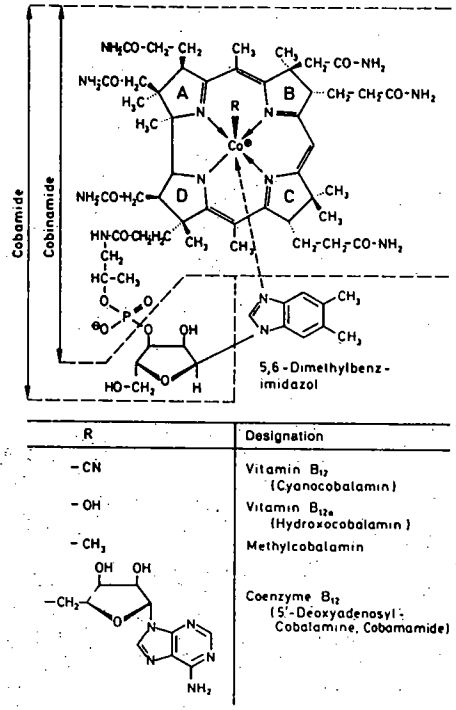


Figure 12.1 Structure of cobalamins

Biosynthesis

The biosynthesis of vitamin B_{12}, which runs parallel with the biosynthesis of porphyrins and chlorophyll up to the formation of uroporphyrinogen III, is diagramed in Figure 12.2. The conversion of uroporphyrinogen III into the corrin ring system is not yet fully understood.

Vitamin B_{12} production based on media containing carbohydrates

Most of the B_{12} fermentation processes use glucose as a carbon source. Several producing strains are known, listed here with their yield of B_{12}: *Bacillus megaterium* (0.45 mg/l); *Butyribacterium rettgeri* (5 mg/l); *Streptomyces olivaceus*

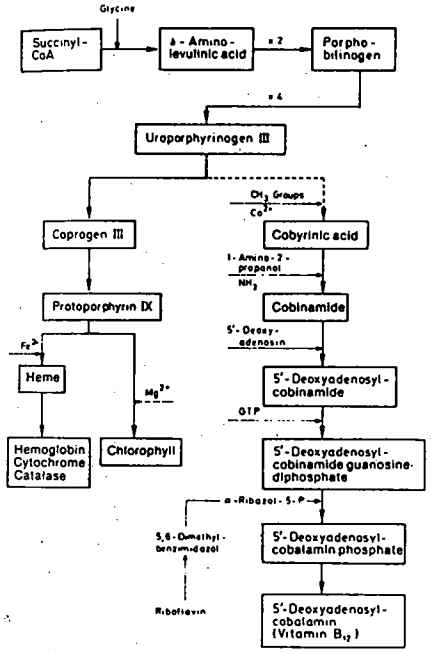


Figure 12.2 Biosynthesis of cobalamins

(3.3 mg/l); *Micromonospora* sp. (11.5 mg/l); *Kl sieila pneumoniae* (0.2 mg/l). Higher yields ha been obtained from *Propionibacterium freud reichii* (19 mg/l), *Propionibacterium sherma* (30–40 mg/l), and (in a process using sugar ca molasses) *Pseudomonas denitrificans* (60 mg/l

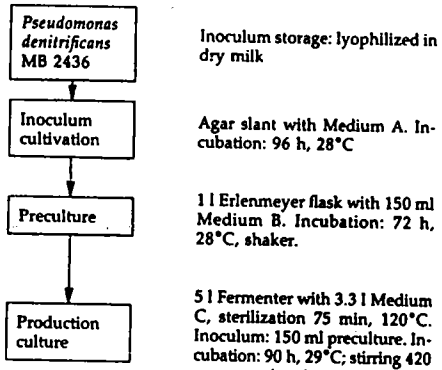
The use of modern genetic engineering te niques is being applied to vitamin producti also. A hybrid strain called *Rhodopseudomo protamicus*, made by the protoplast fusion te nique between *Protaminobacter ruber* and *Rl dopseudomonas spheroides*, produces 135 mg/l tamin B_{12} using glucose as carbon source witho the addition of 5,6-dimethylbenzimidazole.

Processes with Propionibacteria *Propionibact ium freudenreichii* ATCC 6207 and *P. sherma* ATCC 13673, as well as other variants and m tants, are used in a two-stage process with add cobalt (10–100 mg/l). In a preliminary anaerol phase (2–4 days), 5'-deoxyadenosylcobinami is mainly produced; in a second, aerobic pha (3–4 days) the biosynthesis of 5,6-dimethylber imidazole takes place, so that 5'-deoxyaden sylcobalamin (coenzyme B_{12}) can be produce Only traces of other cobamides are synthesiz in this process. As an alternative to the two-sta batch process in a fermenter, both stages can al be operated continuously in two tanks operat in cascade fashion.

During the recovery process, the cobalami which are almost completely bound to the ce are brought into solution by heat treatment (1 30 min at 80–120°C, pH 6.5–8.5). They are th converted chemically into the more stable cyan cobalamin. The raw product (80% purity) is us as a feed additive. Additional purification stag yield a medically usable preparation (95–98 purity). The total yield is 75% of the theoretic value.

Processes with Pseudomonas *Pseudomonas de trificans* has been found to be the most produ tive species among the different vitamin B_{12}-pr ducing pseudomonads. In this one-stage proce vitamin B_{12} is produced during the entire fe

mentation. Cobalt and 5,6-dimethylbenzimidazole must be added as supplements. It has also been found that additions of the compound betaine result in increased yield; sugar beet molasses is used as a low-cost betaine source. Although the mode of action is not known, betaine is assumed to cause an activation of biosynthesis or an increase in membrane permeability. The process shown in Figure 12.3 is one of the most cost-effective processes. After 12 years of strain development, the yields from this process have been increased from 0.6 mg/l to 60 mg/l.



Medium A (g/l): Sugar beet molasses 60; Yeast extract 1; N-Z-Amine 1; $(NH_4)_2HPO_4$ 2; $MgSO_4 \cdot 7\,H_2O$ 1; $MnSO_4 \cdot H_2O$ 0.2; $ZnSO_4 \cdot 7\,H_2O$ 0.02; $Na_2MoO_4 \cdot 2\,H_2O$ 0.005; Agar 25; Tap water; pH 7.4

Medium B (g/l): Medium A, without agar

Medium C (g/l): Sugar beet molasses 100; Yeast extract 2; $(NH_4)_2HPO_4$ 5; $MgSO_4 \cdot 7\,H_2O$ 3; $MnSO_4 \cdot H_2O$ 0.2; $Co(NO_3)_2 \cdot 6\,H_2O$ 0.188; 5,6-Dimethylbenzimidazol 0.025; $ZnSO_4 \cdot 7\,H_2O$ 0.02; $Na_2MoO_4 \cdot 2\,H_2O$ 0.005; Tap water; pH 7.4

Figure 12.3 Laboratory scale production process for vitamin B_{12} using *Pseudomonas denitrificans*. (Merck and Co., Inc., 1971)

Vitamin B_{12} production with other carbon sources

In the last 10 years, studies have been underway with a variety of strains to determine if various alcohol and hydrocarbon substrates could be used for vitamin B_{12} production (Table 12.1). Hydrocarbon and higher-alcohol fermentations resulted in low yields, but those using methanol seem to have considerable promise.

12.3 RIBOFLAVIN

Occurrence and economic significance

Riboflavin (also called lactoflavin or vitamin B_2) was first isolated from whey by Kuhn, György and Wagner-Jauregg in 1933; the structure was confirmed by synthesis by Kuhn and Karrer in 1935. Riboflavin is present in milk as free riboflavin, but is present in other foods (liver, heart, kidney, or eggs) as part of flavoproteins which contain the prosthetic groups FMN (flavin mononucleotide) or FAD (flavin adenine dinucleotide).

A riboflavin deficiency in rats causes stunted growth, dermatitis, and eye damage. Ariboflavinosis is a disease in humans caused by riboflavin deficiency. Appearing as a type of dermatitis, this disease can be counteracted by administering riboflavin at a daily dosage of 1 mg. Riboflavin is available in various preparations for human and veterinary application. In the United States riboflavin, as well as thiamine and nicotinic acid, is frequently added to flour for use in the production of vitamin-enriched bread.

Riboflavin is produced industrially by several processes:

- Chemical synthesis, primarily for pharmaceutical use (20% of world-wide production).
- Biotransformation of glucose to D-ribose by mutants of *Bacillus pumilus* and subsequent chemical conversion of ribose to riboflavin (about 50% of world-wide production).

Table 12.1 Vitamin B_{12} formation with alcohols and carbohydrates as carbon sources

Microorganism	Carbon source		Vitamin B_{12} yield (mg/l)
Bacteria from activated sludge	Methanol	0.3–1.2% (w/v)	35
Methanobacterium soehngenii	Methanol	0.8% (w/v)	8.0
Methanobacillus omelianski	Methanol	0.8% (w/v)	8.8
Protaminobacter ruber	Methanol	1.2% (w/v)	2.5
Methanosarcina barkeri	Methanol, fed-batch culture	0.8% (w/v)	42
Arthrobacter sp.	Isopropanol, fed-batch culture		1.1
Arthrobacter hyalinus	Isopropanol	1% (v/v)	0.56
Nocardia MT 2003	1,2-Propanediol	5% (w/v)	0.78
Klebsiella sp. 101	Ethanol	2% (v/v)	0.02
	1,2-Propanediol	2% (v/v)	0.06
	Methanol	2% (v/v)	0.16
	Methanol, fed-batch culture	0.5% (v/v)	1.3
Pseudomonas sp. ATCC 14718	Ethanol	1% (v/v)	0.05
	1,2-Propanediol	2% (v/v)	0.12
	Methanol	2% (v/v)	0.16
	Methanol, fed-batch culture	0.7–0.8% (v/v)	3.2
Strain XF	Methanol	2% (v/v)	0.23
Pseudomonas ovalis	n-Decane	1% (v/v)	0.08
Pseudomonas aureofaciens	n-Dodecane	1% (v/v)	0.04
Nocardia gardneri	n-Hexadecane	2% (w/v)	4.5
Mixed culture of *Corynebacterium* sp. and *Rhodopseudomonas spheroides*	n-Alkanes	10% (w/v)	2.3

(Florent and Ninet, 1979; Kamikubo et al., 1978)

- Direct fermentation (about 30% of world-wide production).

Total production of riboflavin on a world-wide basis is around 2000 tons per year.

Direct fermentation Riboflavin is synthesized by many microorganisms, including bacteria, yeasts and fungi. High-yielding organisms which could be used in production are listed in Table 12.2. However, only the two ascomycetes listed are important in commercial production. Originally *Eremothecium ashbyii* was used (yield 2 g/l), but after 1946, a process with genetically stable *Ashbya gossypii* was introduced. The riboflavin titer of this process is 10–15 g/l. Despite this yield, there is stiff competition between this microbiological process and the two other processes.

Table 12.2 Microorganisms making riboflavin and the effect of iron on biosynthesis

Microorganism	Riboflavin formation (mg/l)	Optimal iron concentration (mg/l)
Clostridium acetobutylicum	97	1–3
Mycobacterium smegmatis	58	No effect
Mycocandida riboflavina	200	No effect
Candida flareri	567	0.04–0.06
Eremothecium ashbyii	2480	No effect
Ashbya gossypii	6420	No effect

(Perlman, 1979)

Structure

Riboflavin is an alloxazine derivative which consists of a pteridine ring condensed to a benzene ring. The side chain consists of a C_5-polyhydroxy group, a derivative of ribitol. The structure of riboflavin (6,7-dimethyl-9-(D-1′-ribityl)-isoallox-

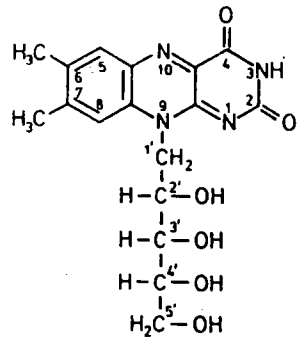


Figure 12.4 Structure of riboflavin

azine) is given in Figure 12.4. The isoalloxazine ring acts as a reversible redox system, as described in textbooks of biochemistry and microbiology.

Biosynthesis

The biosynthetic pathway shown in Figure 12.5 is postulated on the basis of experiments done primarily with yeast and *Ashbya gossypii*. The effect of iron on riboflavin synthesis is not yet understood. The overproduction of riboflavin by *Eremothecium ashbyii* and *A. gossypii* is not affected by iron, but riboflavin production in clostridia and yeasts is inhibited even by very low concentrations of iron. In clostridia, 1 ppm iron causes a 75% inhibition of synthesis. It is assumed that an iron-flavoprotein acts as a repressor of riboflavin synthesis. The overproduction by *E. ashbyii* and *A. gossypii* could be explained by the presence of constitutive riboflavin-synthesizing enzymes.

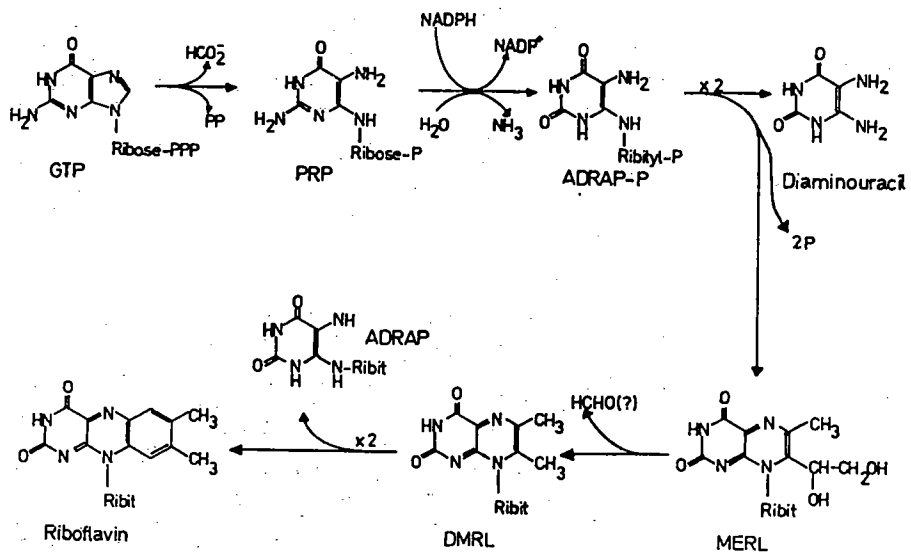


Figure 12.5 Biosynthetic pathway for riboflavin (from Brown and Williamson, 1982). Abbreviations: GTP, Guanosine triphosphate; PRP, 2,5-Diamino-6-keto-4-(5'-phosphoribosylamino)-pyrimidine; ADRAP, 5-Amino-2,5-dioxy-4-(5'-phosphoribitylamino)-pyrimidine; MERL, 6-Methly-7-(1',2'-dihydroxyethyl)-8-ribityllumazine; DMRL, 6, 7-Dimethyl-8-ribityllumazine; Ribit, Ribitol.

Production process

The fermentative production is currently carried out with *Ashbya gossypii* NRRL Y-1056. The high yields of more than 10–15 g/l were attained by strain development as well as by optimization of the nutrient solution, the cultivation of inoculum, and the fermentation conditions. Originally the fermentation used a medium with glucose and corn steep liquor; sucrose and maltose were other suitable carbon sources. When lipids were also used as energy sources, yields markedly increased. Riboflavin production on what is now the basic medium (corn steep liquor 2.25%, commercial peptone 3.5%, soy bean oil 4.5%) has been further stimulated by the addition of different peptones, glycine, distiller's solubles, or yeast extract. By simultaneous feeding of glucose and inositol the rate of formation of riboflavin can be further increased. A careful sterilization of the culture medium is critical for high yields, as is the use of a small inoculum (0.75–2%) of 24- to 48-hour-old actively growing culture. Th fermentation takes 7 days with an aeration rat of 0.3 vvm at 28°C. For foam control, silicon antifoam is applied at first, and soy bean oi which is also metabolized, is added later.

Riboflavin is present both in solution an bound to the mycelium in the fermentatio broth. The bound vitamin is released from th cells by heat treatment (1 hour, 120°C) and th mycelium is separated and discarded. The ribo flavin is then further purified.

Production of riboflavin with an aliphatic hy drocarbon (C_{10}–C_{18}) as carbon source has bee reported using *Pichia guilliermondii*, but yield in formation was not given. Using *Pichia miso*, 5 mg/l riboflavin was obtained on a medium wit n-hexadecane, corn steep liquor, and urea. Stud ies have been carried out on the use of a meth anol-utilizing organism, for example, *Hansenul polymorpha*, for the formation of riboflavin i batch or continuous culture.

Crystalline riboflavin preparations of hig purity have been produced using *Saccharomyce* fermentation with acetate as sole carbon source

Table 12.3 Production processes for several carotenoids

Carotenoid	Organism	Composition of medium	Length of fermentation (days)	Yield (mg/l)
β-Carotene	Mixed culture of *Blakeslea trispora* NRRL 2456(+) and NRRL 2457(−)	see Figure 12.9	8	3000
Lycopene	*Streptomyces chrestomyceticus* subsp. *rubescens*	Starch, soy meal, $(NH_4)_2SO_4$	6	500
	Mixed culture of *Blaskeslea trispora* NRRL 2895(+) and NRRL 2896(−)	Cotton seed meal, corn meal, soy bean oil; addition of triethylamine after 48 hours (suppresses carotenoid cyclization)	5	400
Zeaxanthin	*Flavobacterium* sp.	Glucose, corn steep liquor, palmitate ester, methionine, pyridoxine, Fe^{2+} salts		335
Mixture of various carotenoids	*Mycobacterium phlei*	Sugar beet molasses, urea; exposure to light required	7	300
	S. chrestomyceticus	Glucose, yeast extract	5	500
	Yeast	Sugar, ethanol or alkanes		< 5

(Ninet and Renaut, 1979)

12.4 β-CAROTENE

Occurrence and economic significance

Carotenoids are found in many animal and plant tissues, but originate exclusively from plants or microbes. β-Carotene (provitamin A) is converted into vitamin A in the intestinal mucous membrane and is stored in the liver as the palmitate ester. In human beings, a deficiency of vitamin A (daily requirement 1.5–2 mg) causes night blindness and changes in the skin and mucous membranes; it is particularly necessary for the normal biosynthesis of mucopolysaccharides.

The demand for β-carotene is about 100 tons per year, primarily for use as a food coloring agent. The demand for provitamin A is very low. Other carotenoids, such as lycopene or xanthophylls, which do not have a provitamin A activity, are used as food coloring agents (e.g. margarine, cheese, egg products), for improving color in meat or egg yolk in the poultry industry (citranaxanthine, 3–5 tons per year) or in the salmon industry (astaxanthine). Chemical synthesis is currently the only commercially feasible means of obtaining carotenoids; xanthophylls may also be obtained by extraction of plant material. Carotenoids are produced by many microorganisms (including algae), but fermentative production is not economical in the present market, since the yields of currently available processes (Table 12.3) do not compete with cost-effective chemical synthetic processes. However, depending on the market it appears that a competitive process might be successful, using the halophilic alga *Dunaliella salina* (see Borowitzka et al., 1986).

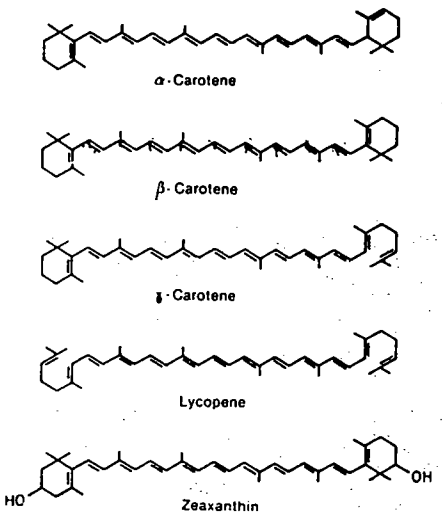


Figure 12.6 Structures of several carotenoids that can be produced by fermentation

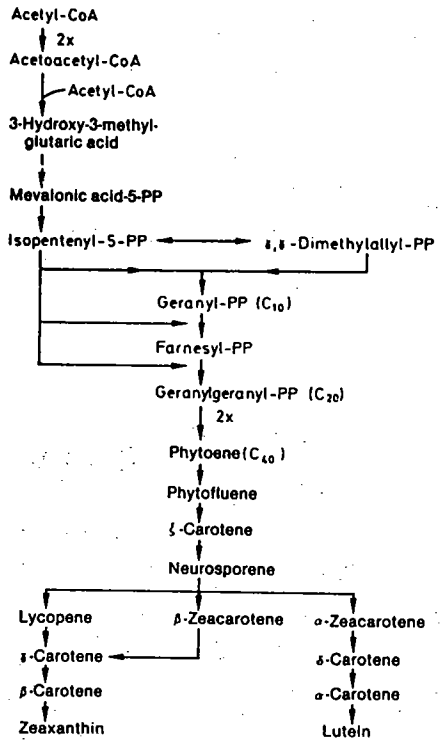


Figure 12.7 Carotenoid biosynthesis

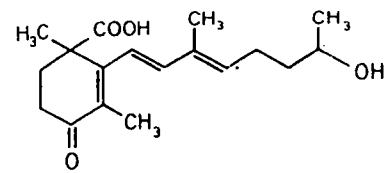


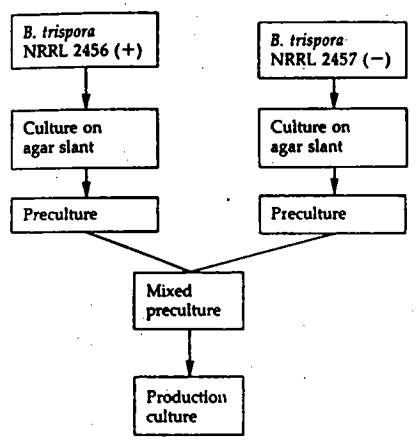
Figure 12.8 Structure of trisporic acid C

Structure

Carotenoids are highly unsaturated isoprene derivatives. Naturally occurring carotenoids are tetraterpenoids consisting of 8 isoprene residues. Over 400 naturally occurring carotenoid compounds are known today. Figure 12.6 shows the impact on process development. When cultures of both sexual forms, (+) and (−) strains, are mixed, a significant increase in carotene product-structures of some carotenoids produced by microbial fermentation. Only compounds with the β-ionone structure (the ring structure found at each end of the β-carotene molecule) are effective as provitamin A. Thus, 2 molecules of vitamin A can be formed from β-carotene; only one molecule of vitamin A can be formed from α- and γ-carotene since each of these molecules has one β-ionone ring.

Biosynthesis

Biosynthesis of β-carotene has been studied mainly in plants and fungi and takes place as shown in Figure 12.7. β-Carotene and related carotenoids are produced primarily by fungi and algae, xanthophylls by bacteria and algae.



Inoculum storage: spores in sterile soil

Incubation: 168 h, 27°C

2 l Erlenmeyer flask with 400 ml Medium A. Incubation: 48 h, 26°C, shaker.

170 l Fermenter with 120 l Medium A. Inoculum: 400 ml of each preculture. Incubation: 40 h, 26°C; stirring 170 rpm; aeration 1.1 vvm

800 l Fermenter with 320 l Medium B. Sterilization 55 min, 122°C. Inoculum: 32 l of mixed preculture. Incubation: 185 h, 26°C; stirring 210 rpm; aeration 1.3 vvm

Medium A (g/l): Corn steep liquor 70; Corn starch 50; KH_2PO_4 0.5; $MnSO_4 \cdot H_2O$ 0.1; Thiamin HCl 0.01; Tap water

Medium B (g/l): Distiller's soluble 70; Corn starch 60; Soy bean meal 30; Cottonseed oil 30; Antioxidant 0.35; $MnSO_4 \cdot H_2O$ 0.2; Thiamin HCl 0.5; Isoniazid 0.6; Kerosene 20 ml; Tap water; pH 6.3. Isoniazid and kerosene are sterilized separately. After 48 h 1 g/l β-ionone and 5 ml/l kerosene are added; glucose feeding (total addition 42 g/l) until the end of the fermentation.

Figure 12.9 Production process for β-carotene using *Blakeslea trispora* (From Ninet and Renaut, 1979)

Production processes for β-carotene

The highest yields have been obtained with the *Blakeslea trispora* process. The observation that carotene production occurs during the process of zygospore formation in this organism has had an impact on process development. When cultures of both sexual forms, (+) and (−) strains, are mixed, a significant increase in carotene production in the (−) strain is achieved. Production is induced by trisporic acids (Figure 12.8), a mixture of closely related substances. Trisporic acids are not precursors of β-carotene but rather they act as (+)-gamones (sexual hormones). They are derived biosynthetically from β-carotene, as shown by labeling experiments. The production of β-carotene (Figure 12.9) is carried out in a submerged fermentation using a mixed culture of (+) and (−) strains. The proportions of the two strains need not be equal and since it is the (−) strain which produces the β-carotene, this strain can be present in great excess. Another activator of β-carotene synthesis is isoniazid, particularly in combination with β-ionone. Alone, β-ionone is toxic to the production organism, but in the presence of plant oils it promotes carotene production. The β-ionones themselves are not incorporated into carotene but affect the synthesis of various of the enzymes involved in the biosynthetic pathway. They can be replaced by a number of other substances, such as terpenes or cyclohexane and cyclohexanone and their trimethyl derivatives. The addition of purified kerosene to the medium doubles the yield by increasing the solubility of the hydrophobic substrate.

Because of the low stability of β-carotene within the cells, the addition of an antioxidant is necessary for the fermentation process.

The carotenoid-rich mycelium can be used directly as a feed additive. To obtain pure β-carotene, the mycelium is removed, dehydrated (for example, with methanol), extracted with methylene chloride (75–92% yield), and the crude product is further purified.

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13

Antibiotics

13.1 INTRODUCTION

Antibiotics are products of secondary metabolism which inhibit growth processes of other organisms even when used at low concentrations. Growth inhibition of one microorganism by another in mixed culture has been known for a long time. The most famous example is the growth inhibition which was observed by Alexander Fleming in 1929, when staphlococcal growth on a petri plate was inhibited by a contaminating *Penicillium notatum* culture. The *Penicillium* contaminant produced the antibiotic penicillin. During World War II, the demand for chemotherapeutic agents to treat wound infections led to the development of a production process for penicillin and the beginning of the era of antibiotic research. This continues to be the most important area of industrial microbiology today. Intensive screening programs in all industrial countries continue to increase the number of described antibiotics: 513 antibiotics were known in 1961, 4076 in 1972, 7650 in 1985, and currently around 8000. In addition, around 3000 antibiotically active substances have been detected in lichens, algae, higher animals, and plants. Each year about 300 new antibiotically active materials are detected, of which 30–35% are secondary components from fermentations with known antibiotics.

Of the large number of known antibiotics of microbial origin, only 123 are currently produced by fermentation. In addition, more than 50 antibiotics are produced as semisynthetic compounds, and three antibiotics, chloramphenicol, phosphonomycin, and pyrrolnitrin, are produced completely synthetically.

The significance of antibiotic production for the producing strain is unclear. Antibiotic production could be of ecological significance for the life of the organism in nature, but solid research to support this hypothesis is very limited. As secondary metabolites, antibiotics could serve regulatory roles during differentiation, perhaps acting as temporary inhibitory agents. To date, most new antibiotics have been detected strictly by

empirical screening methods, with little attention to their possible roles for the producing strains.

The microbial groups producing antibiotics

Antibiotics are produced by bacteria, actinomycetes and fungi; their distribution within the taxonomic groups is shown in Table 13.1.

In the **fungi**, only the antibiotics produced by the Aspergillaceae and Moniliales are of practical importance. The compounds isolated from basidiomycetes have not had any practical use. Only 10 of the known fungal antibiotics are produced commercially and only the penicillins, cephalosporin C, griseofulvin, and fusidic acid are clinically important. In the **bacteria**, there are many taxonomic groups which produce antibiotics. The greatest variety in structure and number of antibiotics is found in the **actinomycetes**, especially in the genus *Streptomyces*. Another important group of substances are the peptide antibiotics, produced by bacteria of the genus *Bacillus*. A number of new compounds have also recently been isolated in other taxonomic groups (see Chapter 2).

The significance of antibiotic production for producer strains is still unclear. The status of the controversy can be found in the references of Zähner and Barabas cited in the bibliography.

Classification of antibiotics

Antibiotics can be classified according to their antimicrobial spectrum, mechanism of action, producer strain, manner of biosynthesis, or chemical structure. There is some overlap in each classification. Table 13.2 shows a simplified classification according to chemical structure. In this chapter, primarily the economically important compounds are covered.

Table 13.1 Numbers of antibiotics produced by major groups of microorganisms

Taxonomic group	Number of antibiotics
Bacteria, other than actinomycetes	950
Actinomycetes	4600
Fungi	1600

Berdy (1985)

Table 13.2 Classification of antibiotics according to their chemical structure. An example of each is given in parentheses.

1. Carbohydrate-containing antibiotics	
Pure sugars	(Nojirimycin)
Aminoglycosides	(Streptomycin)
Orthosomycins	(Evernimomicin)
N-Glycosides	(Streptothricin)
C-Glycosides	(Vancomycin)
Glycolipids	(Moenomycin)
2. Macrocyclic lactones	
Macrolide antibiotics	(Erythromycin)
Polyene antibiotics	(Candicidin)
Ansamycins	(Rifamycin)
Macrotetrolides	(Tetranactin)
3. Quinones and related antibiotics	
Tetracyclines	(Tetracycline)
Anthracyclines	(Adriamycin)
Naphthoquinones	(Actinorhodin)
Benzoquinones	(Mitomycin)
4. Amino acid and peptide antibiotics	
Amino acid derivatives	(Cycloserine)
β-Lactam antibiotics	(Penicillin)
Peptide antibiotics	(Bacitracin)
Chromopeptides	(Actinomycins)
Depsipeptides	(Valinomycin)
Chelate-forming peptides	(Bleomycins)
5. Heterocyclic antibiotics containing nitrogen	
Nucleoside antibiotics	(Polyoxins)
6. Heterocyclic antibiotics containing oxygen	
Polyether antibiotics	(Monensin)
7. Alicyclic derivatives	
Cycloalkane derivatives	(Cycloheximide)
Steroid antibiotics	(Fusidic acid)
8. Aromatic antibiotics	
Benzene derivatives	(Chloramphenicol)
Condensed aromatic antibiotics	(Griseofulvin)
Aromatic ether	(Novobiocin)
9. Aliphatic antibiotics	
Compounds containing phosphorous	(Fosfomycins)

(Berdy, 1985)

Applications of antibiotics

Chemotherapeutic antibiotics can be either broad-spectrum antibiotics, active against many organisms, or narrow-spectrum antibiotics, active against only a restricted range of organisms. Most antibiotics are manufactured as antimicrobial agents for chemotherapy, but some have other applications, as outlined below.

Antitumor antibiotics Such antibiotics are clinically used as cytostatic agents (Table 13.3). Although generally toxic, with careful control of the dose certain of these antibiotics are effective in the treatment of certain kinds of tumors.

Antibiotics for plant pathology Antibiotics may be more useful than synthetic chemicals in the control of plant diseases for the following reasons: they may be applied selectively in low concentrations, they are only slightly toxic to warm-blooded animals and beneficial insects, and they are normally easily broken down by soil microorganisms. Initially, medically useful antibiotics were also used on plants (especially streptomycin to combat plant disease caused by *Pseudomonas* sp. and *Xanthomonas oryzae*). Now antibiotics have been developed which are used exclusively for plant application (Table 13.4).

Table 13.3 The most important antitumor antibiotics

Antibiotic	Category	Organism producing
Aclacinomycin	Anthracycline	*S. galilaeus*
Actinomycin C_1, C_3, D	Chromopeptide	*S. antibioticus*
Adriamycin	Anthracycline	*S. peucetius*
Daunomycin	Anthracycline	*S. peucetius*
Chromomycin A_3	C-Glycoside (oligosaccharide with aromatic chromophore)	*S. griseus*
Mithramycin	C-glycoside (oligosaccharide with aromatic chromophore)	*S. plicatus, S. argillaceus, S. atroolivaceus*
Mitomycin C	Benzoquinone	*S. caespitosus*
Bleomycin A_2, B_2	Glycopeptide	*S. verticillus*
Neocarzinostatin	Peptide	*S. carzinostaticus*

Antibiotics as food preservatives Government regulations in each country directly control the use of antibiotics as food preservatives. The following compounds are available: pimaricin, a fungicide applied to food surfaces; tylosin (effective against *Bacillus* spores) and nisin (effective against clostridia), both used in the canning industry; chlortetracycline, used to maintain freshness in fish, meat and poultry by incorporation into ice (5 ppm) or by addition to an immersion bath (10 ppm). Of these, pimaricin and nisin are currently used commercially.

Antibiotics used as animal growth promoters and in veterinary medicine Animal feed is more efficiently processed in the animal's digestive system if an antibiotic additive is used in subtherapeutic concentrations (1–10 mg/kg feed). Weight gain may also be accelerated. The cause of this growth promotion may be traced to changes in the microflora of the gastrointestinal tract. This is further confirmed by the fact that the growth of animals raised in germ-free conditions is not stimulated by antibiotics. Originally, large quantities of therapeutically useful antibiotics (such as penicillins, tetracyclines, erythromycins, bacitracin, or streptomycin) were added to feed. Because such extensive use of common antibiotics encouraged the risk of rapid development of antibiotic resistance, government regulations have been established to eliminate the parallel use of antibiotics in human medicine and in animal feed. Some new-generation antibiotics which are used solely for nutritional purposes are listed in Table 13.5.

In veterinary medicine, the use of antibiotics is similarly regulated. Antibiotics which are used solely for veterinary work include hygromycin B, thiostrepton, tylosin, and the coccidiostatic agents monensin, lasalocide, and salinomycin.

Antibiotics as tools in biochemistry and molecular biology The use of antibiotics as selective inhibitors has made a vital contribution to the understanding of certain cell functions, such as

Table 13.4 Antibiotics of use in plant pathology

Antibiotic, chemical type in ( )	Organism producing	Uses
Blasticidin S (Nucleoside)	*S. griseochromogenes*	Rice fungicide against *Piricularia oryzae* (rice burn); relatively toxic
Mildiomycin (Nucleoside)	*Streptoverticillium rimofaciens*	Fungicide for mildew
Polyoxin (Nucleoside)	*S. cacaoi* var. *asoensis*	Multi-purpose fungicide
Prumycin (Nucleoside)	*S. kagawaensis*	Fungicide against *Botrytis* and *Sclerotinia* species
Cycloheximide (Amino acid)	*S. griseus*	Leaf fungicide, highly toxic to warm-blooded animals; aids harvesting
Kasugamycin (Aminoglycoside)	*S. kasugaensis*	Rice fungicide against *Piricularia oryzae*
Validamycin (Aminoglycoside)	*S. hygroscopicus* var. *limoneus*	Fungicide against *Rhizoctonia solani* (leaf dropping and stalk diseases); vegetable crops
Tetranactin (Macrotetrolide)	*S. flaveolus*	Insecticide (mites)

Table 13.5 Antibiotics used in animal feed

Antibiotics	Classification	Organism producing
Enduracidin	Peptide antibiotic	*S. fungicidicus*
Mikamycin	Peptide antibiotic	*S. mitakaensis*
Siomycin	Peptide antibiotic	*S. sioyaensis*
Thiopeptin	Peptide antibiotic	*S. tateyamensis*
Thiostrepton	Peptide antibiotic	*S. azureus*
Virginiamycin	Peptide antibiotic	*S. virginiae*
Macarbomycin	Phospho-glycolipid	*S. phaeochromogenes*
Moenomycin	Phospho-glycolipid	*S. bambergiensis*
Quebemycin	Phospho-glycolipid	*S. viridans*
Tylosin	Macrolide	*S. fradiae*
Mocimycin	Heterocyclic compound	*S. ramocissimus*

DNA replication, transcription, translation, and cell wall synthesis. The uses of antibiotics in these areas is discussed in textbooks of biochemistry and microbiology. In addition, some antibiotics (for example, gentamicin) have been added to animal cell cultures to control contamination.

Economic significance of antibiotics

World-wide antibiotic production is over 100,000 tons per year and estimated gross sales for 1980 were $4.2 billion. The annual gross sales in the United States alone is $1 billion, with cephalosporin in the leading position, followed by ampicillin and the tetracyclines. Feed-additive antibiotics are believed to have a world market of $100 million annually.

Objectives of antibiotic research

Before 1960, about 5% of the newly isolated antibiotics were therapeutically useful. In the following years new antibiotics were discovered at an approximately constant rate, but the percentage of the new antibiotics which actually came on the market decreased from 2.6% in 1961–1965 to 1% in 1966–1971. This is primarily because of severe cost increases in development and clinical testing, so that manufacturers produce only those compounds which clearly show promising therapeutic progress. On the average, it takes around 8–10 years to develop a new antibiotic, at an average cost of $10,000,000 to $20,000,000.

Considering the very large number of known compounds, it may seem questionable whether the search for new antibiotics should continue. The reasons for continued research are:

- In many cases the properties of natural antibiotics are not optimal for therapeutic application. The following improvements are needed: greater activity with unchanged or diminished toxicity, decreased side effects, broader antimicrobial range, greater selectivity against certain pathogens, improved pharmacological properties.
- Suitable antibiotics are not available in many fields of human medicine or in nonmedical areas, as shown in Table 13.6. Numerous tests have been made of new and semisynthetic substances, but no significant breakthroughs have been made.
- Since the beginning of chemotherapy, the number of resistant strains has increased. Multiple- and cross-resistance can occur, i.e., if resistance develops to one antibiotic it may simultaneously develop to others having the same mode of operation or uptake mechanism. Careless use of antibiotics has been responsible for much increase in resistance, but even with careful use in chemotherapy, resistance still develops, albeit at a slower rate. Currently, the only alternative for overcoming the resistance problem is the discovery of new antibiotics.

Improved antibiotics can be obtained by modifying known compounds using either

chemical or genetic means (mutasynthesis, pr toplast fusion, recombinant DNA technology see Chapter 3). However, antibiotics with e tirely new basic structures can be expected on from screening, especially by the use of new te procedures and by research on new groups microorganisms (see Chapter 2).

13.2 β-LACTAM ANTIBIOTICS

As shown in Figure 13.1, the β-lactam antibioti can be divided into five distinct classes. The pe icillins and the cephalosporins belong to the mo effective of all therapeutic agents for the contr of infectious diseases. In addition to the deve opment of numerous semisynthetic β-lactan based on the known β-lactam rings, antibioti with completely new β-lactam ring systems ha been isolated in the past few years using ne specific and sensitive screening methods.

Penicillins

Penicillin was described by Fleming in 1929. research group at Oxford under Florey and Cha isolated it from surface cultures of *Penicillium n tatum* in 1940 and the first clinical application penicillin was in 1941. Penicillins are produc by many fungi, particularly *Penicillium* and *A pergillus* species. Natural penicillins are effecti against numerous gram-positive bacteria. Th are labile in acid and may be inactivated by spl

Table 13.6 Possible applications for antibiotics

Application	Many products	Some products	Products needed
Medicine	Gram-positive bacteria	Gram-negative bacteria, including multiple resistance Dermatophytes	Systemic mycoses Viruses Protozoa Parasites Tumors
Nonmedical areas	-	Plant pathology (phytopathogenic fungi) Animal nutrition	Plant pathology Phytopathogenic bacteri and viruses Insects and mites Nematodes Food preservatives

(Zähner, 1978)

Basic structure	Antibiotic	Most important producing strains
Penam	Penicillins	*Penicillium chrysogenum* *Aspergillus nidulans* *Cephalosporium acremonium* *Streptomyces clavuligerus*
Ceph-3-em	Cephalosporins 7-Methoxycephalosporins	*Cephalosporium acremonium* *Nocardia lactamdurans* *Streptomyces clavuligerus*
Clavam	Clavulanic acid	*S. clavuligerus*
Carbapenem	Thienamycins Olivanic acids Epitheinamycins	*S. cattleya* *S. olivaceus* *S. flavogriseus*
Monolactam	Nocardins	*Nocardia uniformis* subsp. *tsuyamenensis*
	Monobactams	*Gluconobacter* sp. *Chromobacter violaceum* *Agrobacterium radiobacter* *Pseudmonas acidophila* *Pseudomonas mesoacidophila* *Flexibacter* sp. *Acetobacter* sp.

Figure 13.1 The basic structures of the naturally occurring β-lactam antibiotics

ting the β-lactam ring with penicillin-β-lactamases (see Figure 15.11). Because of its low toxicity, large doses of penicillin can be used; only a small percentage of patients develops allergies (0.5–2%).

β-Lactam antibiotics are specific inhibitors of bacterial cell wall (peptidoglycan) synthesis. They combine specifically with the so-called **penicillin-binding protein** (PBP) of the bacterial cell and, by inhibiting the enzyme activity of this protein, bring about cell death. In *E. coli* it has been shown that PBP1a and 1b are transpeptidases involved in the cross-linking of the peptidoglycan, PBP3 plays a role in septum formation, and PBP4 and 5 are carboxypeptidases. A further point of attack of the β-lactam antibiotics seems to involve phospholipid synthesis.

Chemical structure The basic structure of the penicillins is 6-aminopenicillanic acid (6-APA), which consists of a thiazolidine ring with a condensed β-lactam ring. The 6-APA carries a variable acyl moiety in position 6, as illustrated in Figure 13.2. If the penicillin fermentation is carried out without addition of side-chain precursors, the **natural penicillins** are produced. From this mixture, only benzylpenicillin is therapeutically useful; the other compounds must be re-

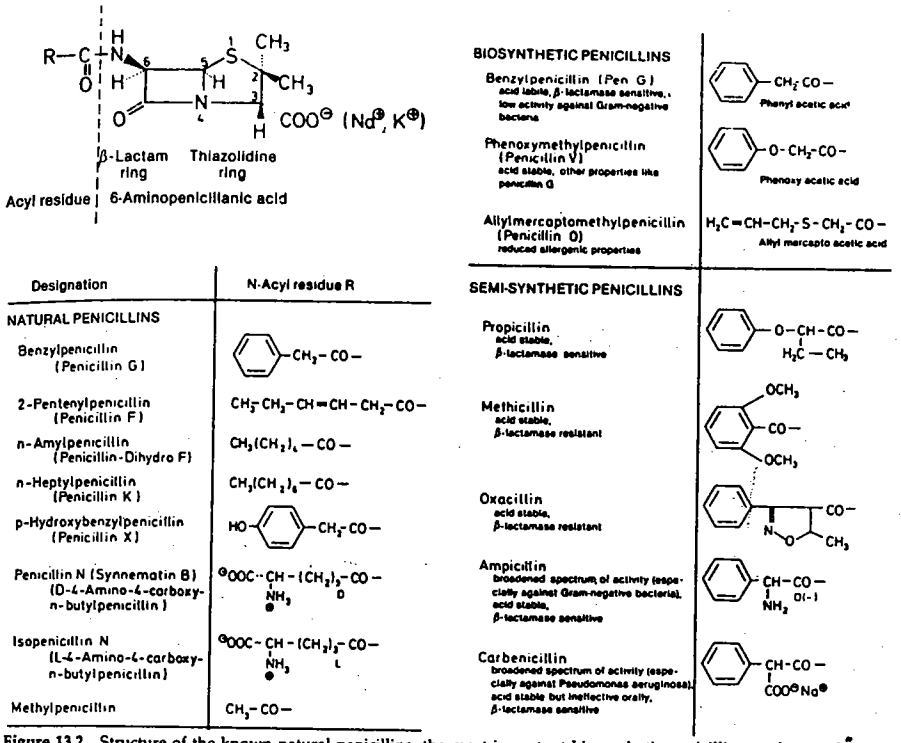


Figure 13.2 Structure of the known natural penicillins, the most important biosynthetic penicillins, and several semisynthetic penicillins

moved at the product recovery stage. The fermentation can be better controlled by adding a side-chain precursor, so that only one desired penicillin is produced. Over 100 **biosynthetic penicillins** have been produced in this way. In commercial processes, however, only penicillin G, penicillin V, and very limited amounts of penicillin O have been produced. In order to produce **semisynthetic penicillins**, penicillin G (sometimes penicillin V is used instead) is chemically or enzymatically split to form 6-APA, which is chemically recycled to make yet another penicillin derivative. (See Section 11.9 for a discussion of the enzyme penicillin acylase). Due to their improved characteristics (acid stability, resistance to plasmid or chromosomally coded β-lactamases, expanded antimicrobial effectiveness), semisynthetic penicillins have come to be extensively used in therapy. Because of their broadened action spectrum, these semisynthetic penicillins are sometimes compared with the so-called third generation cephalosporins.

About 38% of the penicillins produced commercially are used in human medicine (see below), 12% in veterinary medicine, and 43% are used as starting materials for the production of semisynthetic penicillins. In the early 1980's, world-wide production of penicillins amounted

to 12,000 tons, an exceedingly large amount considering how highly active these agents are.

Biosynthesis and regulation The β-lactam-thiazolidine ring of penicillin is constructed from L-cysteine and L-valine. Biosynthesis occurs in a nonribosomal process by means of a dipeptide composed of L-α-aminoadipic acid (L-α-AAA) and L-cysteine or a breakdown product of cystathionine. Subsequently, L-valine is connected via an epimerization reaction, resulting in the formation of the tripeptide δ-(L-α-aminoadipyl)-cysteinyl-D-valine. The first product of the cyclization of the tripeptide which can be isolated is isopenicillin N, but the biochemical reactions leading to this intermediate are not understood. Benzylpenicillin is produced in the exchange of L-α-AAA with activated phenylacetic acid (Figure 13.3). 6-APA, which is not an intermediary product of biosynthesis, is excreted in the absence of a side chain precursor.

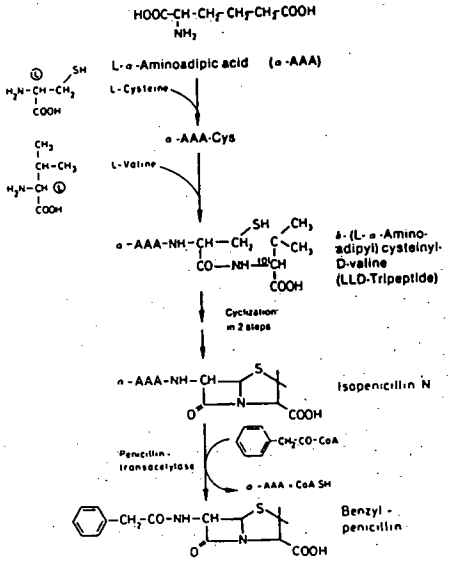


Figure 13.3 Biosynthesis of penicillin in *Penicillium chrysogenum*

Several regulatory mechanisms are known in penicillin biosynthesis. The amino acid lysine is synthesized from a pathway that involves L-α-aminoadipic acid, so that penicillin and lysine share a common branched biosynthetic pathway. Lysine inhibits penicillin synthesis because it is a feedback inhibitor of homocitrate synthase, an enzyme involved in L-α-AAA synthesis. If L-α-AAA is deficient, penicillin cannot be synthesized. However, feedback regulation by lysine does not seem to be a rate-limiting step in penicillin biosynthesis.

Penicillin biosynthesis is affected by phosphate concentration and also shows a distinct catabolite repression by glucose, in addition to a regulation by concentration of ammonium ion, the latter by an unexplained mechanism. Because of the glucose repression, penicillin fermentations were originally done only with the slowly metabolizable sugar lactose.

Strain development The penicillin production of Fleming's isolate was about 2 International units/ml; today's processes yield a penicillin titer of about 85,000 units/ml. This is an increase from 0.0012 g/l to about 50 g/l and well illustrates the value and power of a strain-selection program.

The initial culture improvement came in 1943 with the isolation of *Penicillium chrysogenum* strain NRRL 1951. This organism was better suited for submerged production than the original *P. notatum* strain. Through further mutagenesis, strain Wis Q 176 was isolated, the original strain in the famous Wisconsin culture line. Figure 13.4 shows the genealogy of these strains over a time period of about 10 years. Wis Q 176 was adopted by most penicillin manufacturers and was used as the original strain for the various commercial strain improvement programs, about which little has been published. Newer data have been released only by Panlabs Inc., a corporation which since 1973 has specialized in commercial strain development (Table 13.7).

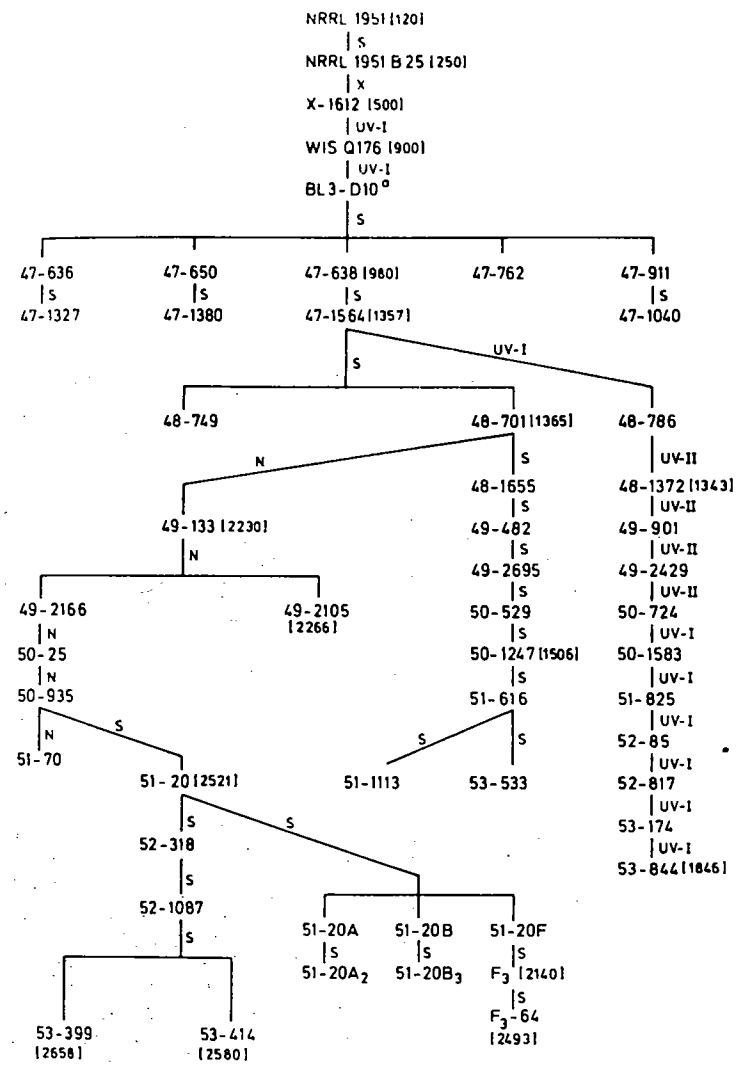


Figure 13.4 Genealogy of the Wisconsin strain of *Penicillium chrysogenum*. S, stages of selection; X, X-ray treatment; UV I, ultraviolet radiation at 275 nm; UV II, ultraviolet radiation at 253 nm; N, treatment with nitrogen mustard. Square brackets show yields in International units/ml. a = pigment-free mutant (From Backus and Stauffer, 1955)

Table 13.7 Increase of penicillin formation in *Penicillium chrysogenum*.

Strain	Penicillin G potassium (mg/ml)	Penicillin yield in glucose equivalents (g Pen.G-K/ g gluc. equiv.) (a)	Productivity P (b)
P- 2	9.0	0.05	0.72
P- 7	16.1	0.09	1.8
P-11	21.6	0.12	2.3
P-13	27.0	0.09	2.6
P-15	29.4	0.12	3.2

(a): (kg fat/oils × 2.5) = kg glucose equivalents. Carbon sources in medium: sugar and plant oils

(b): kg product/1000 l total fermenter capacity × day

Results of strain development and culture medium optimization at Panlabs Inc. (Swartz, 1979)

Yield increases have been the main objective of strain development, but other factors which have an effect on fermentation and efficiency of product recovery have also been optimized. Major advances in processing have resulted through empirical screening of mutants. Until the mid-1960's, the most frequently used mutagens were X-rays, methylbis-(β-chloroethyl)amine (nitrogen mustard), and short-wave ultraviolet radiation. More recently, nitrosoguanidine, alkylating agents, and nitrite have been used as mutagens.

In the early 1970's, strain improvement by using mere mutation had reached its limit. The discovery of a parasexual cycle in *P. chrysogenum* provided a means of utilizing genetic recombination for strain development. Heterozygous diploids were described which had penicillin titers above those of the haploid parent strains. Several such diploids have found use as production strains. However, the highest percentage of strains with increased penicillin production arose from haploid segregants of crosses between mutants of different strain lines.

The protoplast fusion technique has opened up a new approach to the development of high-yielding strains. Yield increases of 8% have been obtained over those obtained by mutation and selection. In addition, some of the resulting strains have had better growth characteristics, such as more stable sporulation and improved growth for inoculum production.

Some of the genes involved in penicillin synthesis in *P. chrysogenum* have been identified by analysis of mutants blocked in penicillin synthesis (*npe*). Twelve *npe* mutants have been mapped into 5 loci (*npe V, W, X, Y,* and *Z*) by means of complementation experiments with heterozygous diploids. Cosynthesis studies showed a defect in the LLD-tripeptide synthesis for *npe X, Y,* and *Z*, a block between the tripeptide and isopenicillin N in *npe W*, and a block between isopenicillin N and penicillin G in *npe V*. The function of *npe Y* was elegantly demonstrated as follows: Protoplasts of the various mutants were fused with liposomes into which the tripeptide had been preloaded, and penicillin synthesis was obtained.

The use of recombinant DNA technology to increase the formation of rate-limiting enzymes via gene amplification or improved transcription has not yet been possible in *P. chrysogenum*, due to the absence of precise biosynthetic data and the absence of good host-vector systems. However, a gene bank for *P. chrysogenum* has been constructed, and a transformation system has been developed.

Production methods Penicillin G and V are produced using submerged processes in 40,000–200,000 liter fermenters. Due to difficulties with the O_2 supply, larger tanks cannot be employed. Penicillin fermentation is an aerobic process with a volumetric oxygen absorption rate of 0.4–0.8 mM/l·min. The required aeration rate is between 0.5–1.0 vvm depending on the strain, on the bioreactor, and on the impeller system; various turbine impellers are used for mixing (120–150 rpm). Some manufacturers use Waldhof fermenters or air-lift fermenters, but this is only possible in mutants which generate low viscosity. Depending on the production strain used, the optimal temperature range is between 25–27°C.

A typical **flow chart** for penicillin production is shown in Figure 13.5.

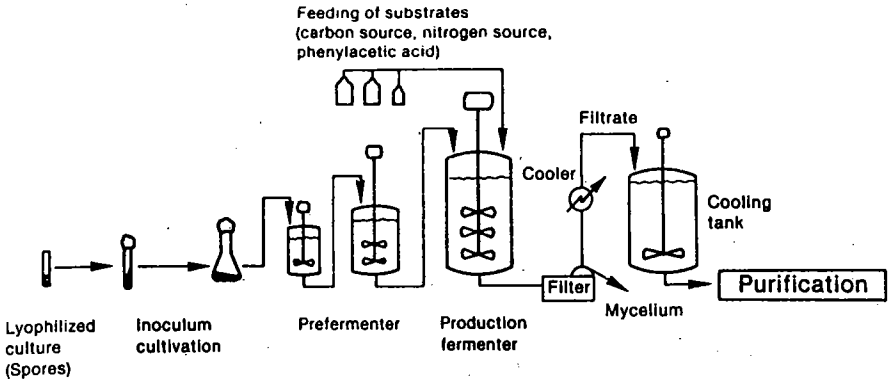


Figure 13.5 Flow chart of the penicillin fermentation (From Swartz, 1979)

The **inoculum** is started using lyophilized spores. Because of the great variability of high-yielding strains, careful strain maintenance is necessary. Spore concentration (optimal 5×10^3/ml) and pellet formation are crucial for the subsequent yield. If an optimal penicillin formation rate is to be achieved, pellets must grow not as compact balls, but in a loose form.

After several stages of growth the production culture is ready. In the typical penicillin fermentation (Figure 13.6), there is a **growth phase** of about 40 hours duration, with a doubling time of 6 hours, during which time the greatest part of the cell mass is formed. The **oxygen supply** in the growing culture is critical, since the increasing viscosity hinders oxygen transfer. This can be resolved by engineering changes in the fermenter or by the use of mutants which develop reduced viscosity. After the growth phase the culture proceeds to the actual **penicillin production phase**. In cases of high penicillin production, growth is sharply reduced (μ = 0.01 h^{-1}). By feeding with various culture medium components, the production phase can be extended to 120–180 hours.

The **medium** of a typical fed-batch culture may vary depending on the strain and usually

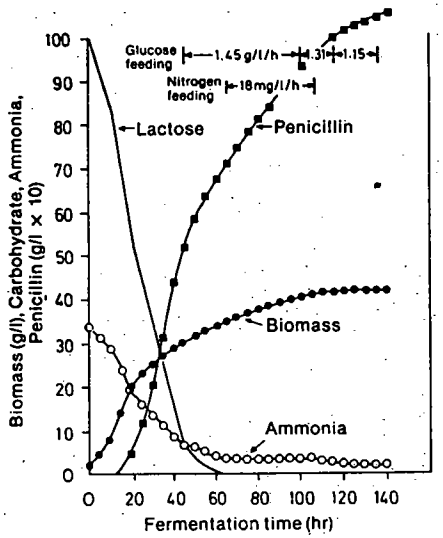


Figure 13.6 Penicillin fermentation with *Penicillium chrysogenum* (From Swartz, 1979)

consists of: corn steep liquor (4–5%, dry weight), which in present processes may be replaced by other nitrogen sources (e.g., a commercial nitrogen source called Pharmamedia); an additional nitrogen source, such as soy meal, yeast extract, or whey; a carbon source (such as lactose); and various buffers. The pH is kept constant at 6.5. Phenylacetic acid or phenoxyacetic acid is fed continuously as a precursor (0.5–0.8% of the total). Processes with glucose or molasses feeding are also successful: feeding rates of 1.0–2.5 $kg \cdot m^{-3} h^{-1}$ with a glucose concentration of 500 $kg \cdot m^{-3}$. About 65% of the metabolized carbon source is used for maintenance energy, 25% for growth, and only 10% for penicillin production. When it is considered that 50% of the production cost derives from the medium ingredients, it is obvious that control of carbon metabolism offers an important point of attack for further optimization of the production process. Yield increases of 25% have been reported by adding glucose and acetic acid. Critical parameters in these fed-batch systems are the rate of sugar utilization and the oxygen supply.

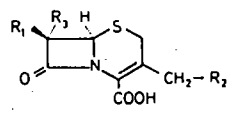
Production with continuous penicillin fermentation has been attempted, but has been difficult due to the instability of the production strains. A "batch fill and draw" system has been suggested as an alternative. In this procedure, 20–40% of the fermentation contents is drawn off and replaced with fresh nutrient solution; this process may be repeated up to 10 times without yield reductions.

Extensive research is being carried out to produce penicillin with immobilized cells. In one laboratory-scale study, the advantage of this approach over the use of a fed-batch system was demonstrated, but this technique has not as yet been introduced commercially.

Penicillin is excreted into the medium and less than 1% remains mycelium-bound. After separation of the mycelium, product recovery is accomplished by means of a two-stage continuous countercurrent extraction of the fermenter broth with amyl or butyl acetate at 0–3°C and pH 2.5–3.0. The yield is around 90%.

Cephalosporins

Cephalosporins are β-lactam antibiotics containing a dihydrothiazine ring with D-α-aminoadipic acid as acyl moiety (Figure 13.7). Cephalosporin C was discovered in culture filtrates of *Cephalosporium acremonium* in 1953. The strain isolated by Brotzu in 1945 was later classified as *Acremonium chrysogenum* and produces several antibiotics: cephalosporin C, penicillin N (a 6-APA derivative with D-α-AAA as side chain), and the steroid cephalosporins P_1–P_5. The cephalosporin antibiotics are also produced by other fungi, such as *Emericellopsis* and *Paecilomyces*. In 1971, in a screening program designed to discover β-lactam antibiotics, the first *cephamycins* (7-methoxycephalosporins) were found, which are produced



Designation	R_1	R_2	R_3
7-Aminocephalosporanic acid (7-ACA)	$-NH_3^+$	$-O-CO-CH_3$	-H
NATURAL CEPHALOSPORINS			
Cephalosporin C	$-CO-(CH_2)_3-(D)CH-NH_3^+$, COO^-	$-O-CO-CH_3$	-H
Deacetyl-3'-carbamoylcephalosporin C		$-O-CO-NH_2$	-H
7-Methoxycephalosporin C		$-O-CO-CH_3$	$-OCH_3$
Cephamycin A		$-OCO-C(CH_3)-CH-$ (phenyl, O, SO_3H)	$-OCH_3$
Cephamycin B		$-OCO-C(O-CH_3)-CH-$ (phenyl, OH)	$-OCH_3$
Cephamycin C		$-O-CO-NH_2$	$-OCH_3$
SEMI-SYNTHETIC CEPHALOSPORINS			
Cephalotin	(thiophene) $-O-CH_2-CO-HN-$	$-O-CO-CH_3$	-H
Cephalexin	(phenyl) $-CH(NH_2)-CO-NH-$	H	-H

Figure 13.7 Structure of cephalosporin C, cephamycins, and several semi-synthetic cephalosporins

from various *Streptomyces* species, such as *S. lipmanii*, *S. clavuligerus*, or *Nocardia lactamdurans*.

Cephalosporins are valued not only because of their low toxicity but also because they are broad-spectrum antibiotics, comparable in action to ampicillin. With about 29% of the antibiotic market, the cephalosporins are the single most important group of antibiotics. Therapeutically only semisynthetic derivatives of cephalosporin and cephamycin are used. For oral use, the first generation cephalosporins have been modified so that they have improved stability against β-lactamases. These second generation antibiotics were then further modified so that they were more active against gram-negative bacteria. Further modifications have led to the commercial development of third generation antibiotics with excellent β-lactamase stability and a broadened action spectrum.

Thirteen therapeutically important semisynthetic cephalosporins are commercially produced. These have been synthesized by chemical splitting to form 7-aminocephalosporanic acid (7-ACA) with subsequent chemical acylation, as well as by modifications on the C-3 site. Cephalosporins are as economically significant as penicillins.

Biosynthesis and regulation As in the case of benzylpenicillin, the first stages of cephalosporin biosynthesis (Figure 13.8) proceed from δ-(α-aminoadipyl)-L-cysteinyl-D-valine to isopenicillin N. In the next stage, penicillin N is produced by transformation of the L-α-AAA side chain into the D-form, via the action of a very labile racemase. After ring expansion to deacetoxycephalosporin C by the so-called "expandase" reaction, hydroxylation via a dioxygenase to deacetylcephalosporin C occurs. The acetylation of cephalosporin C by an acetyl-CoA dependent transferase is the end point of the biosynthetic pathway in fungi. In streptomycetes, further transformations occur: cephalosporin C or the carbamoyl derivative of deacetylcephalosporin C is converted in a two-step reaction with molecular oxygen and S-adenosylmethionine to 7-

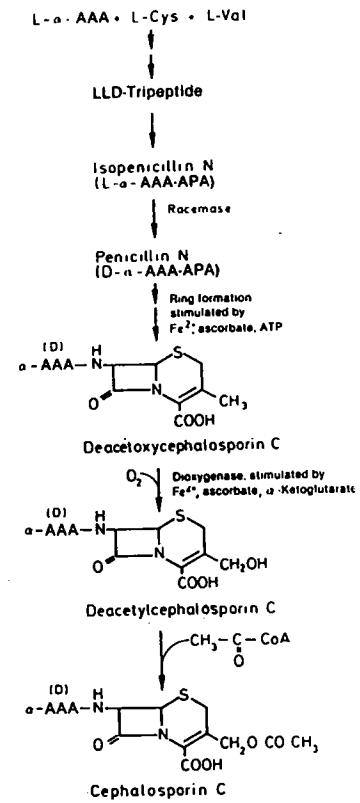


Figure 13.8 Biosynthesis of cephalosporin C by *Cephalosporium acremonium*

methoxycephalosporin or cephamycin C. In contrast to penicillin, the D-α-AAA moiety cannot be changed with precursor feeding.

Cephalosporin production is affected by phosphate regulation as well as by nitrogen and carbohydrate catabolite regulation. Rapidly metabolizable carbon sources, such as glucose, maltose, or glycerol, reduce the formation of cephalosporin. The repression of the "Expandase" seems to be the most significant effect. The addition of lysine in low concentrations promote

cephalosporin production (Figure 13.9), whereas the inhibition caused by higher amounts of lysine can be overcome by addition of DL-α-AAA. Methionine stimulates cephalosporin synthesis in *C. acremonium*, but not in the streptomycetes.

In the biosynthesis of cephamycins by streptomycetes, it should be noted that α-AAA is not a precursor of L-lysine, because streptomycetes synthesize lysine via the diaminopimelic acid pathway. L-lysine is converted by an L-lysine α-ketoglutarate-6-aminotransferase into α-AAA via L-1-piperidine-6-carboxylate as intermediate.

Strain development Strain development has been conducted with the original Brotzu strain, *C. acremonium* CMI 49 137, using mutation, selection, and parasexual techniques. Mutants have also been isolated with defective sulfur metabolism. Some of these regulatory mutants, which were isolated because they were resistant to sulfur analogs, exhibit an increased cephalosporin production or utilize inorganic sulfate as well as methionine. Parasexual recombination with mycelium of *C. acremonium* is difficult because the compartments of the hyphae are almost always mononuclear. By using protoplasts of different strains in fusion experiments, haploid recombinants have been isolated which showed a 40% increase in cephalosporin C production, as well as increased growth and improved sporulation. Protoplast fusion techniques have been combined with parasexual reproduction to develop an overall map of the biosynthetic genes for cephalosporin C. The gene for isopenicillin N synthetase from *C. acremonium* has been cloned in *E. coli* and an effective transformation system for *C. acremonium* has been developed, making it likely that the key steps in cephalosporin production will become subject to analysis and modification.

The cephalosporin C titer of contemporary high-yielding strains, in which the proportion of penicillin N is reduced, is about 20 g/l.

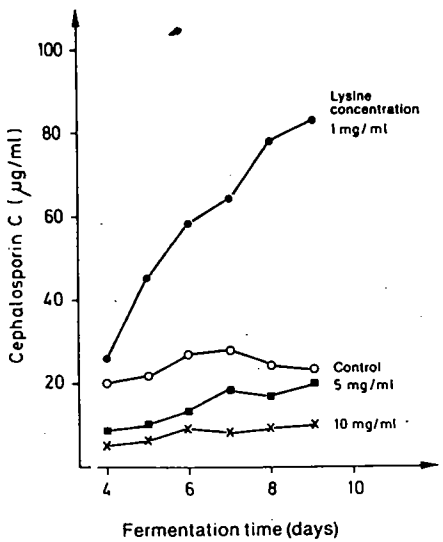


Figure 13.9 Effect of lysine concentration on cephalosporin C formation in *Cephalosporium acremonium* (From Mehta et al., 1979)

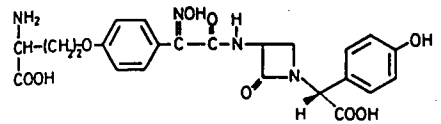
Production method The fermentation of cephalosporin is similar to that of penicillin. Complex media with corn steep liquor, meat meal, sucrose, glucose and ammonium acetate are used. Fermentations are carried out at as fed-batch processes with semicontinuous addition of nutrients at pH 6.0–7.0 and at temperatures between 24–28°C. In the main growth phase, a high aeration rate is necessary, but during the production phase (48–160 h), O_2 consumption decreases sharply.

The chemical synthesis of cephalosporin by ring-expansion of penicillin has been developed in recent years. This process has become especially attractive because of the low price of the penicillin starting material. An example of how this approach has been commercialized is the use of pencillin V to produce oraspor, an orally active cephalosporin.

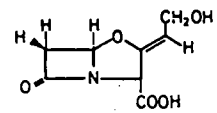
New β-lactam ring systems

New β-lactam ring systems have been discovered since the 1970's by the introduction of screening methods such as using as test organisms β-lactam hypersensitive strains or using enzymatic tests to detect β-lactam inhibitors. The basic structures of these new β-lactams are shown in Figure 13.1, and some specific examples are given in Figure 13.10. Because they may be active as either broad-spectrum antibiotics or β-lactamase inhibitors, these new compounds, especially their semisynthetic derivatives, can be expected to find considerable use in chemotherapy.

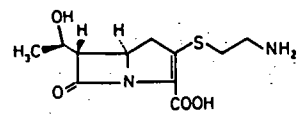
Clavulanic acid, a β-lactam-oxazolidine ring system, is produced along with cephalosporins by *Streptomyces clavuligerus*. Clavulanic acid is only slightly effective antibacterially but inhibits a wide range of β-lactamases in concentrations under 0.1 μg/ml. The inhibition is irreversible; in combination with β-lactamase-sensitive penicillins and cephalosporins, this compound causes a distinct increase in activity of these antibiotics, even against bacteria which are normally resistant to β-lactam antibiotics. Clavulanic acid has been marketed in combination with amoxycillin.



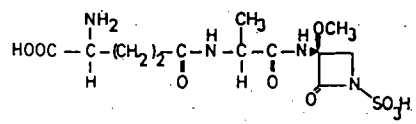
Nocardicin A



Clavulanic acid



Thienamycin



Sulfazecin

Figure 13.10 Newer β-lactam antibiotics

Thienamycins and olivanic acids have a β-lactam-pyrroline ring system in common. Thienamycin, the first in a series of carbapenems with very similar structures, was isolated in 1976 from *Streptomyces cattleya*. Thienamycin acts as a β-lactamase inhibitor and as a broad-spectrum antibiotic against Gram-positive and Gram-negative bacteria, including *Pseudomonas aeruginosa*, but it is extremely unstable.

The semisynthetic N-formimidoylthienamycin, resistant to dehydropeptidases, has good stability. It is the β-lactam with the broadest spectrum and is undergoing clinical trials.

The **olivanic acids** and the closely related epithienamycins show a lower activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Nocardicins are monocyclic β-lactams. From a mixture of seven components, nocardicin A has been isolated as the most active compound. It is weakly active against gram-negative bacteria, but research is being carried out to improve its activity by formation of chemical derivatives.

Monobactams, which are also monocylcic β-lactams, have been isolated from bacterial cultures. However, they are only weakly antibacterial. Compounds with a 3α-methoxy group are resistant to β-lactamases, whereas the rest of the monobactams show varying degrees of sensitivity to β-lactamases. Azthreonam, a semisynthetic monobactam made from threonine, has low toxicity and excellent activity against gram-negative pathogens; it is in clinical trials.

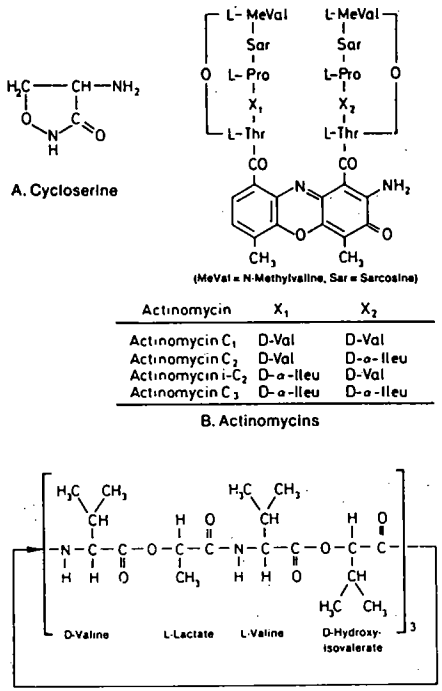
13.3 AMINO ACID AND PEPTIDE ANTIBIOTICS

This group of antibiotics includes amino acid derivatives such as cycloserine and azaserine, most

of the β-lactam antibiotics, which have already been covered in Section 13.2, chromopeptide antibiotics, depsipeptide antibiotics, and linear or cyclic peptide antibiotics. Over 400 peptide antibiotics are currently known.

D-cycloserine

Cycloserine, a D-4-amino-3-isoxazolidone (Figure 13.11 A), may be synthetically produced or may be isolated from cultures of *Streptomyces orchidaceus*, *S. lavendulae*, *S. garyphalus*, or *S. roseochromogenes*. D-Cycloserine, a D-alanine analog, inhibits cell wall synthesis by inhibiting alanine racemase, the enzyme responsible for production of the D-alanine moiety of the peptidoglycan. Cycloserine is very effective against mycobacteria, particularly *M. tuberculosis*, and is used in the treatment of tuberculosis in combination with isonicotinic acid hydrazide (INH) and rifampicin or streptomycin.



Actinomycin	X_1	X_2
Actinomycin C_1	D-Val	D-Val
Actinomycin C_2	D-Val	D-α-Ileu
Actinomycin i-C_2	D-α-Ileu	D-Val
Actinomycin C_3	D-α-Ileu	D-α-Ileu

B. Actinomycins

Figure 13.11 Structure of D-cycloserine (A), several actinomycins (B) and valinomycin (C)

Chromopeptide antibiotics

This group includes the actinomycins (Figure 13.11 B), which are produced as a mixture of very similar substances by different streptomycetes, especially *Streptomyces antibioticus* and *S. chrysomallus*. The following actinomycins have been described: A, B, C_1, C_2, i-C_2, C_3, D, F_1, Z_0–Z_5. All actinomycins which have thus far been isolated have the same phenoxazone-chromophore (actinocin), which is linked to two pentapeptide lactones. These two lactones have varied amino acid sequences, and by adding different amino acids to the fermentation broth, 30 distinct biosynthetic actinomycins have been produced.

The production of actinomycin from 2 molecules of 3-hydroxy-4-methylanthranilic acid pentapeptide lactone is catalyzed by phenoxazine synthase (Figure 13.12). This enzyme is produced only after the transition into the idiophase and shows pronounced catabolite regulation by glucose.

The phenoxazone rings of actinomycins intercalate at a GC pair in 5'-T-G-C-A-3' palindromes of DNA, thus blocking DNA-dependent RNA-polymerases. Actinomycins are very toxic, causing liver and kidney damage, but are beneficial in tumor treatment. The most important actinomycin used in cancer chemotherapy is dactinomycin (actinomycin C_1).

Depsipeptide antibiotics

In depsipeptides, the subunits (amino acids, hydroxy acids) are usually linked with alternating ester and acid amide bonds. **Valinomycin** (Figure 13.11 C), a cyclododecadepsipeptide produced by *Streptomyces fulvissimus*, has no eco-

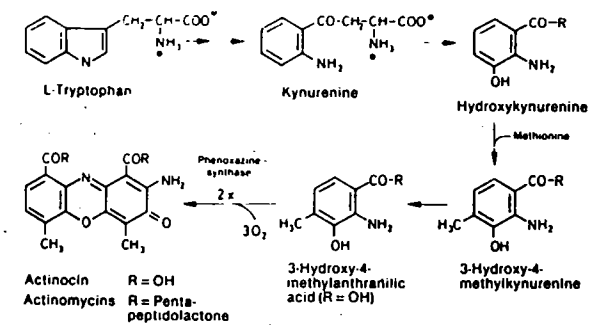


Figure 13.12 Actinomycin biosynthesis in *Streptomyces antibioticus*

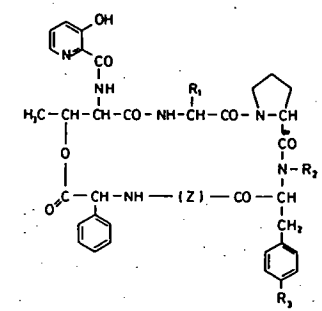
nomic significance but finds wide use in biochemical reasearch as an agent for uncoupling oxidative phosphorylation. It is also useful as a potassium carrier because of its selective binding of K^+ ions.

Antibiotics of the **virginiamycin** family also have a depsipeptide structure. They consist of a mixture of structurally different macrocyclic lactone rings (type A and B). Figure 13.13 shows the structure of virginiamycin S (type B).

Antibiotics of the virginiamycin group are chiefly effective against Gram-positive bacteria and are widely used as growth promoters for poultry, swine, and calves. Seventeen compounds are commercially produced; the most important are the mikamycins and virginiamycins, which are produced by *Streptomyces mitakaensis* or *S. virginiae*.

Linear and cyclic peptide antibiotics

The majority of the antibiotics formed by species of the genus *Bacillus* are peptides; other peptide antibiotics are produced by streptomycetes. The molecular weights of peptide antibiotics are between 270 (bacilysin) and 4500 (licheniformin). Some peptide antibiotics are linear (Figure 13.14), but most are cyclic compounds. They may consist of either amino acids alone (often with D-amino acids and rare amino acids) or they may contain other components in addition to the amino acids (Figure 13.15). The peptide antibiotics produced by bacilli are formed at the onset of sporulation and seem to play a regulatory role in the sporulation process. Table 13.8 lists the peptide antibiotics which are produced commercially. Bialophos, a tripeptide formed by *S. hygroscopicus* from two L-alanine and the L-glu-



		R_1	R_2	R_3	Z
Virginiamycin	S_1	C_2H_5	CH_3	H	4-Ketopipecolinic acid
Virginiamycin	S_4	CH_3	CH_3	H	4-Ketopipecolinic acid
Virginiamycin	S_2	C_2H_5	H	H	4-Hydroxy-pipecolin acid
Virginiamycin	S_3	C_2H_5	CH_3	H	3-Hydroxy-4-keto-pipecolinic acid

Figure 13.13 Structures of the virginiamycin antibiotics

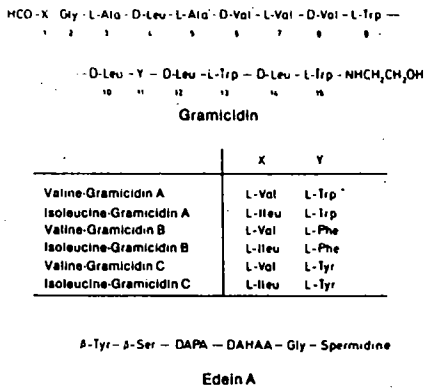


Figure 13.14 Linear peptide antibiotics. Above, gramicidins. Below, edein A (edein B: guanylspermidine). DAPS, Diaminopropionic acid; DAHAS, Diaminohydroxyazelain acid

tamic acid analog phosphinothricin, is being field-tested as an herbicide.

Therapeutic application of peptide antibiotics is limited, due to their toxicity. Gramicidin S, linear gramicidins, tyrocidin, and bacitracin are used topically (for wounds, burns, skin transplants, etc.). Polymyxins are used for infections caused by Gram-negative pathogens (including *Pseudomonas*), and viomycin and capreomycin are used in tuberculosis therapy.

Bacitracin

Bacitracin is the most economically important of the peptide antibiotics. In addition to its use as a topical antibiotic, it is also used as a growth promoter in animal feeds. As a feed supplement, it can be used as the zinc or manganese salt, as the lignin-bacitracin complex, or as bacitracin methylene disalicylate. Total production of bacitracin world-wide was estimated at 500 tons in 1976.

Bacitracin structure Various strains of *B. licheniformis* produce a mixture of bacitracin A, A', B, C, D, E, F, F_1, F_2, F_3 and G. Bacitracin A is the main component of the mixture (about 70%) and has the greatest biological activity. It is a dodecapeptide consisting of 8 L- and 4 D-amino acids and contains both a cyclic hexapeptide and a thiazoline ring structure (see Figure 13.15).

Mechanism of action Bacitracin inhibits the formation of the bacterial cell wall at the level of peptidoglycan biosynthesis. It prevents the dephosphorylation of C_{55}-isoprenylpyrophosphate to C_{55}-isoprenylphosphate.

Biosynthesis and Regulation Peptide formation can occur by either ribosomal or nonribosomal

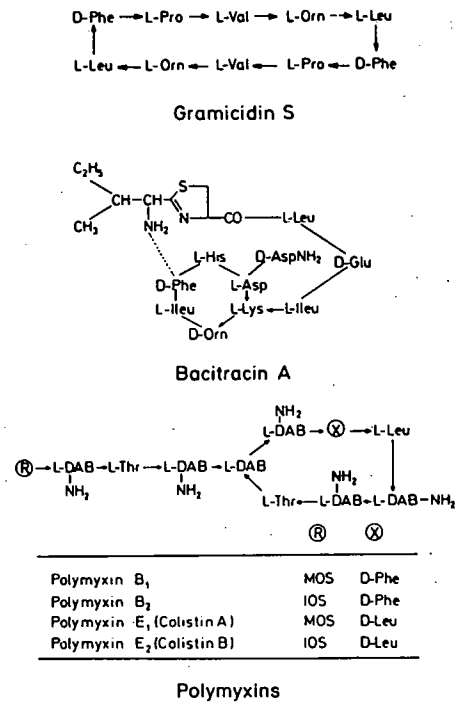


Figure 13.15 Cyclic peptide antibiotics: gramicidin S, bacitracin A, and the polymyxins.
DAB, Diaminobutyric acid; MOS, 6-Methyloctanoic acid; IOS, Isooctanoic acid

Table 13.8 Commercially produced peptide antibiotics

Antibiotic	Organism producing	Activity
Application: Human Therapy		
Amphomycin	*Streptomyces canus*	G⁺
Bacitracin	*Bacillus licheniformis*	G⁺(G⁻)
Capreomycin	*S. capreolus*	My
Gramicidin A	*B. brevis*	G⁺
Gramicidin S (J)	*B. brevis*	G⁺
Iturin A	*B. subtilis-ituriensiens*	G⁺G⁻AF
Polymyxin B	*B. polymyxa*	G⁻
Tyrothricin (bacitracin-tyrocidin complex)	*B. brevis*	G⁺
Tyrocidin	*B. brevis*	G⁺
Viomycin	*S. floridae*	My
Application: Feed additive		
Bacitracin	*B. licheniformis*	G⁺(G⁻)
Enduracidin A	*S. fungicidicus*	G⁺My
Parvulin	*S. parvulus*	G⁺
Siomycin	*S. sioyaensis*	G⁺
Thiopeptin	*S. tateyamensis*	G⁺
Thiostrepton	*S. azureus*	G⁺
Application: Food preservative		
Nisin	*Streptococcus cremoris*	G⁺

G⁺: Gram-positive; G⁻: Gram-negative bacteria; AF: antifungal; My: mycobacteria.
(Perlman, 1979; Kleinkauf and von Döhren, 1985)

mechanisms. The biosynthesis of high molecular-weight antibiotics such as nisin and neocarzinostatin involves ribosomes, mRNA, and tRNA. However, the biosynthesis of most peptide antibiotics does not involve this protein-synthesizing machinery, but takes place instead on a multi-enzyme complex. This nonribosomal peptide synthesis process, which has also been called the "thiotemplate" mechanism, has been described for gramicidin S, tyrocidin, and the linear gramicidins, as well as for bacitracin.

Bacitracin synthetase consists of subunits A, B and C, with molecular weights of 200,000, 210,000 and 380,000, respectively. A simplified version of the process of bacitracin synthesis is given in Figure 13.16. The first step, the activation of amino acids in the presence of ATP and Mg^{2+} to aminoacyl adenylates, takes place at a specific activation site for each amino acid on the enzyme complex. In a transfer reaction, the activated amino acids are then bound to specific thiol groups in thioester linkages. The amino acid sequence of the subsequent peptide is determined by the arrangement of the thiol groups on the enzyme. The racemization to the D-enantiomers probably takes place at this level. The transfer of the growing peptide chain (growth from the N-terminal end toward the C-terminal end) to the next amino acid and to the next enzyme sub-unit is catalyzed by 4'-phosphopantetheine, a cofactor of each sub-unit. The cyclization of the peptide chain takes place in the multi-enzyme complex, but the formation of the thiazoline ring is not yet fully understood.

Production methods Bacitracin was initially obtained from surface cultures; today its production occurs in aerobic submerged culture, like that of all other antibiotic processes. The production process for bacitracin is shown in Figure 13.17.

The yields of this process were increased from about 13 mg/l to about 9 g/l through strain development and medium optimization. A continuous process has also been developed for bacitracin production using immobilized cells.

Chelate-forming peptide antibiotics

Siderochromes and bleomycins are peptide antibiotics which form chelates with metals. **Siderochromes** are iron-containing or iron-binding natural substances containing hydroxamic acid groups, and bring about the formation of ferrihydroxamate complexes (Figure 13.18). Albomycins and ferrimycins have been isolated as antibiotically active siderochromes (sideromycins), but there is no known industrial application for these substances. Sideramines are siderochromes which act as growth stimulants, and ferrioxamine B, a sideramine from *Streptomyces pilosus* ETH 21748, is commercially produced. It is used in human therapy as the desferri compound for the treatment of diseases involving iron storage and also in agriculture in such applications as the elimination of soil iron deficiencies in peach orchards.

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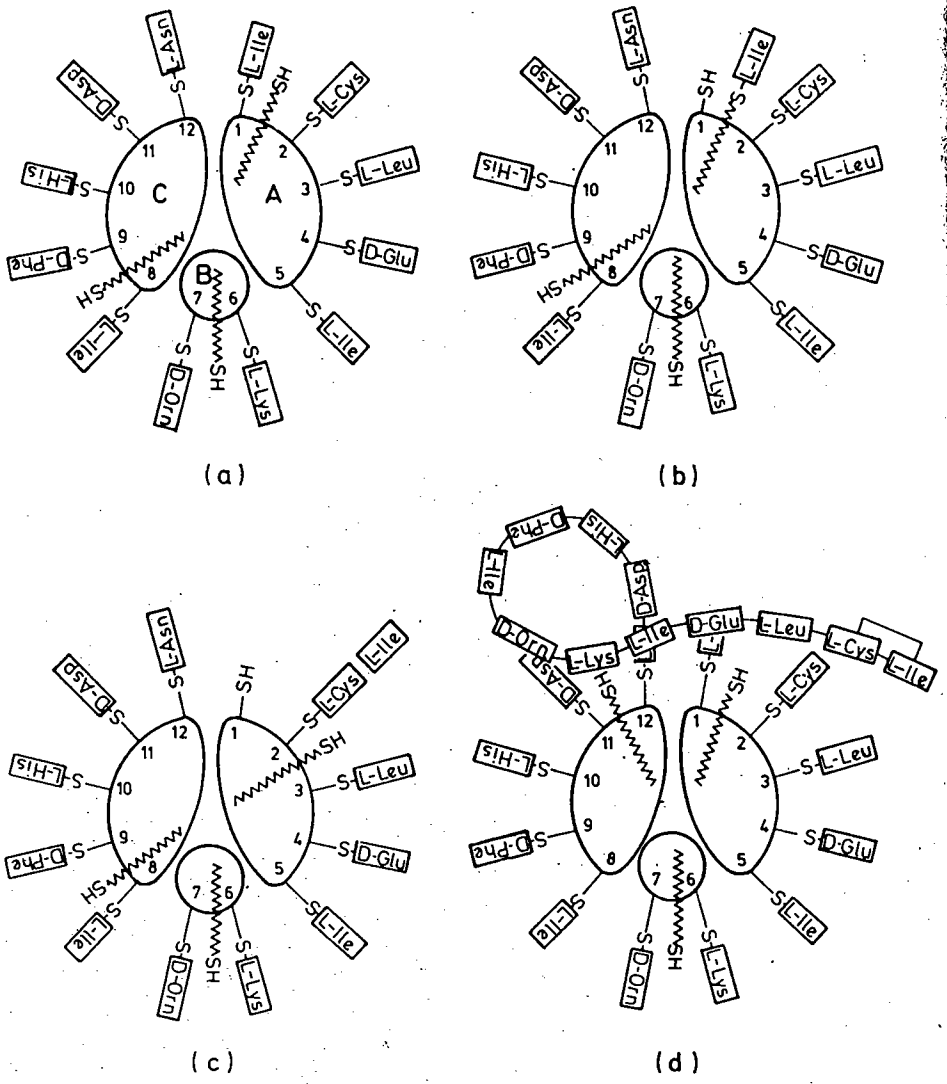


Figure 13.16 Model of nonribosomal peptide synthesis according to the "thiotemplate" model for the formation of bacitracin through the action of bacitracin synthetase in *B. licheniformis* (From Zimmer et al., 1979)

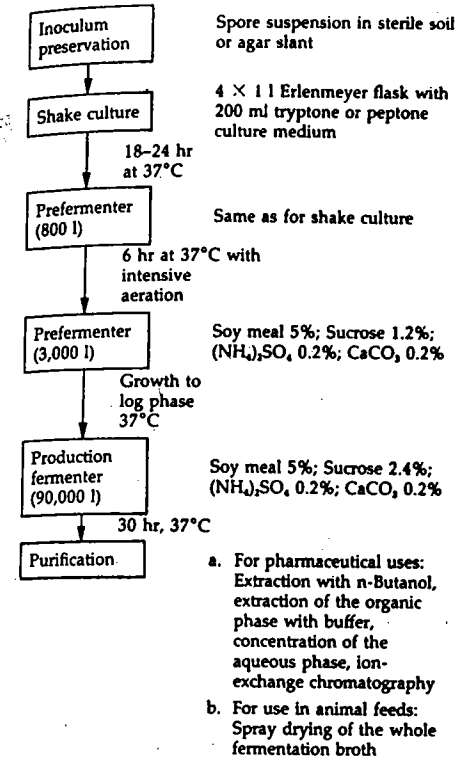


Figure 13.17 Flow scheme for the production of bacitracin by *B. licheniformis* (From Perlman, 1979)

Bleomycins are isolated as Cu(II) complexes from the culture broth of *Streptomyces verticillus*. The glycopeptide exhibits some antitumor action and is marketed as a mixture of bleomycin A_2 and B_2.

13.4 CARBOHYDRATE ANTIBIOTICS

Carbohydrate antibiotics include the therapeutically important aminoglycosides, the orthosomycins, and various sugar derivatives.

Glycosides and sugar derivatives

Several commercially important antibiotics from this heterogenous group are listed in Table 13.9.

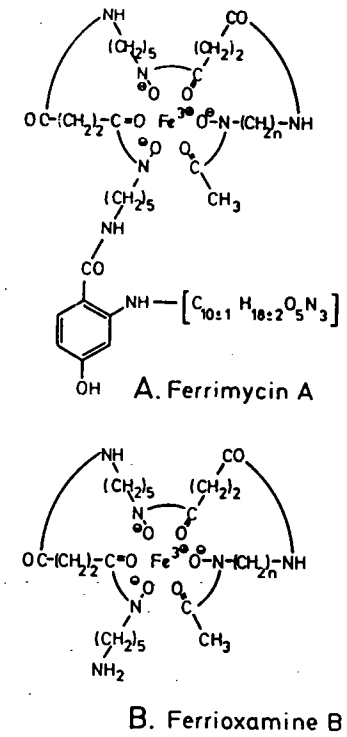


Figure 13.18 Structure of siderochromes

Nojirimycin (Figure 13.19 A), shows antibacterial activity against *Sarcina lutea* and *Xanthomonas oryzae*, and is of interest because of its high inhibitory activity against α- and β-glucosidases and amylases.

Vancomycin, a broad-spectrum antibiotic, consists of the amino sugar vancosamine, two β-hydroxychlortyrosine moieties, three phenylglycine derivatives, N-methyl leucine, and aspartic acid. It is effective against Gram-positive organisms, particularly staphylococci. However, it has adverse side effects in humans and thus is used only to treat infections caused by pathogens which are resistant to other antibiotics or with patients who are hypersensitive to penicillin. No

Table 13.9 Carbohydrate antibiotics. Sugars, glycosides and other commercially important sugar derivatives

Group	Antibiotic	Organism producing
5-Amino sugar	Nojirimycin	*Streptomyces roseochromogenes* *S. lavendulae* *S. nojiriensis*
Glycopeptide	Vancomycin	*S. orientalis*
Sugar amide	Lincomycin	*S. lincolnensis* var. *lincolnensis*
Phosphoglycolipid	Moenomycin A,C,D,E,F,G,H (= Bambermycin)	*S. bambergiensis* *S. ederensis* *S. ghanaensis* *S. geysiriensis*

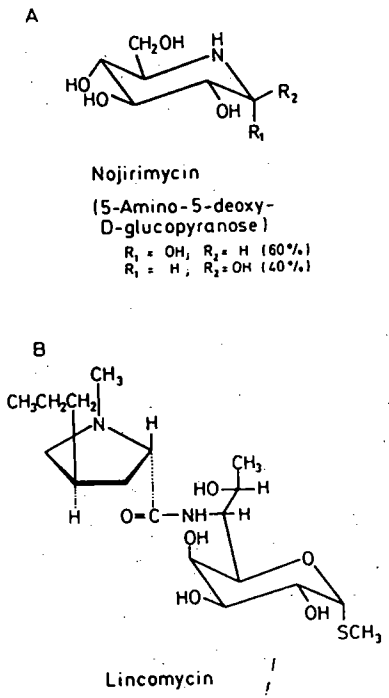


Figure 13.19 Structure of nojirimycin and lincomycin

development of resistance to vancomycin has yet been observed in therapy.

Lincomycin (Figure 13.19 B) is used in the treatment of staphylococcus, streptococcus, and pneumococcus infections; only minor side effects are observed, but during therapy, resistance is acquired rapidly. Clindamycin (7-chlor-7-deoxylincomycin), a semisynthetic derivative with improved pharmacological properties, is principally marketed today. In protein synthesis, the bonding of aminoacyl-tRNA to the A-binding site on the ribosome is inhibited by lincomycin, probably through interaction with the ribosomal protein L6 of the 50S subunit.

Moenomycin is a phosphoglycolipid which shows activity against Gram-positive bacteria as well as Gram-negative bacteria containing a resistance plasmid. Moenomycin A and the related antibiotics macarbomycin and quebemycin are marketed for use as nutritional feed supplements. Phosphoglycolipids inhibit bacterial cell wall synthesis by affecting the transglycosylation reaction in peptidoglycan biosynthesis.

Orthosomycins

The orthosomycins are a class of antibiotics newly discovered in the late 1970's. They are characterized by an oligosaccharide structure and two noncarbohydrate ester groups (Figure 13.20). The following antibiotics have been described: the avilamycins (produced by *Streptomyces viridochromogenes*), the curamycins (*S. curacoi*), the everninomicins (*Micromonospora carbonaceae*), and flambamycin (*S. hygroscopicus*). The avilamycins are being tested for use as animal feed supplements.

Aminoglycoside antibiotics

Aminoglycosides are oligosaccharide antibiotics and consist of an aminocyclohexanol moiety (e.g. deoxystreptamine, streptidine), which is glycosidically linked to other amino sugars. Over 100 aminoglycosides are known, of which those produced industrially as well as some newer com-

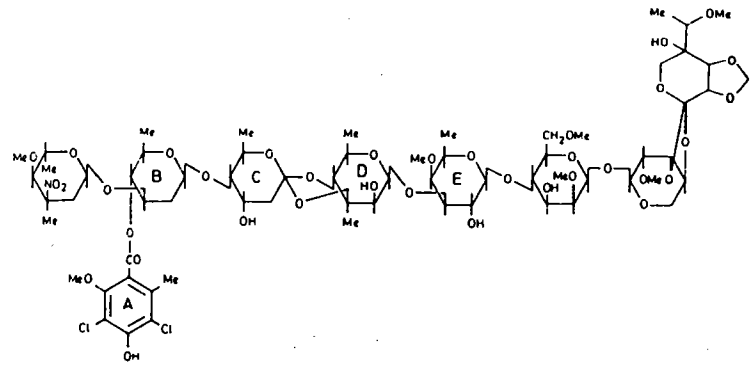


Figure 13.20 Everninomicin B

pounds are listed in Table 13.10; Figure 13.21 shows the most important structures.

Aminoglycosides are primarily used against Gram-negative bacteria in a wide range of applications. Although the semisynthetic cephalosporins are also now widely used for Gram-negative infections, in severe infections the aminoglycosides are often the antibiotics of choice. Although streptomycin and dihydrostreptomycin are broad-spectrum antibiotics, they are primarily used to treat tuberculosis. They are considered reserve antibiotics, mainly due to the fact that resistance can develop rapidly and in a single step by a chromosomal mutation.

In 1976 the world market for aminoglycosides (not including the Soviet Union and China) was estimated at $500 million, with a yearly increase of 10–15%.

All of the aminoglycoside antibiotics cause kidney damage and deafness (nephro- and ototoxicity) as side effects. In addition to resistance development by chromosomal mutation, plasmid-determined resistance due to the formation of inactivating enzymes is known (see Section 15.4). Compounds with reduced toxicity and/or increased effectiveness against resistant organisms are being sought via screening, mutasynthesis, and formation of chemical derivatives. As a result of a screening program, the new group of fortimicins was discovered. This is a group of pseudodisaccharides with a broad spectrum of activity, particularly against aminoglycoside-resistant bacteria. The kanamycin derivatives amikacin and dibekacin (1-N-L(−)-γ-amino-α-hydroxybutyrylkanamycin A and 3′,4′-dideoxykanamycin B), and netilmicin (1-N-ethylsisomicin) are semisynthetic compounds with partial resistance to inactivating enzymes. These compounds have already been introduced for clinical use. The compound 5-*epi*-sisomicin, isolated by mutasynthesis, has been tested clinically because of its action against sisomicin-resistant organisms. However, for cost reasons it is being produced chemically rather than by fermentation.

Besides the antibiotics noted above which are useful for human therapy, kasugamycin and validamycin are used as important antibiotics in rice cultivation.

Mode of action The mode of action of aminoglycosides is on protein synthesis in sensitive bacteria. Streptomycin, the aminoglycoside which has been studied the most, binds to protein S12 of the 30S ribosome and causes misreading of the code and hence inhibition of pro-

Table 13.10 Classification of the most important aminoglycoside antibiotics according to the aminocyclohexanol moiety. Production strain, spectrum of activity, and application are given for commercially produced antibiotics

Group	Antibiotic	Organism producing	Spectrum of activity[a]	Application
I Streptamine derivatives				
Streptidine	Streptomycin	*Streptomyces griseus*	G^+G^-My	Human therapy
	Dihydrostreptomycin	*S. humidus*[b]	G^+G^-My	Human therapy
Actinamine (N,N-dimethylstreptamine)	Spectinomycin[c]	*S. spectabilis*	G^+	Human therapy
		S. flavopersicus		(Penicillin-resistant gonococci)
	Bluensomycin	*(S. bluensis)*		
II 2-Deoxystreptamine (DOS) derivatives				
A. 4,5-Disubstituted DOS				
Neomycin group (Pseudotetrasaccharides)	Neomycins B, C	*S. fradiae*	G^+G^-	Human therapy
	Paromomycins I, II	*S. rimosus* forma *paromomycinus*	G^+G^- Protozoa	Human therapy
	Lividomycins A, B	*S. lividus*	G^+G^-	Human therapy
Ribostamycin group (Pseudotrisaccharides)	Ribostamycin	*S. ribosidificus*	G^+G^-	Human therapy
	Butirosins	*(Bacillus circulans)*		
B. 4,6-Disubstituted DOS				
Kanamycin group	Kanamycins A, B, C	*S. kanamyceticus*	G^+G^-My	Human therapy
	Tobramycin (= Nebramycin-factor 6)	*S. tenebrarius*	G^+G^-	Human therapy
	Seldomycins 1, 2, 3, 5	*(S. hofunensis)*		
Gentamicin group	Gentamicins C_1, C_{1a}, C_2	*Micromonospora purpurea* *M. echinospora*	G^+G^-	Human therapy
	Sagamicin = Micronomicin	*(M. sagamiensis*, etc.)	G^+G^-	Human therapy
	Sisomicin	*M. inyoensis*	G^+G^-	Human therapy
	Verdamicin	*(M. grisea)*		
C. Monosubstituted DOS	Hygromycin B	*S. hygroscopicus*	G^+G^-F worms	Feed additive
	Destomycins A, B, C	*(S. rimofaciens)*	G^+G^-My worms	Feed additive
	Apramycin (= Nebramycin-factor 5)	*(S. tenebrarius)*		
III Fortamine derivatives	Fortimicin	*M. olivoasterospora*	G^+G^-	Human therapy
	Sannamycins A, B	*(S. sannanensis* sp. nov.)		
	Sporaricins A, B	*(Saccharopolyspora hirsuta* subsp. *kobensis)*		
	Istamycins A, B	*(S. tenjimariensis* nov. sp.)		
IV Other aminocyclohexanol derivatives	Validamycins	*S. hygroscopicus* var. *limonensis*	G^+G^-F	Plant pathology
	Kasugamycin	*S. kasugaensis*	G^+G^-F	Plant pathology

[a] G^+: antibiotically effective against gram-positive organisms, G^-: against gram-negative organisms; My: against mycobacteria; F: antifungal antibiotics.
[b] Dihydrostreptomycin is almost exclusively produced via chemical reduction of streptomycin.
[c] Spectinomycin has unusual status: it contains one aminocyclitol moiety but no amino sugar.
(Berdy, 1985)

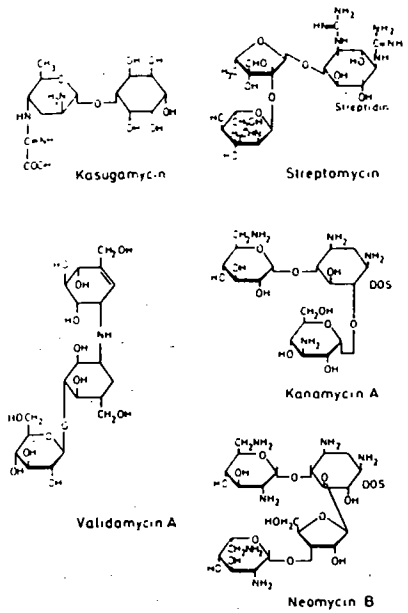


Figure 13.21 Aminoglycoside antibiotics (structure of validamycin according to Suami et al., 1980). For the structure of neomycin/ribostamycin see Figure 15.9; gentamicin complex see Figure 15.10

tein synthesis. S12 is a component of the A site at which the amino-acyl-tRNA and fMet-tRNA bind. 2-Desocystreptamine-containing antibiotics inhibit the translocation process of the peptidyl-tRNA. In addition, aminoglycoside antibiotics cause damage to the bacterial cell membrane, thus bringing about loss from the cell of low-molecular weight constituents.

Biosynthesis of aminoglycosides As shown by labeling experiments, glucose is the precursor of all the subunits of those aminoglycoside antibiotics which have been examined. Although a number of enzymatic reactions have been uncovered, the exact biosynthesis of any aminoglycoside antibiotic is not yet fully understood. Figure 13.22 shows the pathway of **streptomycin biosynthesis**. Over 30 separate enzymatic steps are known. One of the principal components of streptomycin, **streptidine**, is synthesized from glucose-6-phosphate via *myo*-inositol. A number of enzymatic steps, involving oxidation, amination, phosphorylation, carbamidinylation, and dephosphorylation, lead to the formation of streptidine-6-P. The second dephosphorylation step takes place during the final biosynthetic step to streptomycin synthesis. Although the pathway for **streptose** biosynthesis is known (see Figure 13.22), that for **N-methyl-L-glucosamine** is still unclear. The combination of the three subunits involves two steps. Only in the last step is the phosphate moiety removed, leading to the formation of the biologically active streptomycin from the biologically inactive streptomycin phosphate.

In the DOS-aminoglycosides (see Table 13.10), the biosynthesis of 2-deoxystreptamine occurs from glucose (Figure 13.23).

Regulation of biosynthesis Only few data are available on the induction and regulation of aminoglycoside biosynthesis. Streptomycin-producing strains of *S. griseus* and *S. bikiniensis* show regulation via the so-called factor A, 2 (S)-isocapryloyl-3-(S)-hydroxymethyl-γ-butyrolactone shown in Figure 13.24. This compound controls streptomycin biosynthesis, streptomycin resistance, and sporulation. It is active at the extremely low concentration of 10^{-9}M. Factor A^- mutants that are unable to produce the antibiotic can be restored to streptomycin production by addition of factor A.

In addition, streptomycin production is influenced by nitrogen and carbohydrate catabolite regulation, as well as phosphate regulation.

Strain development Strain-development programs have been conducted to increase yields for all industrially produced aminoglycoside antibiotics, since yields of wild strains range only from 10–200 μg/ml, which is not sufficient for a cost-effective process. The following yields have been published: kanamycin 2000 μg/ml; tobra

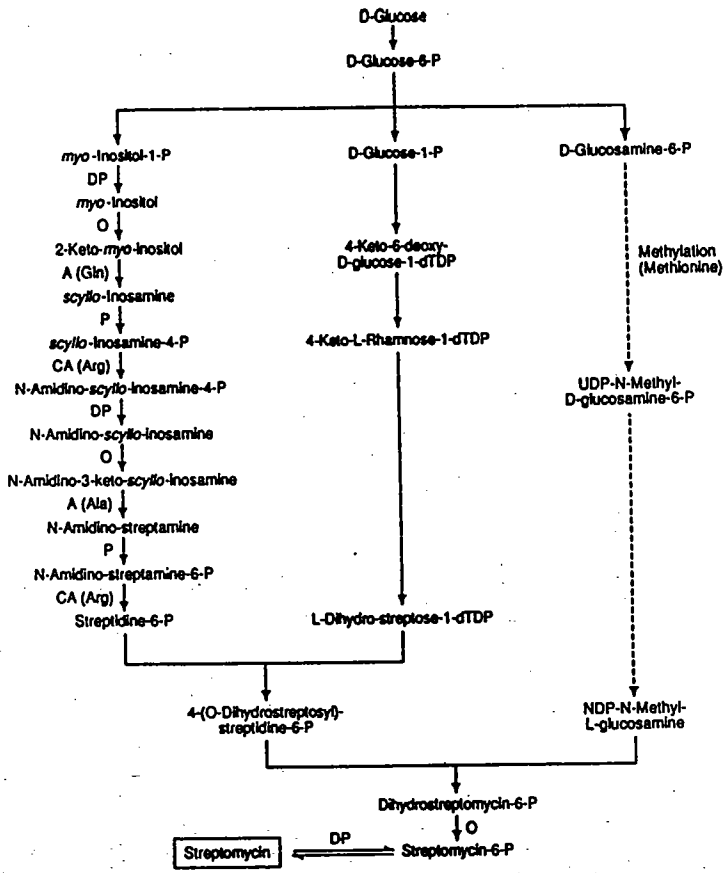


Figure 13.22 Streptomycin biosynthesis (based on Florent, 1985). O, oxidation; A, amination; P, phosphorylation; CA, carbamidinylation; DP, dephosphorylation; Ala, alanine; Arg, arginine; Glu, glutamine.

mycin 1500 μg/ml; neomycin 2000 μg/ml; gentamicin 1400 μg/ml. Streptomycin yields have been increased from 100–200 μg/ml to about 15 mg/ml by strain-improvement techniques. The current yields of production processes are probably far more than the published values in many cases.

Mutation and selection are the principal methods which have been used in strain development; there have also been some cases in which recombination (conjugation and protoplast fusion) has been employed. The strain development of kasugamycin is a typical example. Using the "agar disk" method (see Section 3.3), in one year 650,000 isolates were screened after mutagenesis, resulting in a yield increase from 200 μg/ml to about 7000 μg/ml. Conjugation of auxotrophs produced hybrid strains, whose off-

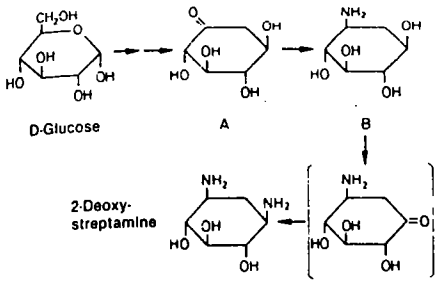


Figure 13.23 Biosynthesis of 2-deoxystreptamine in *Bacillus circulans* S-11. A, 2-Deoxy-scyllo-inosose; B, 2-Deoxy-scyllo-inosamine (From Fujiwara et al., 1980)

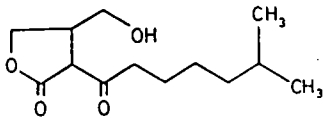


Figure 13.24 Structure of Factor A from *S. griseus*

spring in one case produced up to 26% more kasugamycin (9300 μg/l) than the initial prototroph strain (Figure 13.25). Cell fusion and recombinant DNA methods have been used in several cases to produce higher-yielding strains. Protoplast fusion has been used to increase yield of sisomicin producers and amplification of a resistance gene, 6′-N-acetyltransferase, has increased production of kanamycin and neomycin.

Production methods Aminoglycoside antibiotics are produced in fermenters with volumes up to 150,000 l. Optimal oxygen supply is required (aeration rate 0.5–1.0 vvm) and temperatures are held between 28–30°C with the pH in the neutral range. The length of fermentation is between 4–7 days, depending on the strain.

Glucose in combination with starch or dextrin serves as the carbon source. Originally, the antibiotic butirosin could be produced only with glycerol as carbon source, due to catabolite repression by glucose, but the isolation of mutants not subject to catabolite regulation has made it possible to use glucose. In several processes, continuous feeding of glucose has been successfully used. Soy meal is an ideal nitrogen source for aminoglycoside production because of its slow catabolism. NaCl (1–3 g/l) must be added to the streptomycin fermentation. The production of gentamicin, sisomicin, and fortimicin A is significantly accelerated by addition of Co^{2+}, which is active in the methylation steps during biosynthesis. Addition of Zn^{2+} increases the yield of nebramycin and addition of Mg^{2+} stimulates kanamycin and neomycin production.

Aminoglycoside processes are typical secondary metabolite fermentations with trophoand idiophases, as shown in Figure 13.26 for the butirosin fermentation.

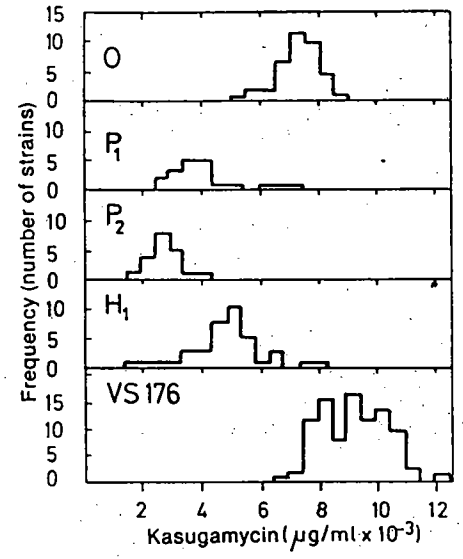


Figure 13.25 Effect of genetic recombination on kasugamycin formation. O, prototrophic original strain (kasugamycin formation, 7300 μg/ml; P_1, auxotrophic *(ilv)* mating partner (4400 μg/ml); P_2, auxotrophic *(ser)* mating partner (2900 μg/ml); H_1, prototrophic hybrid strain *(ilv × ser)* (5100 μg/ml); VS176, selection from H_1 (9300 μg/ml) (From Ichikawa et al., 1971)

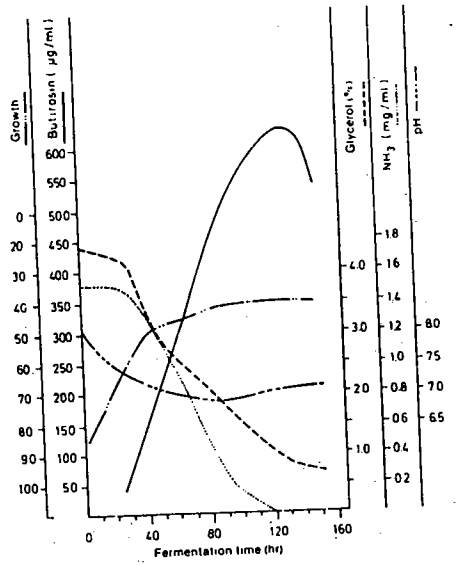


Figure 13.26 Butirosin formation with *Bacillus circulans*. Medium: glycerol 4%; soy meal 1%; Wilson's peptone 1.75%; NH_4Cl 0.4%; $CaCO_3$ 0.5% (Howells et al., 1972)

Most of the aminoglycoside antibiotics are excreted and are present in the culture supernatant, from which they are removed by adsorption to ion-exchange columns. Cell-bound aminoglycosides, such as gentamicin, sisomicin, and fortimicin, must first be released by acidification to pH 2–2.5 with H_2SO_4.

13.5 MACROCYCLIC LACTONE ANTIBIOTICS

The macrolide group of antibiotics consists of compounds with large lactone rings. In addition to those antibiotics conventionally called **macrolides**, this group includes **polyenes, macrotetrolides**, and a heterogeneous group of atypical macrolides.

Macrolide antibiotics

Macrolides are hydrophobic, usually basic compounds. They consist of 12-, 14-, 16-, or 17-membered lactone rings with 1–3 sugars glycosidically linked with the aglycone (lactone ring) and with each other. The sugars are amino sugars and/or 6-deoxyhexoses. The structures of erythromycin and oleandomycin are examples of 14-membered macrolides (Figure 13.27); the 16-membered macrolides leucomycin and tylosin are illustrated in Figure 13.28. Macrolides are produced by 1–3% of all actinomycete isolates; the compounds listed in Table 13.11 are commercially available.

Macrolides are very effective against Gram-positive bacteria, particularly staphylococci, and mycoplasmas and were frequently used against penicillin-resistant organisms, but because of the development of the newer β-lactam antibiotics (see Section 13.2), the macrolides are no longer of as great medical significance. The aglycone and sugar moieties are both necessary for anti-

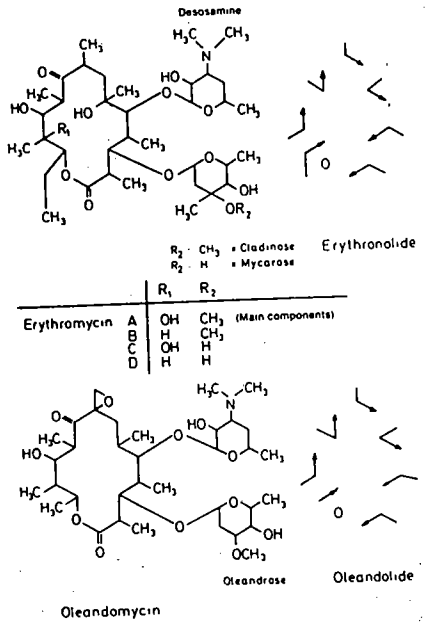
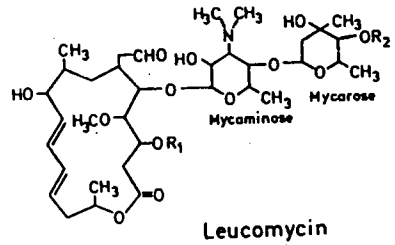
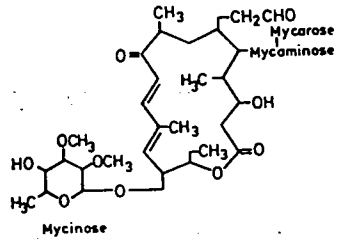


Figure 13.27 Structure of erythromycin and oleandomycin. Straight arrow, acetate residue; bent arrow, propionate residue



	R_1	R_2
Leucomycin A_1	H	$COCH_2CH(CH_3)_2$
A_3 (Josamycin)	$COCH_3$	$COCH_2CH(CH_3)_2$
A_4	$COCH_3$	$COCH_2CH_2CH_3$
A_5	H	
A_6	$COCH_3$	$COCH_2CH_3$
A_7	H	
A_8	$COCH_3$	$COCH_3$



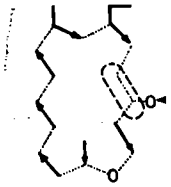


Figure 13.28 Structure of leucomycin and tylosin. Straight arrow, acetate residue; bent arrow, propionate residue; upper right bent arrow, butyrate residue; dotted region, position of C-3, 4 unknown

Table 13.11 Commercially produced macrolide antibiotics

Macrolide antibiotic	Organism producing	Activity	Application
Carbomycin A	*Streptomyces halstedii*	G⁺	Was marketed for several years but discontinued du ineffectiveness
Erythromycin	*S. erythreus*	G⁺	Human therapy, feed additive
Josamycin (= Leucomycin A_3)	*S. narbonensis* var. *josamyceticus*	G⁺	Human therapy
Leucomycins A_1–A_4 (= Kitasamycins)	*Streptoverticillium kitasatoensis*	G⁺	Human therapy
Maridomycin	*S. hygroscopicus*	G⁺	
Midekamycin	*S. mycarofaciens*	G⁺	
Oleandomycin	*S. antibioticus*	G⁺	Human therapy
	S. olivochromogenes		
Spiramycins I, II, III	*S. ambofaciens*	G⁺	Human therapy
Tylosin	*S. fradiae*	G⁺, Myco-plasmas	Feed additive, veterinary medicine
	S. hygroscopicus		

bacterial activity. Therapy with macrolides results in a rapid buildup of resistance and there is also cross-resistance between the individual antibiotics. Because of the low toxicity, efforts are being made to isolate new compounds of this class, to chemically modify known macrolides, and to modify known macrolides through biotransformation.

All macrolide antibiotics are inhibitors of protein synthesis, binding to the 50 S subunit of bacterial ribosomes. Erythromycin is known to inhibit the elongation-factor G-dependent release of deacylated tRNA from the P site of the ribosome.

Biosynthesis and regulation The aglycone moiety of macrolides is built either of propionate subunits or of a combination of acetate and propionate subunits, sometimes with the participation of butyrate or related compounds. One of the first intensively studied macrolides was erythromycin; the biosynthesis of this antibiotic was studied by labeling experiments and from the behavior of mutants of *Streptomyces erythreus* blocked in antibiotic biosynthesis. Analogous to fatty acid synthesis, erythronolide production proceeds from propionyl-CoA as a starting material, onto which 6 molecules of 2-methylmalonyl-CoA condense. This polyketide synthesis takes place on a multi-enzyme complex called *lactone synthase*, which is very similar to the fatty acid synthetase of *S. erythreus*. After cyclization to the aglycone, the first free compound, 6-deoxyerythronolide B, can be isolated. The remaining glycosylation steps are diagrammed in Figure 13.29.

A number of observations have been made about the regulation of macrolide biosynthesis. Biosynthesis of the macrolide tylosin is stimulated by long-chain fatty acids; glucose inhibits the metabolism of fatty acids and tylosin production, probably by inducing an acetyl-CoA deficiency. Phosphate regulation is also observed with many macrolide antibiotics, as well as nitrogen (NH_4^+) catabolite regulation.

Erythromycin biosynthesis is regulated at various levels. For instance, erythronolide B inhibits its own production, the synthesis of 6-deoxyerythronolide B, and presumably also the activity of the lactone synthase. End-product inhibition of the transmethylase by erythromycin A has been observed. This enzyme catalyzes the methylation of mycarose to cladinose as a last step of the biosynthetic pathway. In high-yielding strains used for industrial production, this regulation is eliminated.

When propanol (0.2–0.5%) is added to the fermentation broth of *S. erythreus*, it induces the synthesis of acetyl-CoA-carboxylase and causes a 100% increase in erythromycin production.

Production method Macrolide antibiotics are produced in aerobic submerged fermentations. Yields in large-scale industrial processes are around 20 g/l. A typical medium for *S. erythreus* contains glucose 50 g, soy meal 30 g, $(NH_4)_2SO_4$ 3 g, NaCl 5 g, $CaCO_3$ 6 g, tap water 1 l, pH 7.0. Complex culture medium ingredients may also be used, such as starch, corn steep liquor, yeast extract, and oils (e.g., soy bean oil). Fermentation temperatures for most macrolides are 25–28°C and for erythromycin 33°C. The length of the fermentation is 3–7 days.

The macrolide tylosin can be produced in either batch or continuous fermentation. The specific production rate is increased to 1.1 mg/g biomass·h if glycerol is used instead of glucose and if the feeding rate of sodium glutamate is optimized at a low specific growth rate of 0.03/h (27% of the μ_{max}).

Polyene macrolide antibiotics

Polyenes consist of 26- to 38-membered lactone rings with 3–7 conjugated double bonds. Depending on the length of the chromophore, polyenes are classified as trienes, tetraenes, pentaenes, hexaenes, or heptaenes. Many polyenes also contain a glycosidically-bound amino sugar (mycosamine or perosamine). Some heptaenes carry p-aminoacetophenone or N-methyl-p-aminoacetophenone as an aromatic moiety on the carbon atom which is involved in lactone formation. The structures of two polyenes are given in Figure 13.30.

More than 90 polyenes have been characterized, primarily from streptomycetes. These antibiotics are active against fungi and yeast but they are ineffective against bacteria. Polyenes affect membrane sterols of sensitive eucaryotes, causing a change in membrane permeability. Some polyenes are relatively toxic (nephro- or hepatotoxic) but they are widely used commercially for topical application because no better antifungal antibiotics are available.

Polyenes are not suitable for plant-disease control because they are sensitive to light and

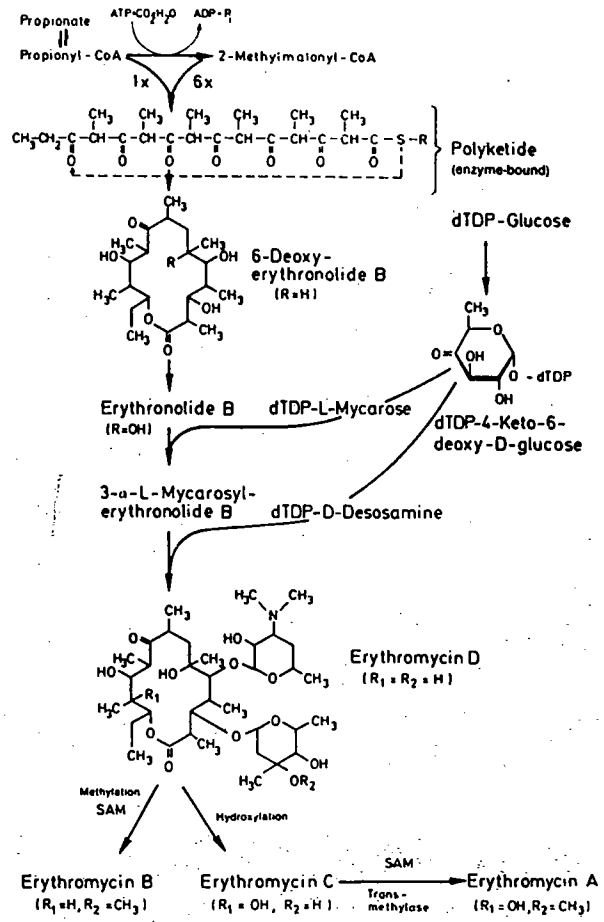
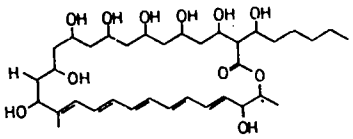
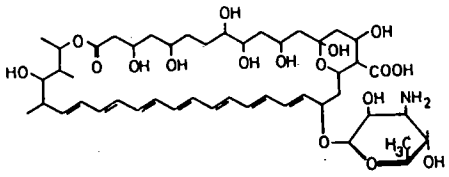


Figure 13.29 Biosynthesis of erythromycin in *S. erythreus*. SAM: S-adenosyl-L-methionine



Filipin



Amphotericin B

Figure 13.30 The polyene antibiotics filipin (pentaene) and amphotericin B (nonaromatic heptaene)

heat. Table 13.12 lists the commercially produced polyenes.

Biosynthesis and regulation Like macrolides, polyene antibiotics are produced from C_2 and C_3 units via the polyketide biosynthetic pathway. Among the aromatic polyenes, p-aminobenzoyl-CoA, the precursor of p-aminoacetophenone and N-methyl-p-aminoacetophenone, seems to function as the starting point for polyketide polymerization. The chromophore consists solely of acetate units. The amino sugar is derived from glucose, with thymidine diphosphate 4-keto-6-deoxy-D-glucose as an intermediate. Polyene biosynthesis shows phosphate regulation, catabolite repression, and feedback inhibition.

Production methods Glucose is usually the best carbon source for polyene production. Most fermentations using glucose are continuous, because catabolite repression occurs at the required glucose concentration (for candicidin 7–9.6%). The candicidin production phase can be extended to 300 h by glucose feeding. The addition of acetate, propionate, malonate, or n-propanol stimulates polyene production, and soy meal or soy peptone can be used as nitrogen sources.

For maximal polyene production, careful control of aeration is essential. During the growth phase, the required oxygen transfer rate is around 0.8 mmole O_2/l·min, but during the production phase, O_2 concentration should not exceed 40% of saturation. Figure 13.31 shows the relation of candicidin production to various fermentation parameters.

Since polyenes are not water-soluble, they accumulate as crystals or microparticles at the surface of the cell or in the medium, from which they can be extracted with appropriate solvents.

Table 13.12 Commercially produced polyene macrolide antibiotics

Antibiotic	Organism producing	Polyene type	Application
Amphotericin B	*Streptomyces nodosus*	Heptaene, M	F, Histoplasmosis
Candicidin B	*S. griseus*	Heptaene, M, AAP	F, Prostate hypertrophy
Candidin	*S. viridoflavus*	Heptaene, M	F
Filipin	*S. filipinensis*	Methylpentaene	F
Fungimycin (=Perimycin)	*S. coelicolor* var. *aminophilus*	Heptaene, P, MAAP	F
Hamycin	*S. primprina*	Heptaene, M, AAP	F
Levorins	*S. levoris*	Heptaene, M, AAP	F, Prostate hypertrophy
Mycoheptin	*Streptoverticillium mycoheptinicum*, *S. netropsis*	Heptaene, M	F
Nystatin A_1, A_2, A_3	*S. noursei*	Tetraene, M	F, *Candida albicans*; slightly toxic
Pimaricin	*S. natalensis*	Tetraene, M	F, Food preservative
Trichomycin A, B	*S. hachijoensis*	Heptaene, M, AAP	F, *Trichomonas vaginalis*

M=Mycosamine; AAP=p-Aminoacetophenone; P=Perosamine; MAAP=N-Methyl-p-aminoacetophenone; F=Antifungal activity.

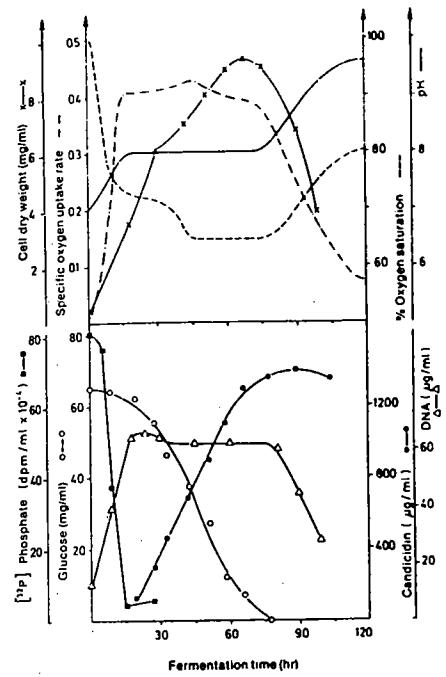


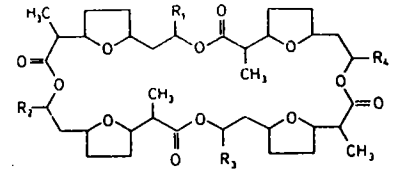
Figure 13.31 Relationship of candicidin formation to different fermentation parameters

Macrotetrolides

Macrotetrolides are cyclic polylactones which function as ionophores. These sugar-free compounds are synthesized from four tetrhydrofuranyl hydroxy acids (Figure 13.32). Tetranactin, produced by *S. flaveolus*, is used as an insecticide.

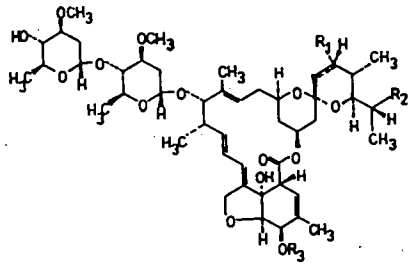
Atypical macrolides

This is a heterogeneous group, classified together because all structures contain the lactone ring. Examples include avermectin, milbemycin, venturicidin, bafilomycin, and irumamycin. Avermectin, whose structure is shown in Figure 13.33, is produced by *S. avermitilis*. It has found use in veterinary medicine because of its high activity against nematodes and arthropods. Irumamycin is undergoing field trials in agriculture as a fungicide.



Nonactin	S werraensis	$R_1=R_2=R_3=R_4=CH_3$
Monactin	S sp ETH 23112	$R_1=R_2=R_3=CH_3$ $R_4=C_2H_5$
Dinactin	S sp ETH 23112	$R_1=R_2=CH_3$ $R_3=R_4=C_2H_5$
Trinactin	S sp ETH 23112	$R_1=CH_3$ $R_2=R_3=R_4=C_2H_5$
Tetranactin	S. flaveolus	$R_1=R_2=R_3=R_4=C_2H_5$

Figure 13.32 Macrotetrolides



Avermectin	R_1	R_2	R_3
A_{1a}		C_2H_5	CH_3
A_{1b}		CH_3	CH_3
A_{2a}	OH	C_2H_5	CH_3
A_{2b}	OH	CH_3	CH_3
B_{1a}		C_2H_5	H
B_{1b}		CH_3	H
B_{2a}	OH	C_2H_5	H
B_{2b}	OH	CH_3	H

In those avermectins without an R_1 group, a double bond is present.

Figure 13.33 Structures of the avermectins

Ansamycins

Ansamycins are macrolactam antibiotics with an aromatic chromophore to which an aliphatic polyketide derivative is bound at both ends, via amide, ether, and/or carbon-carbon bonds, the so-called "ansa chain" (Figure 13.34). Ansamycins are distinguished by their chromophore. Those compounds with a benzene ring, such as geldanamycin or ansamitocin, act mainly on eucaryotes, whereas those with a naphthalene structure are antibacterially effective. In this latter class are the rifamycins, halomycins, and streptovaricins.

Rifamycins are important in chemotherapy. They are very effective against Gram-positive bacteria and mycobacteria, but less effective against Gram-negative bacteria; they show no cross resistance with other antibiotics. They are produced by *Nocardia mediterranei*. Approximately 20 different rifamycins have been isolated from culture solutions of *Nocardia* mutants, and genetic studies have shown that the genes for the last steps in the biosynthetic pathway lie in a single cluster. The wild strain produces rifamycin B as a major component and smaller amounts of the so-called rifamycin complex (A, C, D, E). The production of this complex can be largely suppressed by the addition of sodium diethylbarbiturate. Rifamycin B is completely inactive but during its breakdown or via chemical modification, the biologically active rifamycins O, S, and SV are formed. Rifamycin S and SV are biosynthetic precursors to rifamycin B. Mutants have been isolated which excrete rifamycin SV due to a block in the subsequent steps. The ansa chain of rifamycin is formed from two malonyl-CoA and eight methylmalonyl-CoA units and methionine. The polyketide condensation process proceeds from an aromatic C_7 unit. The biosynthesis of the naphthohydroquinone chromophore branches off from the aromatic biosynthetic pathway between 3-deoxy-D-arabinoheptulose-7-P and shikimate.

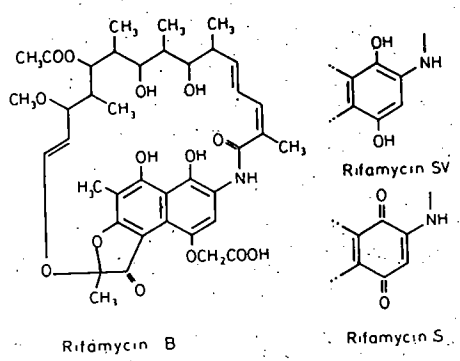


Figure 13.34 The rifamycins B, S and SV

Rifampicin, a semisynthetic compound derived from rifamycin SV, is widely used in the treatment of tuberculosis. It is a specific inhibitor of bacterial DNA-dependent RNA polymerase. It binds with the β-subunit of the polymerase, and RNA polymerases of higher organisms are not affected.

13.6 TETRACYCLINES AND ANTHRACYCLINES

Tetracyclines

The tetracyclines are important antibiotics with widespread medical use. **Chlortetracycline**, the first antibiotic of this group, was isolated from cultures of *Streptomyces aureofaciens* in 1945. Tetracycline itself was first prepared by dehalogenation of chlortetracycline but was subsequently found to be produced by cultures of *S. viridifaciens* as well. Presently about 20 streptomycetes are known which produce a mixture of different tetracyclines. Several strains are listed in Table 13.13.

The basic structure of the tetracyclines consists of a naphthacene ring system. Figure 13.35 shows the structures of several therapeutically important compounds. There are also semisynthetic derivatives on the market as well as the fermentatively produced tetracyclines.

Table 13.13 Streptomycetes producing tetracycline

Species	Products[a]
S. alboflavus	CTC, TC, OTC, actinomycin
S. antibioticus	OTC, TC
S. aureofaciens	CTC, TC
S. aureus	TC
S. californicus	TC, actinomycin
S. cellulosae	OTC, actinomycin
S. feofaciens	TC
S. flaveolus	OTC, TC, actinomycin
S. flavus	CTC, TC, OTC, actinomycin
S. lusitanus	CTC, TC
S. parvus	OTC, TC, actinomycin
S. platensis	OTC
S. rimosus	OTC, TC, rimocidin
S. sayamaensis	TC, CTC
S. vendargensis	OTC, vengacide
S. viridifaciens	CTC, TC

[a] CTC 7-chlortetracycline; TC tetracycline; OTC oxytetracycline
(Perlman, 1979)

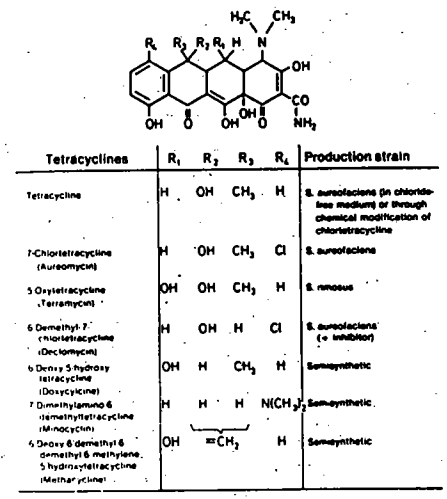


Figure 13.35 Structure of clinically important tetracyclines

Tetracyclines are broad-spectrum antibiotics, which are effective against Gram-positive and Gram-negative bacteria, as well as rickettsias, mycoplasmas, leptospiras, spirochetes, and chlamydias. There is a marked cross-resistance between different tetracyclines. After cephalosporin and penicillin, the tetracylcines are the most widely used antibiotics. Chlortetracycline and oxytetracycline are commonly used in human and veterinary medicine. In many countries they are also widely used as nutritional supplements in poultry and swine production and in some countries they are also used to preserve fish, meat and poultry.

Tetracyclines act as **inhibitors of protein synthesis**. Their site of action is the 30S ribosome, where binding of aminoacyl-tRNA to the ribosomal A-site is inhibited.

Biosynthesis and regulation Chlortetracycline and oxytetracycline are the tetracyclines most frequently produced by the streptomycetes, tetracycline itself usually being only a minor component. However, *S. aureofaciens* mutants with a block in the chlorination reaction excrete tetracycline as the major product. A simplified version of chlortetracycline biosynthesis is illustrated in Figure 13.36. The biosynthesis can be subdivided into three sections:

- Glucose is converted into acetyl-CoA.
- Malonyl-CoA is produced in the transformation of acetyl-CoA by means of acetyl-CoA-carboxylase. After transamination to malonamoyl-CoA, which is bound to an enzyme complex ("anthracene synthase"), this compound condenses with 8 molecules of malonyl-CoA. The polyketide presumably formed from this condensation has not been isolated. An alternative pathway to malonyl-CoA is via the production of oxalacetate catalyzed by phosphoenol pyruvate carboxylase, oxalacetate being converted to malonyl-CoA by oxidative decarboxylation. Cyclization into the tricyclic intermediate also takes place on the enzyme anthracene synthase.
- The step-by-step transformation of the assumed tricyclic intermediate, which has not yet been isolated, into the variety of known tetracyclines.

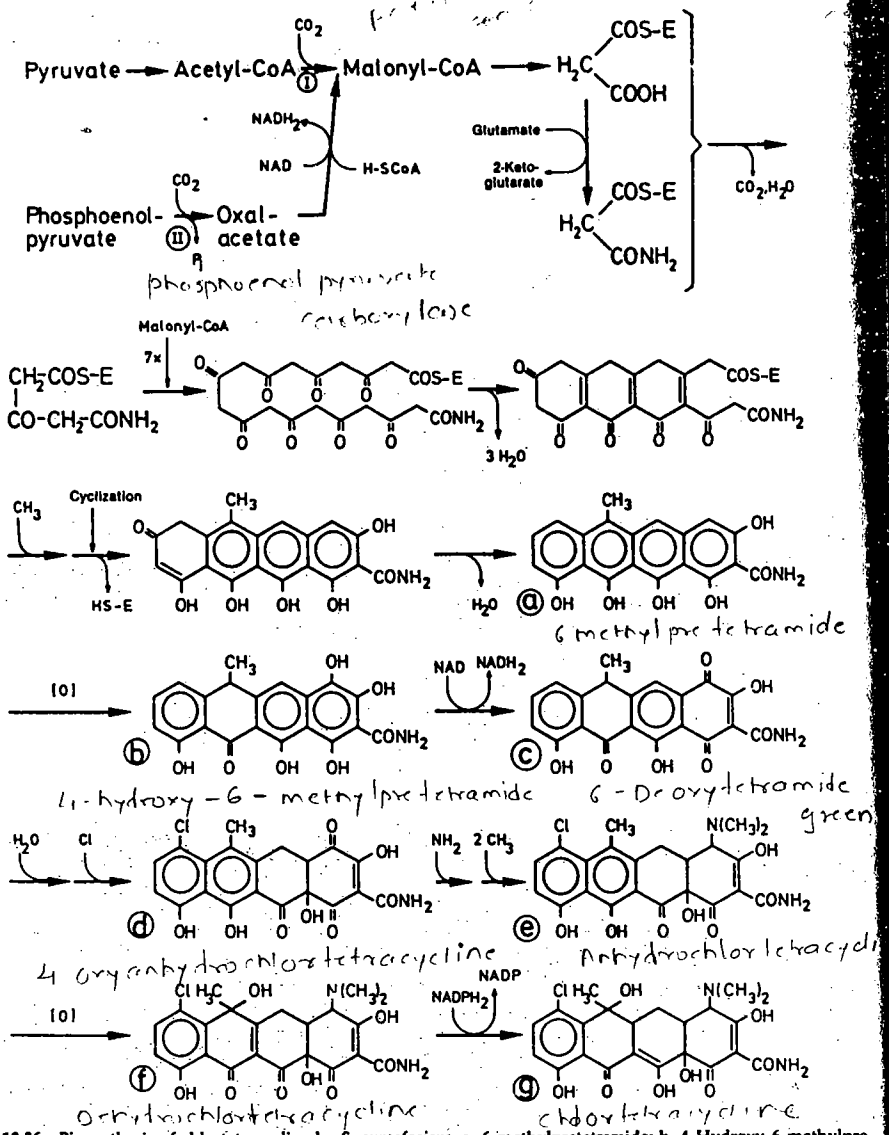


Figure 13.36 Biosynthesis of chlortetracycline by *S. aureofaciens*. a, 6-methylpretetramide; b, 4-Hydroxy-6-methylpretetramide; c, 6-Deoxytetramide green; d, 4-Oxyanhydrochlortetracycline; e, Anhydrochlortetracycline; f, Dehydrochlortetracycline; g, Chlortetracycline; I, Acetyl-CoA carboxylase; II, Phosphoenolpyruvate carboxylase (From Vanek et al., 1971; Hoštalek et al., 1979)

The scheme of chlortetracycline biosynthesis, still mainly hypothetical, has been deduced from studies of mutants blocked in tetracycline biosynthesis and from cosynthesis studies with *S. aureofaciens*. There are 72 intermediate products; 27 substances have been characterized but 45 have not yet been isolated and the chlorination reaction itself is not yet understood.

There is a clear correlation between tetracycline production and carbohydrate catabolism. High-yielding strains have a lower glycolysis rate than strains with low tetracycline production. The addition of 0.5–3 μg/ml benzylthiocyanate, an inhibitor of sugar uptake, causes a 50% increase in chlortetracycline production; simultaneously, the activity of the pentose-phosphate cycle increases. In the case of tetracycline biosynthesis, phosphate regulation is well understood. The addition of P_i to the producing culture decreases the rate of tetracyline formation by inhibition of ATC oxygenase. The rate of tetracycline synthesis is directly proportional to the activity of this enzyme, which is only detectable in the culture after phosphate has been completely depleted.

Strain development Studies on the genetics of *S. aureofaciens* have shown that over 300 genes are involved in the biosynthesis of chlortetracycline. Considering also the dearth of knowledge of bic synthesis and regulation in this pathway, it i understandable that a rational approach to strai improvement has not been possible. So far, a developments of higher-yielding strains hav come from empirical mutagenesis studies.

Ultraviolet radiation, alone or in combinatio with other mutagens, has brought about th greatest number of higher-yielding strains usin *S. aureofaciens* and *S. rimosus*. Table 13.14 show the effect of different mutagens on the variabilit of chlortetracycline production, and Figure 13.3 shows the increase in chlortetracyline productio with each step in the course of strain develop ment. Strains with increased tetracycline pro duction have been isolated by selecting reve tants of mutants blocked in antibiotic synthes and from prototrophic revertants of auxotroph They may also be isolated from mutants showin increased resistance to their own product. Th highest published yield for tetracycline is 25 g liter.

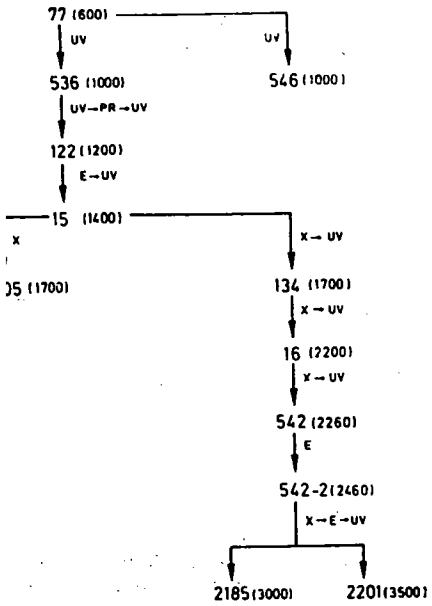
Hybridization has not resulted in strains wit increased product formation, but in combinatio with mutation it has caused an increase in strai variability. Protoplast fusion has been used increase yields.

A genetic map has been compiled for the wi type *S. rimosus* (ATCC 10970), as illustrated

Table 13.14 Spontaneous and induced variability of chlortetracycline production in *S. aureofaciens*

Mutagen[a]	Distribution of productivity (% of control)[b]		Frequency of strains with improved productivity (%)[c]
	Total range	Most frequent class	
—	40–110	80–100	5.3
Ultraviolet radiation	0–130	90–110	17.4
X-rays	0–110	70– 90	7.1
γ-Rays	0–110	0– 10	1.8
Nitrosoguanidine	0–120	40– 80	9.4
N-Mustard	0–110	80–100	5.8

[a] Research was conducted with conidia at death rates >99%. Ultraviolet 50 sec; X-rays and γ-rays 100 kR; N-methyl-N′-nitro-N-nitrosoguanidine 1 mg/ml, 60 min, 0.02 M phosphate buffer, pH 6.7; N-mustard 0.01 M, 30 min, 0.15 M phosphate buffer, pH 8.0.
[b] Productivity of initial strain = 100%.
[c] Percent of the tested isolates.
(Hoštalek et al., 1974)



gure 13.37 Genealogy of the chlortetracycline pro-
icer *S. aureofaciens*. UV, ultraviolet radiation; PR, pho-
reactivation; E, ethyleneimine treatment; X, X-rays. In
Chlortetracycline titers in μg/ml (From Hoštalek et al.,
)74)

gure 13.38. This map has some similarities to
ıat of *S. coelicolor*, the streptomycete best
ıown genetically. The genes for the first steps
f oxytetracycline biosynthesis are located be-
veen *ribB* and *cysD* in a narrow area of the *S.*
mosus chromosome. The genes for the steps
om anhydrotetracycline to oxytetracycline are
ıcated in a second cluster between *proA* and
deA. Location of the genes in clusters facilitates
enetic engineering research, and recombinant
)NA methodology has been developed for ox-
tetracycline-producing strains of *S. rimosus*. Al-
ıough plasmids of *S. rimosus* have been detected
hich function as fertility factors and which code
or self-resistance, the role of extrachromosomal
)NA in the regulation of tetracycline biosyn-
ıesis is not yet clear.

Production methods The production of tetracyclines is carried out in stirred fermenters with volumes up to 150,000 l. Figure 13.39 illustrates a typical process for **chlortetracycline** production. If glucose is used, continuous feeding is necessary; starch can be used as an additional carbon source. Because of phosphate repression, tetracycline fermentations are run with phosphate limitation.

For maximal yield, an optimal oxygen supply is important, particularly in the first hours (6–12 h) of the fermentation. Aeration rates of 1 vvm are customary; yield increases result when oxygen-enriched air is used.

There are several routes to **tetracycline**:

- By a chemical process using chlortetracycline.
- By fermentation in chloride-free culture medium, which can be made by pretreatment with ion exchangers.
- By adding chlorination inhibitors to the medium, such as mercaptobenzothiazole, 2-thiouracil, or thiourea. Bromide inhibits chlorination in some cultures, but causes formation of 7-bromtetracycline in other strains.
- With mutants which are blocked in the chlorination reaction.

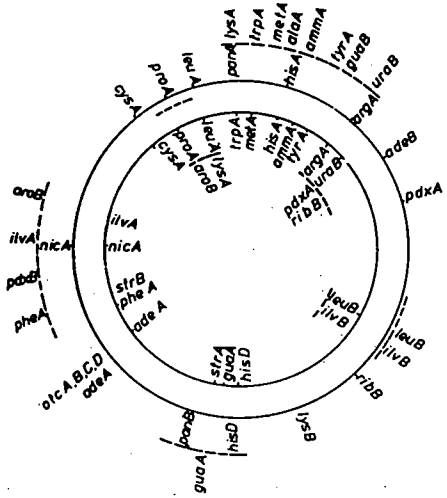


Figure 13.38 Outer circle, genetic map of *S. rimosus* R 7 (ATCC 10970). Inner circle, genetic map of *S. coelicolor*. The correct orders for the sites outside the dotted lines have not yet been determined (From Alačevič, 1976)

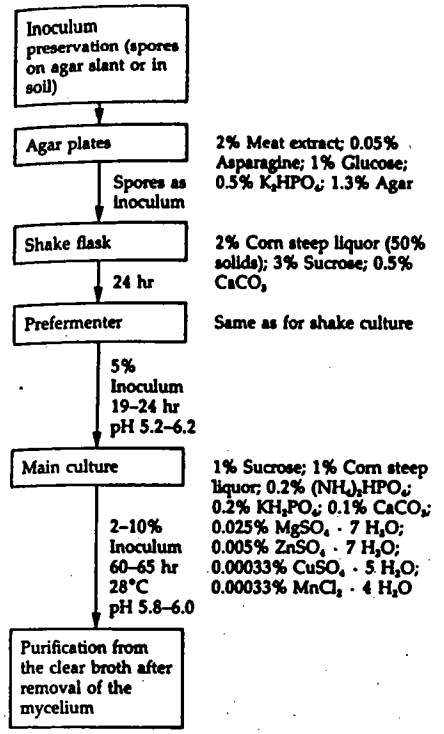


Figure 13.39 Production chart for chlortetracycline with *S. aureofaciens* (Perlman, 1979)

Anthracyclines

Anthracyclines are glycosides which consist of 7,8,9,10-tetrahydro-5,12-naphthacenequinone as an aglycone combined with 1–3 sugar residues. Anthracyclines are very **toxic compounds**. They intercalate into DNA and inhibit DNA-dependent DNA polymerase in DNA replication.

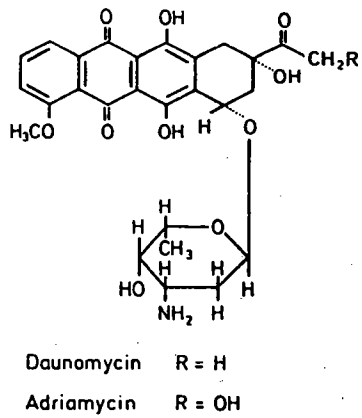


Figure 13.40 Anthracyclines. Structure of adriamycin and daunomycin

Daunomycin (daunorubicin) and **adriamycin** (doxorubicin), produced by *Streptomyces peucetius*, are used clinically in cancer treatment. The structure of both compounds is diagramed in Figure 13.40. Daunomycin acts against certain forms of leukemia; adriamycin, which has low toxicity, is effective in treating various tumors. However, these compounds have significant side effects and research is under way, using mutasynthesis and protoplast fusion as well as by the formation of chemical derivatives, to produce compounds with decreased problems.

13.7 NUCLEOSIDE ANTIBIOTICS

About 200 antibiotics with nucleoside-like structures are known. Because of the diversity of structures, marked differences in biological activity are seen. Aminoacyl nucleosides used as fungicide antibiotics in plant protection include **blasticidin S** from *Streptomyces griseochromogenes*, **mildiomycin**, from *Streptoverticillium rimofaciens*, and the **polyoxins** from *S. cacaoi* var. *asoensis*. Polyoxins (Figure 13.41 A) inhibit the

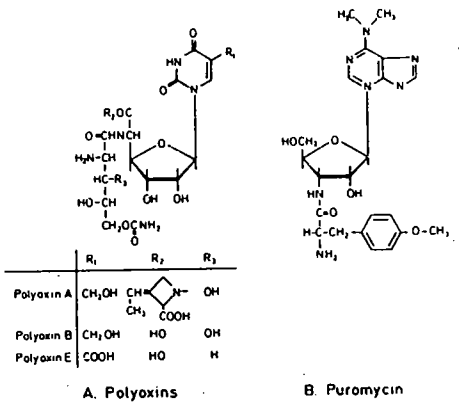


Figure 13.41 Nucleoside antibiotics

incorporation of N-acetyl-D-glucosamine into the chitin moiety of fungal cell walls by competitive inhibition of chitin synthase.

The dapiramines as well as nikkomycin, which also act as chitinase inhibitors, are undergoing field tests as possible herbicides. Several compounds with cytostatic or antiviral activity are under development; for example, neplanosin A from cultures of *Ampullariella regularis*.

Puromycin (Figure 13.39 B) from *S. alboniger* has found wide use as a tool in studying ribosomal function in protein biosynthesis but has no clinical significance. Puromycin resembles the aminoacyl end of transfer RNA. In both procaryotes and eucaryotes it binds to the A-site of the ribosome and is transferred to the growing polypeptide chain, thus causing a break in the polymerization process.

13.8 AROMATIC ANTIBIOTICS

This is a heterogenous group of antibiotics with aromatic rings in the molecule. The biosynthesis of individual compounds differs considerably. Three antibiotics of this group are produced commercially.

Chloramphenicol

Chloramphenicol (Figure 13.42) is produced by *Streptomyces venezuelae*, *S. phaeochromogenes* var. *chloromyceticus*, *S. omiyaensis*, and other streptomycetes. Chloramphenicol is a broad-spectrum antibiotic which acts on Gram-positive and Gram-negative bacteria, actinomycetes, rickettsias, and chlamydias. It causes significant **side effects**, particularly bone-marrow damage, but risk of toxicity can be reduced if therapy is conducted carefully. Because of its potential toxicity, chloramphenicol has not been as widely used as would be expected from its antimicrobial spectrum and it is presently considered a reserve antibiotic. Nevertheless, chloramphenicol is indispensible in the treatment of persistent *Salmonella* infections.

Chloramphenicol binds specifically to the 50S subunit of 70S ribosomes and blocks the peptidyl transferase reaction, causing a premature chain break. In molecular biology, chloramphenicol is used as a research tool to inhibit the translation process in procaryotes without affecting the synthesis of nucleic acids.

The pathway for chloramphenicol biosynthesis is via the aromatic pathway from chorismic acid. However, chloramphenicol has been produced by chemical synthesis since about 1950 and even improved fermentation processes have not succeeded in replacing this chemical process.

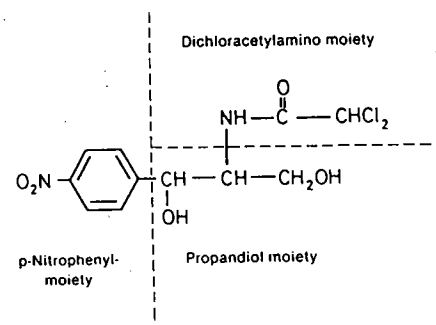


Figure 13.42 Chloramphenicol

Griseofulvin

Griseofulvin, a benzofuran derivative (Figure 13.43), is produced by *Penicillium griseofulvum*, *P. patulum*, and other species of *Penicillium*. Griseofulvin is a fungistatic antibiotic but acts only on fungi with chitinous cell walls. In humans, griseofulvin is administered orally for the treatment of fungal skin infections (dermatomycoses). Although the antibiotic has been used in plant pathology in the treatment of leaf diseases due to *Botrytis*, *Alternaria solani*, and species of mildew-causing fungi, it is currently not in wide use here because of the high cost (due to the high production costs). Griseofulvin affects the morphogenesis of many fungi and in concentrations of 10–1000 ppm causes a characteristic "curling" effect of the hyphae. The exact mechanism of action has not yet been determined, but mitosis and chitin biosynthesis are presumed to be affected.

The biosynthesis of griseofulvin occurs on a multi-enzyme complex. The antibiotic is produced from acetate, 6 molecules of malonate, and 2 methyl groups derived from methionine. An intermediate stage in the biosynthetic process is a 14-member polyketide, from which the antibiotic is produced via cyclization, chlorination, and methylation reactions.

The chemical synthesis of griseofulvin has been achieved, but production by fermentation with *P. patulum* is more cost-effective. Griseofulvin production is carried out as an aerobic submerged process in a glucose-rich medium. In some processes, glucose feeding is used. Starch, lactose, or sucrose can also be used as carbon sources and usually $NaNO_3$ is used as a nitrogen source. The addition of potassium chloride (0.1–0.3%) stimulates production. The fermentation take place at 25°C for 7–9 days within an optimal pH range between 6.8–7.2. High aeration (1 vvm O_2) is required when media with high carbon or nitrogen concentrations are used. The entire culture must be processed for recovery, since griseofulvin is only partially mycelium-bound.

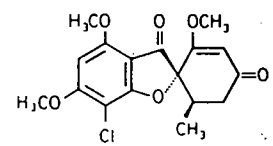


Figure 13.43 Griseofulvin

Novobiocin

Novobiocin is a coumarin glycoside (Figure 13.44), which can be isolated from culture filtrates of *Streptomyces spheroides*, *S. niveus*, *S. griseus*, or *S. griseoflavus*. Novobiocin is effective against Gram-positive bacteria, particularly staphylococci, against Gram-negative meningococci and gonococci, and also against *Haemophilus* and some *Proteus* strains. Novobiocin shows no cross resistance with other antibiotics, but organisms do develop rapid resistance, a marked disadvantage for its medical use. Novobicin acts as a specific inhibitor of DNA gyrase during DNA replication.

The biosynthesis of the coumarin moiety is via aromatic metabolism. L-Tyrosine is incorporated into ring A and B; ring C is derived from glucose. The fermentation is carried out in a medium with glucose or sucrose as a carbon source. Organic acids, such fumaric acid, can be added, and peptone and meat extract are favorable nitrogen sources. p-Aminosalicylic acid can be used as a precursor and addition of tyrosine and phenylalanine stimulate the formation of the antibiotic. Fermentations take place over about 90 h at 25–28°C and at a slightly alkaline pH range, with aeration rates of up to 1.5 vvm.

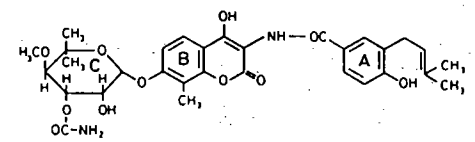


Figure 13.44 Novobiocin

13.9 OTHER COMMERCIALLY PRODUCED ANTIBIOTICS

Some antibiotics are discussed here which are used in therapy or have other uses. Their structural affiliation with certain classes of antibiotics was given in Table 13.2.

Fusidic acid

Among the steroid antibiotics, only fusidic acid (Figure 13.45) is therapeutically important. This compound, isolated from culture filtrates of *Fusidium coccineum*, inhibits the ribosomal translocation process during protein synthesis in both procaryotes and eucaryotes. Fusidic acid finds special use for the treatment of staphylococcal infections, especially wound infections and those of the skin.

Polyether antibiotics

Due to the numerous tetrahydropyran and tetrafuran moieties in these antibiotics, they are called polyethers. Over 150 different compounds are known. Their biosynthesis is derived from a polyketide derivative which is produced on a multienzyme complex. **Monensin** (Figure 13.46), commercially the most important compound of this group, consists of 5 acetate, 4 propionate and 3 butyrate units. Because of their ability to transport mono- or divalent cations through biological or artificial membranes, these antibiotics are classified with the **ionophores**.

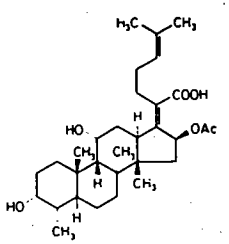


Figure 13.45 Fusidic acid

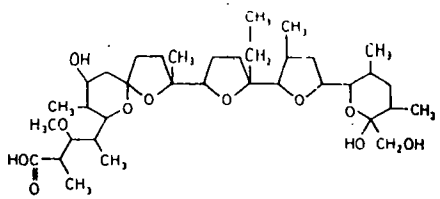
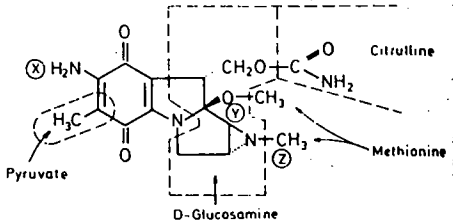


Figure 13.46 Monensin



	X	Y	Z
Mitomycin A	CH_3O	OCH_3	H
Mitomycin B	CH_3O	OH	CH_3
Mitomycin C	NH_2	OCH_3	H

Figure 13.47 Structure of mitomycins A–C and biochemical precursors of mitomycin C

Antibiotics in this group which are commercially prepared include monensin, produced by *Streptomyces cinnamonensis*, **lasalocide**, produced by *S. lasaliensis*, and **salinomycin**, produced by *S. albus*. Salinomycin yields of 60 g/l are attained on media containing a fatty acid. Polyethers are active against coccidia (protozoa), as well as Gram-positive bacteria, mycobacteria, and fungi. They are added to poultry feed (about 100 ppm) to prevent coccidioses. Monensin, which was first introduced in 1971, has about 80% of the market; current annual gross world sales are estimated at $100 million.

Mitomycins

The mitomycins, produced by *Streptomyces caespitosus*, are benzoquinones; Figure 13.47 gives their structure and biochemical origin. Erythrose and pyruvate have been detected as precursors of the methylbenzoquinone. Mitomycin C is very effective against tumors, but it is relatively toxic. Although this compound was first described in the late 1950's, it was not marketed until 1974. Mitomycin reacts with double-stranded DNA, producing cross-linkages which inhibit DNA replication. Although various derivatives have been produced with the aim of achieving reduced toxicity, success along these lines has not yet been achieved.

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14

Ergot alkaloids

14.1 OCCURRENCE AND SIGNIFICANCE

Alkaloids, some of which have therapeutic applications, are generally derived from plants, but the ergot alkaloids are produced by fungi. Ergot alkaloids were first obtained from the sclerotium of the parasitic ascomycete *Claviceps purpurea*, which develops on rye and other grasses. The term *ergot* is used to refer both to the fungus developing in the rye plant and to the alkaloids which are produced by the fungus. Two related groups of ergot alkaloids are known, those based on lysergic acid and those based on clavin (see later). Presently there are over 40 known ergot alkaloids produced by various *Claviceps* strains. Although tests have been conducted on over 1000 different species of various filamentous fungi, lysergic acid alkaloids have been found only in species of the genus *Claviceps*. However, clavine alkaloids were found in species of the genera *Aspergillus*, *Penicillium*, and *Rhizobium*, as well as in *Claviceps*. Several of the most important alkaloid producers are listed in Table 14.1. Ergot alkaloids have also been found in higher plants, but they seem to be limited to several species of the plant family Convolvulaceae.

Some **pharmacological properties** of ergot alkaloids include the stimulation of the sympathetic nervous system, an effect on the smooth muscles (uterus, artery walls), and antagonism to adrenalin and serotonin. Chronic ergot poisoning (ergotism), which used to occur frequently due to consumption of bread made from grain containing ergot alkaloid, results in cramps, vomiting, and gangrene, and can be fatal in severe cases. In the Middle Ages, ergotism was common in northern Europe, where it was called St. Anthony's Fire, but it has been eliminated by modern methods of grain cleaning.

Some lysergic acid alkaloids are used therapeutically in obstetrics, including ergotamine, ergobasine, and the semisynthetic methylergobasine, all of which act to stimulate labor and cause the uterus to contract. They are also employed during the period after birth to contract the post-

Table 14.1 Several producers of ergot alkaloids and the alkaloids produced by them

Organism	Plant host	Alkaloid content (%)	Alkaloids produced (main components)
Claviceps purpurea	Rye, wheat, barley and others	0.1 –0.5	Ergotoxine group: Ergocornine, Ergocryptine, Ergocristine; Ergotamine group: Ergotamine, Ergosine
C. fusiformis	*Pennisetum typhoides*	0.3	Ergometrine, Agroclavine, Elymoclavine, Chanoclavine
C. gigantea	*Zea mais*	0.03	Festuclavine, Pyroclavine, Dihydroelymoclavine, Chanoclavine
C. paspali	*Paspalum* spp.	0.003	D-Lysergic acid, α-Hydroxyethyl-lysergamide

(Mantle, 1975)

partum uterus. Hydrogenation of the $\Delta^{9,10}$-double bond in the D-ring reduces the contracting action on the smooth muscles; dihydroergotamine is thus used to treat migraine headaches. Also, a mixture of dihydro compounds from ergocristine, ergocryptine, and ergocornine is used for treatment of disturbances of the peripheral and central circulation system. Finally, a few naturally occurring alkaloids, particularly the semisynthetic compound lysergic acid diethylamide (LSD), are very effective **hallucinogens**.

14.2 DEVELOPMENTAL CYCLE OF *CLAVICEPS*

The *Claviceps* fungus lives as a parasite on grasses. During flowering of the host plant, the infection cycle begins. Hyphae from germinating ascospores or conidia grow into the plant ovary and within a week the ovary tissue is penetrated by a filamentous mycelium (sphacelia), which produces mononuclear conidia for asexual reproduction. No alkaloids are produced during this stage. About two weeks after infection, production of conidia stops, the filamentous mycelium is replaced with a plectenchymous, nonsporulating tissue, and the sclerotium appears. It is during this latter developmental phase that alkaloids are produced, usually as part of a mixture of different substances. The sclerotia survive until the host plant blooms again; hyphae grow out from the sclerotia and long-stalked stromata grow on the ends of the hyphae. The stromata contain perithecia with asci; the products of this sexual stage are 8 needle-shaped ascospores per ascus. The ascospores germinate on the plant and the cycle begins again, as illustrated in Figure 14.1.

14.3 STRUCTURE

The active ingredients of ergot were characterized in 1935 as derivatives of lysergic acid and the structural formulas were confirmed through synthesis in 1954. Ergot alkaloids are classified among the **indole alkaloids** and are derived from the tetracyclic ergoline ring system (Figure 14.2).

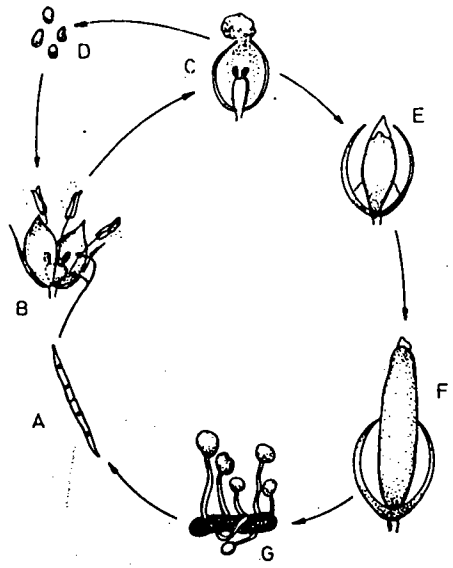
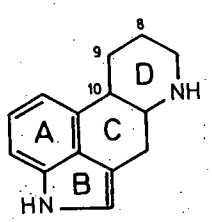


Figure 14.1 Development cycle of *Claviceps purpurea*. A, ascospore (1×100 μm); B, infection of grass flower; C, growth of filamentous mycelium in the ovary; D, asexual spores excreted with honey dew cause additional infections but no alkaloid formation; E, filamentous mycelium is replaced by plectenchymatic tissue; alkaloid formation is initiated; F, the sclerotium ripens within 2 months; G, sexual stage with perithecium formation (From Mantle, 1975)



Ergoline

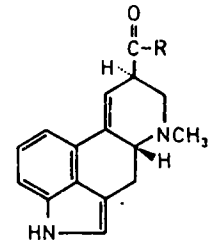
Figure 14.2 Structure of the ergoline ring system

Two main classes of ergot alkaloids have been recognized. The **clavine alkaloids** or **clavines** have ergoline as a basic structure, but contain no peptide components. Some substances of this group are intermediates in lysergic acid biosynthesis (see Figure 14.5). Various *Claviceps* species produce clavines, particularly in culture away from the plant, but traces of different clavines mixed with lysergic acid alkaloids have also been found in parasitic growth. Despite their pharmacological effect, clavines have not been used therapeutically.

The other class of alkaloids are the **lysergic acid alkaloids** or "Ergo" alkaloids. These are the classical alkaloids which are isolated from sclerotia. In these compounds, D-lysergic acid (or its stereoisomer D-isolysergic acid) is linked with a tricyclic peptide or an amino alcohol by an amide bond. The best known member of the latter group is ergometrin, which has 2-aminopropanol-(1) as a side chain. The structure of this compound, as well as the structures of several other of the simpler lysergic acid alkaloids, are shown in Figure 14.3.

Two groups of peptide alkaloids have been isolated, the classical **ergopeptins** and the more recently discovered **ergopeptams**. The ergopeptins consist of a lysergic acid molecule connected by a peptide bond to a tricyclic peptide which consists of an α-hydroxy-γ-amino acid derived from L-valine or L-alanine, with another amino acid and L-proline (Table 14.2; Figure 14.4). The first ergopeptams were only isolated in 1982. They are ergocristine, α-ergocryptine, and ergocornine. In these compounds, the tripeptide moiety, an acyclic lactam, is composed of two amino acids (which can be identical to the amino acids of the ergopeptins) and D-proline.

Lysergic acid and its derivatives epimerize easily at C-8, leading to the formation of the pharmacologically ineffective D-isolysergic acid compounds. They are distinguished by the ending "-inine", so that ergocristinine is the C-8 epimer of ergocristine.

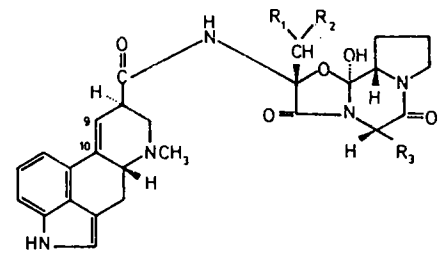


Name	R
Ergometrine (Ergobasine)	$-HN-C(CH_3)(CH_2OH)-H$
α-Hydroxyethylysergamide	$-HN-CH(CH_3)-OH$
Lysergic acid	$-OH$
$\Delta^{8,9}$-Lysergic acid[x]	$-OH$

Figure 14.3 Structure of several simply constructed lysergic acid alkaloids (x = double bond in the $\Delta^{8,9}$ position)

Table 14.2 Amino acid sequence of the tripeptide moiety in ergotoxine and ergotamine alkaloids (ergopeptins)

Alkaloid	Amino acid sequence of the tripeptide
Ergotoxine-alkaloids:	
Ergocristine	—L-Valine—L-Phenylalanine—L-Proline
Ergocornine	—L-Valine—L-Valine—L-Proline
α-Ergocryptine	—L-Valine—L-Leucine—L-Proline
β-Ergocryptine	—L-Valine—L-Isoleucine—L-Proline
Ergotamine-alkaloids:	
Ergotamine	—L-Alanine—L-Phenylalanine—L-Proline
Ergosine	—L-Alanine—L-Leucine—L-Proline



Name	R_1	R_2	R_3
Ergotamine	H	H	$CH_2-C_6H_5$
Ergosine	H	H	$CH_2CH(CH_3)_2$
Ergocristine	CH_3	CH_3	$CH_2-C_6H_5$
α-Ergocryptine	CH_3	CH_3	$CH_2CH(CH_3)_2$
β-Ergocryptine	CH_3	CH_3	$CH(CH_3)CH_2CH_3$
Ergocornine	CH_3	CH_3	$CH(CH_3)_2$
Ergostine	H	CH_3	$CH_2-C_6H_5$

Figure 14.4 Naturally occurring ergot alkaloids of the peptide type (ergopeptins)

14.4 BIOSYNTHESIS

Alkaloid synthesis occurs upon the endoplasmic reticulum in *Claviceps* when it is grown in submerged culture. About 95% of the clavines and the simple lysergic acid derivatives are excreted into the medium; the peptide alkaloids remain mostly mycelium-bound.

The formation of the ergoline ring proceeds from mevalonic acid and tryptophan, as outlined in Figure 14.5. In a series of reactions analogous to those involved in isoprenoid synthesis, mevalonic acid is transformed via mevalonic acid pyrophosphate and isopentenyl pyrophosphate

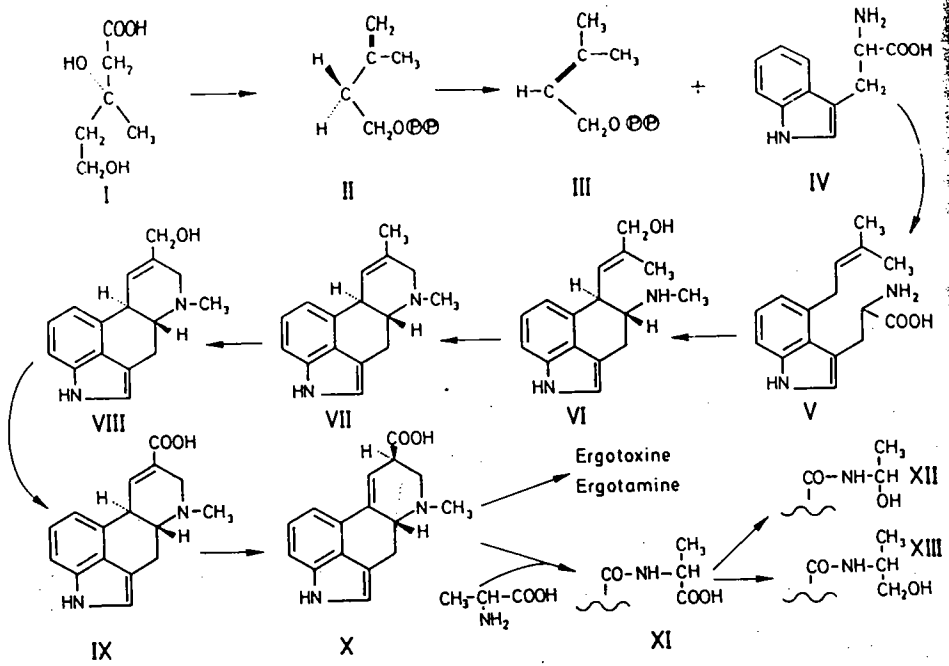


Figure 14.5 Biosynthesis of the ergoline ring system. I, Mevalonate; II, Isopentenylpyrophosphate; III, Dimethylallylpyrophosphate; IV, Tryptophan; V, 4-Dimethylallyltryptophan; VI, Chanoclavin-I; VII, Agroclavine; VIII, Elymoclavine; IX, $\Delta^{8,9}$-Lysergic acid; X, Lysergic acid; XI, Lysergylalanine; XII, α-Hydroxyethyllysergamide; XIII, Ergometrine

into dimethylallyl pyrophosphate. The first reaction specifically for alkaloid synthesis is the linkage of dimethylallyl pyrophosphate with L-tryptophan (in position C-4), tryptophan being converted into 4-dimethylallyl tryptophan. In the next intermediate step, chanoclavin-I, a clavine with an open D-ring, is produced. The methyl group on the nitrogen in position 6 is derived from methionine. The ring closes after oxidation to chanoclavin-I-aldehyde and the tetracyclic compound agroclavine is formed. Hydroxylation of the methyl group of agroclavine to form elymoclavine is carried out by a peroxidase or oxygenase in the presence of cytochrome P-450. The further transformation from elymoclavine into derivatives of lysergic acid occurs via $\Delta^{8,9}$-lysergic acid, which in contrast to lysergic acid occurs freely in relatively high concentrations in the medium.

Biosynthesis of tripeptide moieties of the peptide alkaloids is not fully understood. Attempts to incorporate ^{14}C-labeled precursors showed that lysergic acid and various amino acids are incorporated as single compounds. From results of experiments with inhibitors, ergopeptin formation is postulated to take place on a multienzyme complex, analogous to the biosynthesis of peptide antibiotics (see Section 13.3). The proposed biosynthetic pathway, which begins with the linkage of L-proline to the enzyme, is illustrated in Figure 14.6. It has been possible to obtain incorporation of the appropriate amino acids

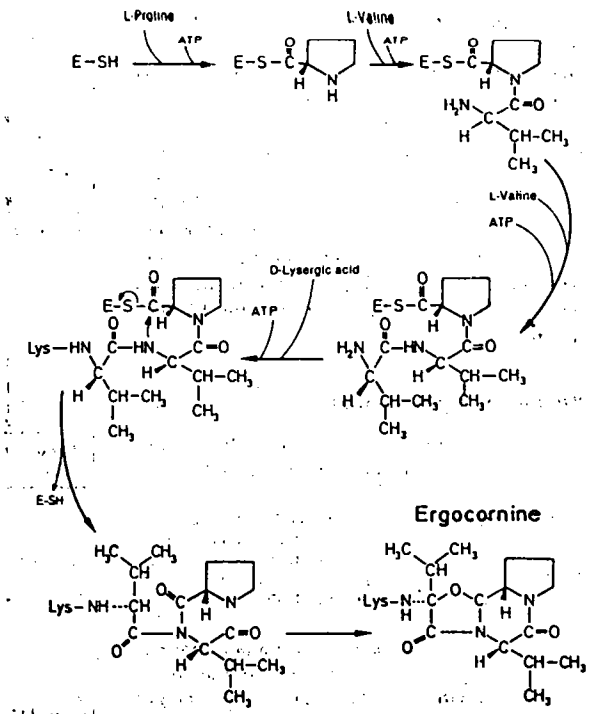


Figure 14.6 Hypothetical scheme for the formation of the peptide side chain of ergopeptines on a multi-enzyme complex (From Floss, 1976)

into the respective ergopeptin in a cell-free system in ergosine or ergotamine-producing strains of *Claviceps purpurea*, and experiments are underway to characterize the "cyclol-synthetase complex" which is involved. It is likely that ergopeptams are formed by irreversible epimerization as intermediate products before ring closure.

14.5 PRODUCTION OF ERGOT ALKALOIDS

Annual world production of peptide alkaloids was 4000 kg in 1976, and lysergic acid production was 12,000 kg.

There are a number of methods of obtaining ergot alkaloids: by **chemical synthesis**, by **culture** of *Claviceps* strains on the respective host plants, by **microbial fermentation**, either in surface culture or with immobilized cells. Culture on the host plant or in submerged culture are the processes most used commercially.

Chemical synthesis

Total chemical synthesis of ergot alkaloids is possible, but currently this is not cost-effective. Lysergic acid which is produced fermentatively can be chemically transformed into the desired alkaloids. Ergoline derivatives used medically are produced commercially using this method, with the exception of peptide alkaloids.

Obtaining alkaloids from sclerotia

In 1976, more than 95% of the peptide alkaloids were prepared by extraction from sclerotia,

chiefly in Switzerland and eastern European countries. In this process, rye flowers are mechanically inoculated with conidia. The fungus grows in the ovary tissue and forms conidia which then serve as the inoculum for an extensive plant infection. About 200–500 kg of sclerotia can be harvested from 1 hectare (2.47 acres) of rye. Disadvantages of this process are that the harvest is only possible once a year and the extent of infection is extremely dependent on the weather. Since 1978, this production method has been replaced in Switzerland with the fermentation method.

Production by fermentation

Three species of *Claviceps* are currently used in the production of alkaloids by fermentation:

Claviceps paspali	α-Hydroxyethyl lysergamide, $\Delta^{8,9}$-Lysergic acid, ergometrine
C. fusiformis	Clavines (chiefly agroclavine)
C. purpurea	Ergotamine, ergosine, ergocornine, ergocristine, ergocryptines

Research with *C. paspali* at the Italian pharmaceutical company Farmitalia led to the initial breakthrough in fermentative production of ergot alkaloids. Whereas the initial isolate grown in submerged culture produced about 20 mg alkaloid/l, strain development and culture medium optimization resulted in a commercial process which had alkaloid titers of 5 g/l, with α-hydroxyethyl lysergic amide as the main product.

The stages of the fermentation process are outlined in Figure 14.7. After hydrolytic splitting, D-lysergic acid can be used in the production of semisynthetic alkaloids. Chemical conversion to the peptide alkaloids has also been accomplished.

A second simplified process for the production of lysergic acid has been developed by the Swiss company, Sandoz. An isolate of *C. paspali* produces high yields of $\Delta^{8,9}$-lysergic acid which can be rearranged by isomerization into D-lysergic acid (with the double bond in the $\Delta^{9,10}$ position). Production of the alkaloid agroclavine is possible with *C. fusiformis*, which in semicontinuous culture can achieve yields of 6g/l.

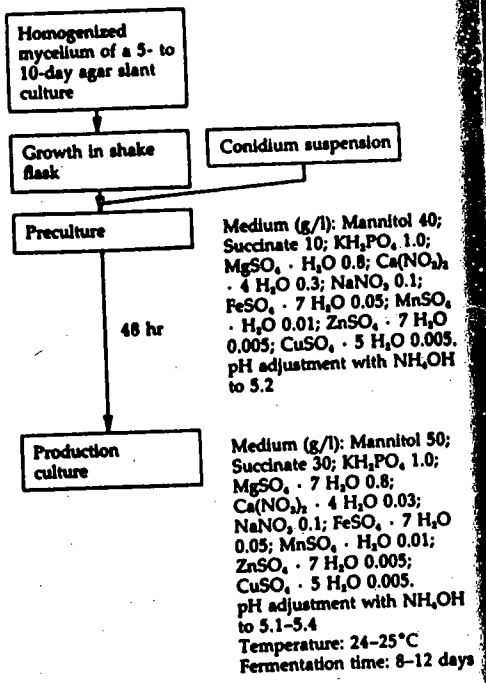


Figure 14.7 Flow scheme for the production of α-hydroxyethyl lysergamide with *C. paspali* (From Kelleher, 1970)

Processes for the production of therapeutically important peptide alkaloids have also been developed. Table 14.3 lists commercially produced compounds.

Alkaloid composition is affected by the strain used and the culture medium, as shown in Table 14.4. In *C. purpurea* the relative proportions of ergocornine and ergocryptine can be altered from 2:1 to the more desirable 1:1 by addition of L-valine to the fermentation medium. Good alkaloid formation seems to be linked to the ability to metabolize high sucrose and citrate concentrations, but at the same time, the medium must not contain any phosphate. Synthesis proceeds in parallel with lipid and sterol biosynthesis. The progress of a typical fermentation is illustrated in Figure 14.8 for *C. purpurea*.

Table 14.3 Production of ergot alkaloids by fermentation

Alkaloid	Manufacturers 1	2	3	4
Ergocornine	+	+	+	+
Ergocristine	+	+		+
Ergocryptine	+	+	+	+
Ergotamine		+	+	
Ergometrine			+	
Lysergic acid		+		
$\Delta^{8,9}$-Lysergic acid	+			+

Manufacturers: 1. Biochemie GmbH, Kundl (Austria); 2. Farmitalia S.p.A. (Italy); 3. Gedeon Richter (Hungary); 4. Sandoz-Wander AG (Switzerland).
(Rehacek, 1980)

Scale-up has been a problem, since the production strains are sensitive to the shearing action of the impeller but at the same time exhibit a high oxygen requirement. Maintenance of sterility is also difficult for long fermentation periods. Very slight alterations in the composition of the medium or the addition of antifoam agents usually cause lower yields. In addition, the degeneration of high-yielding strains frequently occurs, so that a careful strain maintenance program is necessary.

Surface culture

A process has been described for the large-scale production of ergot alkaloids using surface cultures of *C. purpurea* grown under sterile conditions. The advantage of this process is that a higher proportion of the more desirable ergotamine and ergotoxine alkaloids are formed.

Semicontinuous transformation with immobilized mycelium

A process has been developed for semicontinuous alkaloid production using immobilized mycelium of *Claviceps purpurea* and *C. fusiformis*. In *C. purpurea* CBS 164.59, which forms ergometrin and a mixture of chanoclavin, agroclavin and elymoclavin, the best results have been obtained by immobilization in 4% calcium alginate gel. At concentrations of alginate up to 8%, the overall yield of alkaloids was increased by 35%, but because of the reduced O_2 diffusion into the alginate beads, the main product was agroclavin. To prevent contamination problems during the long incubation period with the immobilized mycelium (200–400 days), an antibiotic such as chloramphenicol was added.

14.6 REGULATION OF ALKALOID PRODUCTION IN CULTURES

To produce alkaloids, the medium must always contain an organic acid of the TCA cycle or a related compound as well as a carbohydrate. Mannitol and succinate serve for the production of lysergic acid, and sucrose and citrate for the

Table 14.4 Ergot alkaloid production by various strains of *C. purpurea*

Characteristics	275 FI	FI 32/17	FI 43/14	FI S 40
Alkaloid produced	Ergotamine	Ergocryptine Ergotamine	Ergocornine Ergosine	Ergocristine
Alkaloid yield (mg/l)	1150	1800	1000–1100	1000–1100
Culture medium	T 25[a]	T 25[a]	T 25[a]	TS[b]
Sucrose consumed (g/l)	160	75	145	100
Citric acid consumed (g/l)	14	15	6	
Lipid production (mg/l)	14	24	47	18.5
Sterol production (mg/l)	316	444	393	510
Fermentation time (days)	14	16	12–14	8–10

[a] Medium T 25 (g/l): Sucrose 300; citric acid 15; KH_2PO_4 0.5; $MgSO_4 \cdot 7 H_2O$ 0.5; KCl 0.12; $FeSO_4 \cdot 7 H_2O$ 0.007; $ZnSO_4 \cdot 7 H_2O$ 0.006; yeast extract 0.1. Tap water; pH adjusted to 5.2 with NH_4OH. Sterilization at 120°C, 20 min.
[b] Medium TS (g/l): Sucrose 100; asparagine 10; KH_2PO_4 0.5; $MgSO_4 \cdot 7 H_2O$ 0.3; $FeSO_4 \cdot 7 H_2O$ 0.007; $ZnSO_4 \cdot 7 H_2O$ 0.006; yeast extract 0.1. Distilled H_2O; pH adjusted to 5.2 with NaOH. Sterilization at 110°C, 20 min.
(Amici et al., 1969)

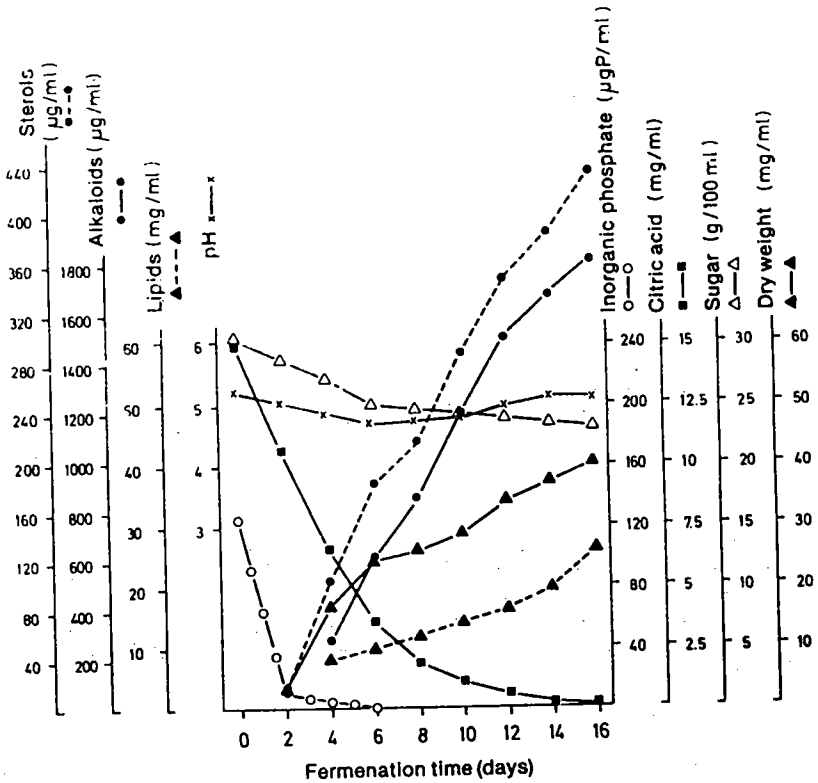


Figure 14.8 Fermentation kinetics of the ergocryptine/ergotamine producer *C. purpurea* FI 32/17 in medium T 25 (composition in Table 14.4)

production of ergotamine or ergocristine. Both carbon sources are metabolized simultaneously without any evidence of a diauxy effect, although glucose itself represses alkaloid production.

Ergot alkaloid production exhibits a typical **phosphate regulation**, as shown in Figure 14.9. In the trophophase (3–4 days), after free phosphate has been used up growth ceases and the culture enters the idiophase. Tryptophan, which induces alkaloid synthesis and simultaneously serves as a precursor, accumulates. At the same time, the inducible enzymes of secondary metabolism appear, such as 4-dimethylallyl tryptophan synthase, which is induced by tryptophan or trytophan analogs (such as 5-methyltryptophan). The phosphate inhibition mentioned above can be counteracted by the addition of tryptophan. During the transition from the trophophase to the idiophase, another key enzyme of secondary metabolism, chanoclavin-I-cyclase, appears.

Measurements of the enzyme activities of intermediary metabolism have indicated the predominant role of the TCA cycle at the beginning

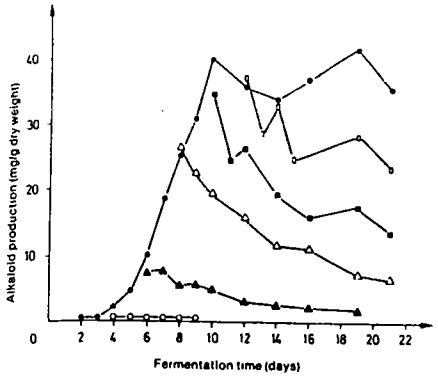


Figure 14.9 Clavine formation with *Claviceps* SD 58 after a single addition of phosphate (concentration in the medium: 1.1 g/l KH_2PO_4) at different times: • — • control; ○ — ○ addition after 3 days; ▲ — ▲ addition after 5 days; △ — △ addition after 7 days; ■ — ■ addition after nine days; and □ — □ addition after 11 days (From Robbers et al., 1978)

of the submerged fermentation process. In contrast, the glyoxylate cycle prevails during the alkaloid production phase. Acetyl-CoA can be produced via an anaplerotic sequence (glycerate pathway) of the dicarboxylic acid cycle. The activity of phosphofructokinase, a key enzyme of glycolysis, is reduced. The changeover from the TCA to the glyoxylate cycle is stimulated by the addition of citrate, succinate, or malate. Moreover, excess citrate causes inhibition of citrate synthase, making acetyl-CoA available for conversion to mevalonic acid, a key precursor of the ergoline ring system. Increased amounts of acetyl-CoA flow to fatty acid biosynthesis during activation of acetyl-CoA carboxylase, thus explaining the parallelism of lipid and alkaloid production. Since the enzymes of the pentose phosphate cycle are derepressed, a sufficient quantity of $NADPH_2$ is produced, which is necessary for lipid synthesis.

In submerged culture, synthesis of the alkaloids is increased considerably when asparagine is used as a **nitrogen source** instead of ammonium salts. Measurements during the growth phase showed there was an increase in asparaginase activity at the same time that an accumulation of ammonium ions occurred. The initiation of alkaloid formation is coupled with a significant decrease in the intracellular ammonium concentration brought about by ammonium assimilation via glutamine synthetase. Since glutamine is the amino group donor, this lead to an increase in the intracellular concentration of tryptophan, an ergolin precursor.

14.7 STRAIN DEVELOPMENT

In nature the production of ergot alkaloids is linked exclusively to the sexual phase of the developmental cycle, which does not occur in vitro. In submerged culture, the alkaloid-producing mycelium has morphological similarities with the hyphae in sclerotia. These thick-walled, isodiametric cells have a high fatty acid content. The composition of the culture medium affects the differentiation into sclerotic cell types. Increasing the sucrose concentration from 10 to 30% or adding citrate, $(NH_4)_2SO_4$, or high Ca^{2+} concentrations promotes the formation of the sclerotial cell types. Most alkaloid-producing strains are homocaryotic. In several strains that produce primarily peptide alkaloids, heterocaryosis leads to an increase in product formation. **Plasmids** have been demonstrated so far only in wild type strains. These plasmids are linear genetic elements (5.6–6.3 kb) which are present in free form in the mitochondria.

A frequently occurring problem in strain preservation is the **degeneration** of high-yielding strains. The proportion of unproductive variants after a single transfer was 3% in an ergotoxine-producing *C. purpurea* strain, but after 7 passages it was 78%. Thus, strains must be carefully maintained in storage (mycelium fragments or conidia in nutrient solution containing 10–20% glycerol can be stored in liquid nitrogen). Occasional reisolation of high-yielding variants may also be necessary in order to guarantee alkaloid production.

Yield increases have been achieved in genetic research primarily by use of mutation and selection. Conidia, mycelium fragments, or protoplasts have been treated with mutagens, such as ethyl methanesulfonate, nitrosoguanidine, nitrous acid, ethylenimine, or ultraviolet radiation. Such studies have led in every case to increased yields: for example, a 160-fold increase in an ergocristine producer. Mutagenesis has resulted primarily in changes in the amount of alkaloids produced rather than in the proportion of the various products. However, in *C. purpurea* shifts in the proportions of ergotamine to ergotoxine, ergocornine/ergocryptine to ergocrystine, or ergotamine to ergosine. Due to the absence of the sexual phase in vitro, for a long time there was no means of performing genetic analysis or using genetic recombination for strain development, but **protoplast fusion** has overcome this difficulty. Fusion of an ergocristine producer with an ergocornine/ergocryptine producer resulted in strains which appeared to be hybrids, as judged by their nutrient requirements (citrate or succinate) and by the amount of alkaloids produced, which ranged between the values of the parent strains.

Experiments with **mutasynthesis** (see Section 3.6) using *C. purpurea* show promise of obtaining new pharmacologically useful alkaloids. Auxotrophs of an ergocristine-producing strain (Phe^-) and an ergocornine/ergocryptine-producing strain (Leu^-) produced the appropriate alkaloid analogs after the addition of phenylalanine analogs (p-chlorophenylalanine, p-fluorophenylalanine) or leucine analogs (norleucine, norvaline). By feeding synthetic amino acid analogs it has been possible to exchange both of the other amino acids of the tripeptide moiety.

For the development of a **cloning system**, mitochondrial (mt) DNA from *C. purpurea* has been used as a vector. Chromosomal DNA of *C. purpurea* containing the gene for the phosphoribosylanthranilate isomerase, has been cloned in *E. coli*. This raises the possibility of using protein engineering to increase the action of the rate-limiting enzymes in the biosynthetic sequence. It can be anticipated that these new genetic techniques may lead not only to further increases in yield but to the discovery of new structural variants of the ergot alkaloids.

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15

Microbial transformations

15.1 INTRODUCTION

Microorganisms have the ability to chemically modify a wide variety of organic compounds. Such changes are called **biological** or **microbial transformations**, or more generally **bioconversions**. In these enzymatic reactions, the substrate may be metabolized, but in some cases the conversion may take place without energy gain (co-metabolism). In general, an industrial process can be implemented either by chemical synthesis or by bioconversion, but bioconversion is often preferable for the following reasons:

- Substrate specificity: Only one specific reaction step is normally catalyzed by an enzyme.
- Site specificity (regiospecificity): If several functional groups of one type are present in the molecule, only one specific position may be affected.
- Stereoselectivity: If a racemic mixture is used as starting material, only one specific enantiomer is converted. If a center of asymmetry appears as a result of the enzyme reaction, the reaction product is normally optically active.
- Reaction conditions: Enzymatic reactions do not cause destruction of sensitive substrates, due to the mild conditions of conversion. Several reactions can be combined, either in one fermentation step using an organism with suitable enzyme systems, or by step-wise conversions using different microorganisms. The reactions cause less environmental hazard, as they take place chiefly in water.

15.2 TYPES OF BIOCONVERSION REACTIONS

The most important microbial transformation reactions are outlined in Table 15.1. Chemicallly, these transformations can be grouped under the following categories: oxidation, reduction, hydrolysis, condensation, isomerization, formation of new C—C bonds, introduction of hetero functions. Oxidation reactions are particularly useful

Table 15.1 Bioconversion reactions of various types

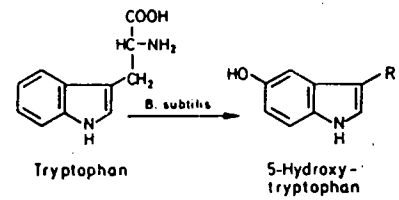
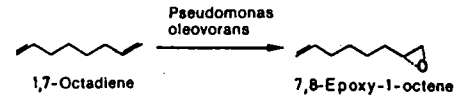
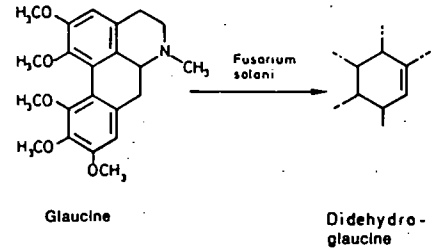
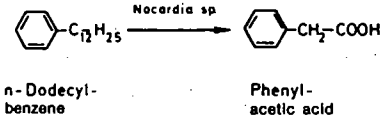
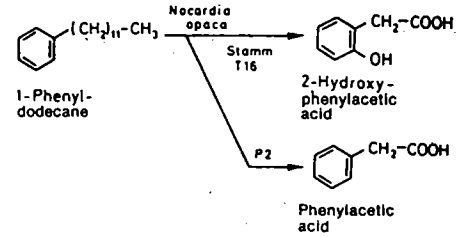
Type of reaction	Example	Conversion efficiency (%)
OXIDATIONS		
Hydroxylation		100
Epoxidation		~25
Dehydration of -CH–CH-		60
Oxidation of aliphatic side chains with the formation of aldehydes, ketones or carboxyl functions		80
Oxidative breakdown of alkyl side chains		? 67–85

Table 15.1 (continued)

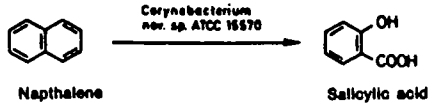
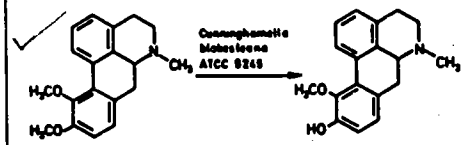
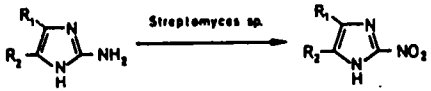
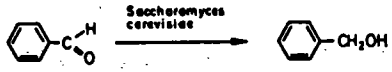
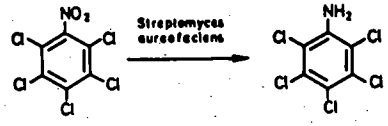
Type of reaction	Example	Conversion efficiency (%)
Oxidative splitting of aromatic rings	 Napthalene → Salicylic acid	70
Oxidative splitting of substituents (oxidative deamination, N–CH_3-demethylation, O–CH_3-demethylation)	 10,11-Dimethoxy-aporphine → Isoapocodeine	100
Oxidation of heterofunctions (amino groups to nitro groups; formation of N-oxides and sulfoxides)	 2-Amino-4-alkyl-imidazole → 2 Nitro-4-alkyl-imidazole	
	R_1 = H; R_2 = H	50
	$-CH_3$ H	25
	$-C_2H_5$ H	36
REDUCTIONS		
Reduction of carboxyl functions	 Benzaldehyde → Benzyl alcohol	50
Reduction of heterofunctions (particularly $-NO_2$)	 Nitropenta-chlorbenzol → Pentachlor-aniline	?

Table 15.1 (continued)

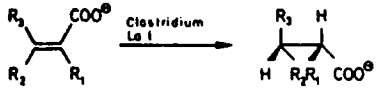
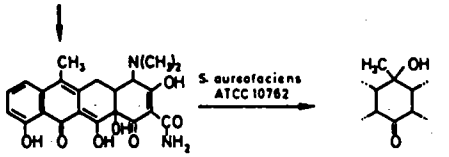
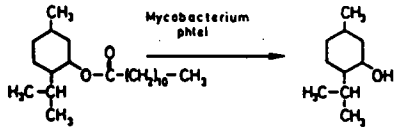
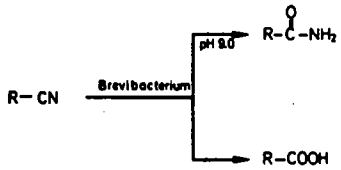
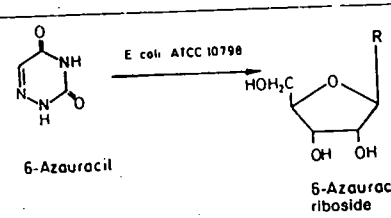
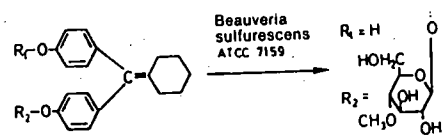
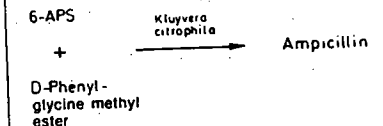
Type of reaction	Example	Conversion efficiency (%)
Hydration of carbon-carbon double bonds	 α,β-unsaturated carboxylic acids	?
HYDROLYTIC REACTIONS		
Hydration of carbon-carbon double bonds	 Anhydrotetracycline → Tetracycline	55
Hydrolysis of carboxylic acid esters	 d,l-Menthyl laureate → l-Menthol	?
Hydrolysis of N-derivatives	 R–CN → R–C(=O)–NH_2	?
	R–CN → R–COOH	?
CONDENSATIONS		
Phosphorylation	Streptomycin $\xrightarrow{\text{S. griseus}}$ Streptomycin-P	?

Table 15.1 (continued)

Type of reaction	Example	Conversion efficiency (%)
N-Glycosidation		16
O-Glycosidation		60
N-Acylation		63

? = data unavailable or substances in mixture not isolated.

in industrial production. To a lesser extent, isomerization, reduction, hydrolysis, and condensation also have industrial application.

15.3 PROCEDURES FOR BIOTRANSFORMATION

Spores, growing cultures, resting cells, enzymes, immobilized cells, or immobilized enzyme systems can be used in the microbial conversion of organic compounds.

In processes with **growing cultures**, the strain used is cultivated in a suitable medium and a concentrated substrate solution is added after suitable growth of the culture (6–24 h). A variant of this procedure is to use a very large inoculum and to add the concentrated substrate immediately without allowing for a growth period. Emulsifiers such as Tween or water-miscible solvents with low toxicity (ethanol, acetone, dimethyl formamide, dimethyl sulfoxide) may be used to help solubilize poorly soluble compounds. Steroid conversions, in which the low solubility limits the amount of substrate which can be added, are commonly carried out at substrate concentrations between 0.1–10 g/l medium, although in some cases, up to 30 g/l can be converted. Solvent concentrations between 5 and 15 ml per liter of medium can be used. In some steroid transformations, the substrate is

added and converted in fine crystalline form. These so-called pseudo-crystalline fermentations can be carried out with relatively high concentrations of substrate (for example, 15–50 g/l with progesterone).

For the biotransformation of lipophilic materials it is possible to employ a polyphase system. The aqueous phase containing the cell material or the enzyme is overlayed with a water-immiscible fluid phase in which the substrate has been dissolved. The substrate passes slowly into the aqueous phase and as the transformation reaction proceeds, the product passes back into the solvent phase. In some cases, the actual transformation occurs at the interface of the aqueous and solvent phases.

Conversion time is related to the type of reaction, the substrate concentration, and the microorganism used. Oxidation and dehydration reactions using bacteria are often completed in a few hours; conversions with yeast and especially fungi can take several days. Hydrolysis reactions with most kinds of microorganisms can be accomplished in a few hours.

Transformation reactions in large-scale equipment are carried out under sterile conditions in aerated and stirred fermenters, the conversion process being monitored chromatographically or spectroscopically. The process is terminated when a maximal titer is reached. Sterility is necessary because contamination can suppress the desired reaction, induce the formation of faulty conversion products, or cause total substrate breakdown.

If enzyme induction by the added substrate is not necessary, **resting cells** may be used. This has the considerable advantage that growth inhibition by the substrate is eliminated. High cell densities, which promote increased productivity, may be used; at the same time, risk of contamination is reduced. Since the transformation reaction occurs predominantly in the buffer solution, the recovery of the product is relatively easy. A number of transformation processes employ **immobilized cells**, offering the advantage that the process can be carried out continuously and the cells can be used over and over again. Immobilized bacterial cells, which catalyze one stage or multi-stage reactions, are presently used commercially in the production of aspartic acid, L-alanine, and malic acid.

Cell-free enzyme extracts, generally involving the use of **immobilized enzymes** (see Section 11.11), are usually employed in biotransformation reactions when undesirable side reactions or further breakdown of the reaction products must be avoided or when the rate of the reaction is hindered by transport of substrate or product through the cell membrane. The use of carrier-bound enzymes also makes possible the development of continuous processes. We discussed in Chapter 11 the use of immobilized enzymes for several processes which could be considered biotransformations, for example penicillin acylase, glucoamylase, and glucose isomerase. A pilot-scale process has been developed for the synthesis of amino acids by the reductive amination of α-keto acids by amino acid dehydrogenases. This system permits the regeneration of the required NADH cofactor.

The end products of transformation reactions are usually extracellular and may occur in either dissolved or suspended form. For further processing, bacteria and yeasts are generally not separated, whereas fungal mycelium is usually removed by filtration. In all cases, separated cell material must be washed repeatedly with water or organic solvents since a significant amount of the reaction product can be adsorbed to the cells. Depending on the solubility of the product, recovery is performed by precipitation as the calcium salt, by adsorption to ion exchangers, by extraction with appropriate solvents, or, for volatile substances, by direct distillation from the medium.

15.4 APPLICATIONS OF BIOCONVERSIONS

Although a vast array of biotransformations have been described, only a few of these processes have found industrial application. Some pro-

esses have insufficient yields and for others the market is too limited. In the future, a wide range of applications is expected to arise in connection with new technology, such as the more cost-effective processes using immobilized cells or enzymes. Other improvements are expected with the use of strains which have been genetically optimized for specific processes.

15.5 TRANSFORMATION OF STEROIDS AND STEROLS

Naturally occurring steroids have **hormone properties**. Examples are the adrenal cortex hormones (glucocorticoids and mineral corticoids), androgens, estrogens, and hormones active during pregnancy, such as progesterone. All steroids have the same basic structure, a cyclopentanoperhydrophenanthrene. Figure 15.1 shows the structure of the most important types. Estrogens, progesterone, and androgens are used therapeutically; derivatives of progesterone and estrogens are also used as contraceptives. In addition, steroids are used as sedatives, in antitumor therapy, and as veterinary products. The glucocorticoids are valuable compounds with wide therapeutic uses. Cortisone is especially useful because of its anti-inflammatory action in such conditions as rheumatoid arthritis and skin diseases. By altering the structure, specifically by introducing a 1,2 double bond in ring A of the cortisol or cortisone molecule to produce prednisolone or prednisone, substances can be produced with markedly increased anti-inflammatory effect. Addition of fluorine and methyl groups leads to the formation of compounds with reduced mineral corticoid activity (Na^+ retention, K^+ excretion), such as 16α-hydroxy-9α-fluoroprednisolone (triamcinolone) or 6α-methylprednisolone (medrol). By means of transformation, anabolic steroids with reduced androgenic effects have been developed, such as 1-methyl-Δ^1-androstenolone.

At the present, the available steroids serve most of the medical requirements quite well so that further development of new steroid transformation processes is limited. Research is now primarily directed at the optimization of the existing processes, primarily by use of immobilized cells or enzymes or by the optimization of the reaction system, such as by the use of polyphase systems. Further optimization work is in the direction of finding better starting materials or reducing the degradative side reactions. Genetic engineering research on the microorganisms used for steroid transformations is also under investigation. Additionally, studies are under way on the use of plant cell cultures for steroid transformations.

Types of transformations

Because the steroid molecule contains several asymmetric centers, **total synthesis** is very difficult. The original chemical process involved 31 separate reaction steps and yielded 1 g cortisone acetate from 615 g deoxycholic acid. More recent chemical processes are simpler and deoxycholic acid (from ox bile) is presently used as substrate in several production processes.

Preliminary research on the 11α-hydroxylation of progesterone pointed to the possibility of the microbial introduction of oxygen into the steroid nucleus in a site-specific and stereospecific manner without prior activation. These reactions worked well, and cost-effective production of cortisone became possible. The oxygen atom at C-11 is essential for the anti-inflammatory effect of cortisone. In 1949, 1 g of cortisone cost $200 to produce, but as a result of the introduction of the microbial process for the 11-α-hydroxylation of progesterone, the cost had decreased to under $1 by 1979. Currently, almost all positions of the steroid molecule can be specifically hydroxylated by different microorganisms and the number of transformation reactions is larger than the number carried out by animal tissue (Figure 15.2). The microbial hydroxylation reactions are carried out by highly specific monooxygenases; some examples are an 11α- or 11β-hydroxylase, a 17α-hydroxylase and a 21-hydroxylase.

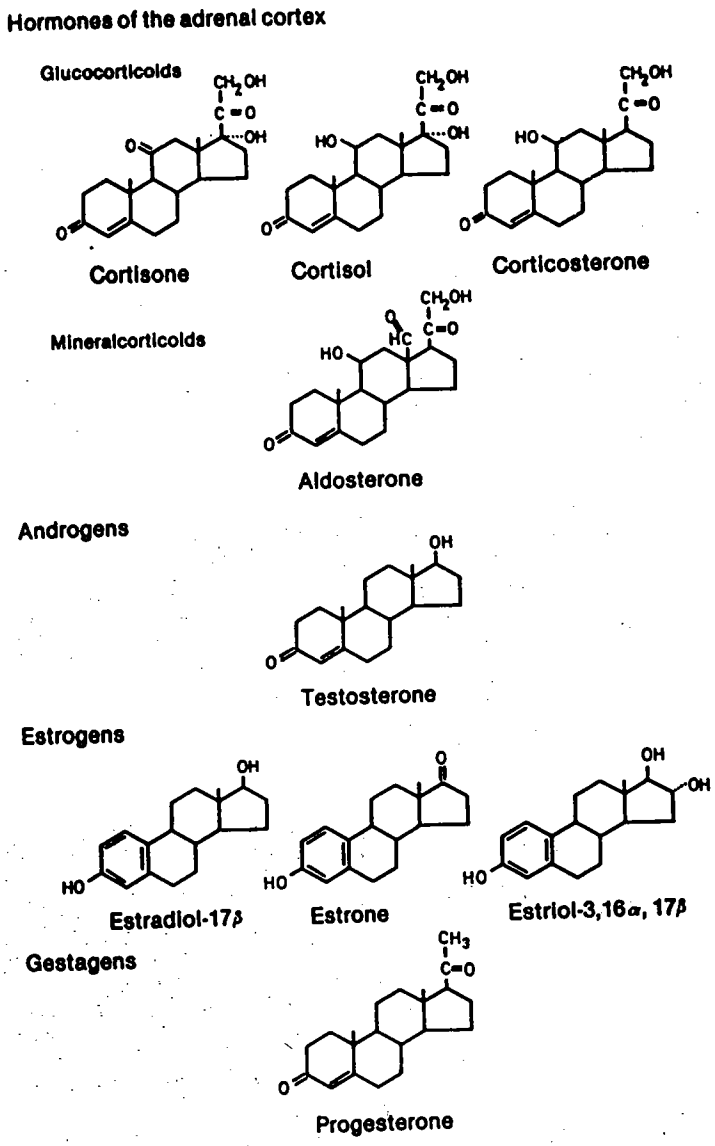
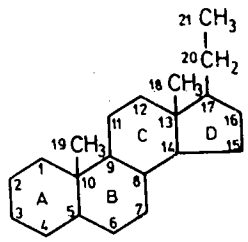


Figure 15.1 Structure of several naturally occurring steroid hormones



5β-Pregnane

Microorganisms			Animal tissue	
1α	7α	15α	1α	15α
1β	7β	15β	2α	16α
2α	9α	16α	2β	17α
2β	10β	16β	6α	18
3β	11α	17α	6β	19
5α	11β	18	7α	21
5β	12α	19	11β	
6β	12β	21	12α	
	14α			

Figure 15.2 Steroid hydroxylation by microorganisms as contrasted with the process in animal tissue

As outlined in Table 15.2, microorganisms are capable of carrying out a wide variety of other steroid transformation reactions besides hydroxylation.

Economically important transformations

In recent decades, thousands of modified steroids produced by a combination of chemical and microbial reaction steps have been tested for their therapeutic effectiveness. A typical example of such a combined synthesis is the production of cortisone and its 1-dehydro-derivatives from diosgenin via Reichstein's Substance S (11-deoxycortisol), as shown in Figure 15.3.

The microbial reaction steps listed in Table 15.3 are of great economic significance. Moreover, progesterone transformation to a C_{19}-steroid is used industrially in testosterone and estrogen production and the microbial dehydration

Table 15.2 Reaction types of microbial transformations from steroids

A. Oxidations
- Conversion of secondary alcohols to ketones
- Introduction of primary hydroxyl groups into the steroid side chain
- Introduction of secondary hydroxyl groups into the basic steroid framework
- Introduction of tertiary hydroxyl groups into the basic steroid framework
- Dehydration of ring A in positions 1(2) and 4(5)
- Aromatization of ring A
- Oxidation of the methylene group to the keto group
- Splitting of the side chain of pregnane at C-17 during production of a ketone
- Splitting of the side chain of pregnane at C-17 and opening of ring D during production of testololactone
- Splitting of the steroid side chain during production of a carboxyl group
- Splitting of the side chain of pregnane at C-17 during production of a secondary alcohol
- Production of epoxides
- Decarboxylation of acids

B. Reductions
- Reduction of ketones to secondary alcohols
- Reduction of aldehydes to primary alcohols
- Hydration of double bonds in position 1(2) in ring A
- Hydration of double bonds in position 4(5) in ring A and 5(6) in ring B
- Elimination of secondary alcohols

C. Hydrolysis
- Saponification of steroid esters

D. Ester production
- Acetylation

(Sebek and Perlman, 1979)

of ring A is used in estrogen production. The conditions for several bioconversion reactions of this type are given in Table 15.4 (examples 1–6). Steroid transformations have thus far been conducted as batch fermentations, but progress is being made with the use of immobilized cells or enzymes. Advantages of the latter include reduced risk of contamination, simplified product recovery, shorter conversion times, and increased substrate concentrations. In several processes more than one biochemical step can be combined. For instance, immobilized mycelium of *Curvularia lunata* or immobilized cells of *Arthrobacter simplex* have been used to carry out the

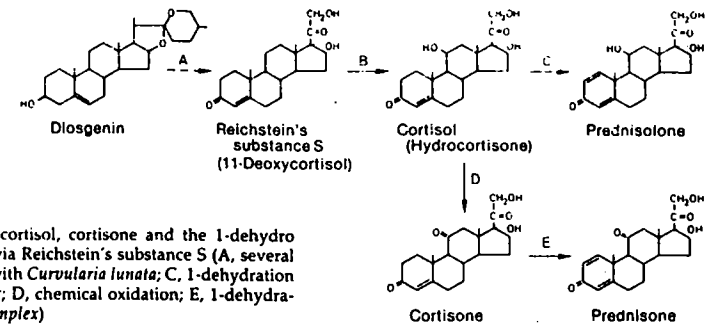


Figure 15.3 Production of cortisol, cortisone and the 1-dehydro compounds from diosgenin via Reichstein's substance S (A, several steps; B, 11β-hydroxylation with *Curvularia lunata*; C, 1-dehydration with *Corynebacterium simplex*; D, chemical oxidation; E, 1-dehydration with *Corynebacterium simplex*)

Table 15.3 Examples of commercial steroid processes

Reaction	Substrate → Product	Microorganism	Manufacturer
11α-Hydroxylation	Progesterone → 11α-Hydroxyprogesterone	*Rhizopus nigricans*	Upjohn Company
11β-Hydroxylation	Component S → Cortisol	*Curvularia lunata*	Pfizer Inc., Gist-Brocades
16α-Hydroxylation	9α-Fluorocortisol → 9α-Fluoro-16α-hydroxycortisol	*Streptomyces roseochromogenes*	E. R. Squibb and Sons, Lederle Laboratories
1-Dehydrogenation	Cortisol → Prednisolone Diendiol[a] → Triendiol[b]	*Arthrobacter simplex* *Septomyxa affinis*	Schering Corp. Upjohn Company
1-Dehydration, side chain splitting	Progesterone → 1-Dehydrotestololactone	*Cylindrocarpon radicicola*	E. R. Squibb and Sons
Side chain splitting	β-Sitosterol → Androstadiendione, and/or 9α-hydroxyandrostendione (see Fig. 15.5 for structure)	*Mycobacterium* sp., *M. fortuitum* mutants	G. D. Searle and Co. Upjohn Company

[a] Diendiol = 11β,21-dihydroxy-4,17(20)-pregnadiene-3-one
[b] Triendiol = 11β,21-dihydroxy-1,4,17(20)-pregnatriene-3-one (precursors in the production of 6α-methylprednisolone).
(Sebek and Perlman, 1979)

two-step reaction from Reichstein's component S to prednisolone. In several processes, fungal spores are being used directly to catalyze the transformation. Since most steroid substrates are not very soluble, transformation conditions have been developed for some steroids in a solvent system which is water-immiscible, e.g., for testosterone with immobilized cells of *Nocardia rhodochrous*. Since the organic solvent is often toxic to the cells or enzyme, an alternative is the use of an aqueous two-phase system. For example, the 1-dehydration of cortisol to prednisolone can be carried out by cells of *Arthrobacter simplex* in a system consisting of 25% (w/w) polyethylene glycol (PEG) 8000 and 6 (w/w) dextran T40.

Microbial breakdown of sterol side chains

The growing demand for steroids caused a shortage of steroid precursors for bioconversion, such as the compound diosgenin, which is obtained from the Mexican yam root (*Dioscorea composita*) or the South African plant *Testudinaria sylvatica*. Intensive studies were conducted on the use of low-cost sterols of animal origin, such as cholesterol, or of plant origin, such as β-sitosterol

Table 15.4 Conditions for operation of several steroid and sterol transformations

No.	Substrate	Product	Yield (weight %)	Microorganism	Medium	Conditions used
1	Progesterone	1-Dehydrotestololactone	50	*Cylindrocarpon radicicola*	a	72 h, 25°C
2	Progesterone	1,4-Androstadiene-3,17-dione	85	*Fusarium solani*	b	96 h, 25°C
3	Progesterone	15α-Hydroxy-4-pregnene-3,20-dione	11	*Streptomyces aureus*	c	72 h, 25°C
4	4-Androstene-3,17-dione	11α-Hydroxy-4-androstene-3,17-dione	25	*Rhizopus arrhizus*	d	96 h, 28°C
5	Progesterone	11-α-Hydroxy-progesterone	91	*Aspergillus ochraceus*	e	120 h, 28°C
6	Hydrocortisone	Prednisolone	93	*Arthrobacter simplex*	f	120 h, 28°C Pseudo-crystal fermentation
7	Cholesterol	1,4-Androstadiene-3,17-dione	90	*Arthrobacter simplex*	g	44 h, 30°C + chelating agents
8	β-Sitosterol Cholesterol Stigmasterol Campesterol	9α-Hydroxy-4-androstene-3,17-dione	Data unavailable	*Mycobacterium fortuitum*	h	336 h, 30°C mutant with block in breakdown of steroid nucleus

Media in Table 15.4

a. 3 g Corn steep liquor (dry weight), 3 g $NH_4H_2PO_4$, 2.5 g $CaCO_3$, 2.2 g soy bean oil, 0.5 g progesterone, distilled H_2O to 1 l, pH 7.0.
b. 15 g Peptone, 6 ml corn steep liquor, 50 g glucose, distilled H_2O to 1 l, pH 6.0; 0.25 g progesterone, added after 48 h.
c. 2.2 g Soy bean oil, 15 g soy meal, 10 g glucose, 2.5 g $CaCO_3$, 0.25 g progesterone, distilled H_2O to 1 l.
d. 20 g Peptone, 5 ml corn steep liquor, 50 g glucose, tap water to 1 l, pH 5.5–5.9; 0.25 g androstendione, added after 27 h.
e. 10 g peptone, 5 g yeast extract, 30 g sucrose, tap water to 1 liter; pH 6.5; 40 g progesterone dissolved in acetone added to the growing culture after 24 h.
f. 5g Peptone, 5 g corn steep liquor, 5 g glucose; distilled H_2O 1 l; pH 7.0; 1–50% finely ground hydrocortisone, suspended in ethanol, added to a 24 hour old culture.
g. 10 g Corn steep liquor, 2 g meat extract, 5 g glucose, 0.5 g K_2HPO_4; distilled H_2O 1 l; pH 7.0; cholesterol (1 g) added after 20 h (dispersed in water); 0.8 mM α,α'-dipyridyl (an iron chelating agent) in ethanol added after 26 h.
h. 10 g Glycerol, 8.4 g Na_2HPO_4, 4.5 g KH_2PO_4, 2 g NH_4Cl, trace elements; distilled H_2O 1 l; 1 g soy meal and 10 g sitosterol are added.

(Sebek and Perlman, 1979)

and stigmasterol (from soy beans) or campesterol (produced in great amounts as a byproduct of paper manufacture).

The objective of these studies was the selective removal of the aliphatic side chain without further breakdown of the steroid nucleus. However, a screening procedure with cholesterol as the substrate led only to strains carrying out a total breakdown of sterol to CO_2 and H_2O. The breakdown of the side chain to yield a C-17 ketocompound, shown in Figure 15.4, involves a mechanism which is similar to that of the β-oxidation of fatty acids. A C-1(2)-dehydration and 9α-hydroxylation are mandatory for further

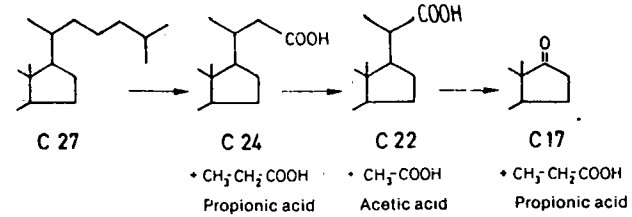


Figure 15.4 Side chain breakdown of sterols

breakdown of the steroid ring. As shown in Figure 15.5, the breakdown product 3-hydroxy-9,10-secoandrostatriene-9,17-dione is produced from cholesterol via an opening of the B ring, with the production of two useful intermediate products, androstendione and androstadiendione. In order to modify the steroid nucleus without breaking any of the rings, methods for selectively blocking attack on the ring are needed. Several methods are available to selectively block the breakdown of the steroid nucleus:

- The breakdown reaction can be blocked by chemical modification of the substrate.
- The conversion can take place in the presence of inhibitors which prevent C-1(2)-dehydration or 9α-hydroxylation, such as compounds which chelate Fe^{2+} or Cu^{2+}, bivalent ions which replace Fe^{2+}, or substances which block sulfhydryl functions, such as Ni^{2+}, Co^{2+}, and Pb^{2+}.
- Mutants with inactive C-1(2)-dehydrogenase and/or 9α-hydroxylase can be used. *Mycobacterium* mutants which transform cholesterol, stigmasterol, and sitosterol into the main product androstenedione and the secondary product androstadiendione have been isolated. With these mutants, there is no further breakdown of these latter compounds.

Some of the processes just mentioned are used commercially (see Table 15.4, examples 7, 8).

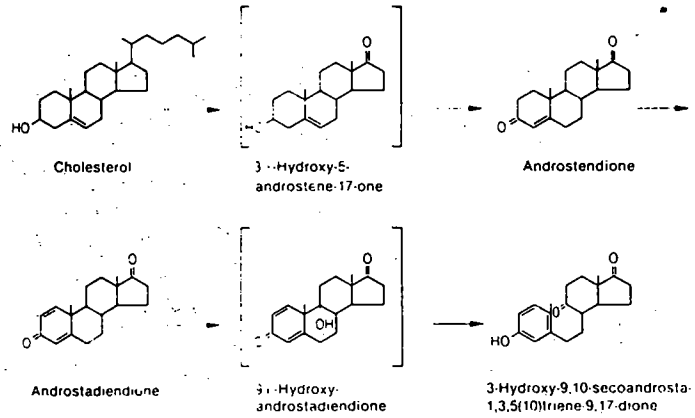


Figure 15.5 Biotransformation of cholesterol to androstadiendione and androstatriendione by mycobacteria

15.6 TRANSFORMATION OF NONSTEROID COMPOUNDS

In addition to the steroid transformations, microbial transformations of alkanes, of alicyclic, aromatic, and heterocyclic compounds, of terpenes, and of alkaloids have been described. In some cases, microbial transformations are difficult to control. For example, in experiments with gibberellins, carotenoids, and camphor, breakdown reactions predominate and there is only slight accumulation of utilizable intermediary products; attempts to isolate mutants blocked in the breakdown reactions have been unsuccessful. In other cases, as in alkaloid biotransformations, reaction rates have been too low for commercial processes. However, some transformation reactions are economically important; some of these have already been covered earlier (e.g., gluconic acid, kojic acid, in Chapter 8; manufacture of L-amino acids through racemic separation or hydantoin cleavage, in Chapter 9; penicillin acylase, glucose isomerase, in Chapter 11). A few more commercially significant bioconversions among the many known reactions are discussed here.

L-Ascorbic acid (Vitamin C)

The established process for producing L-ascorbic acid is the so-called Reichstein-Grüssner synthesis, shown in Figure 15.6. This process consists of several chemical steps and one microbial conversion. L-ascorbic acid is used in vitamin preparation or as an antioxidant in food manufacture and world production by this process is about 40,000 tons per year. The oxidation stage from D-sorbitol to L-sorbose is carried out by *Acetobacter suboxydans* in a submerged process at 30–35°C with vigorous stirring and aeration. Sorbitol is added at an initial concentration of 20% to a nutrient solution consisting of 0.5% yeast extract or corn steep liquor, and $CaCO_3$. A quantitative conversion is completed after about 2[illegible] hours; higher sorbitol concentrations prolong the conversion time. Today this process is carried out continuously in two stages; there are even some installations in which polyacrylamide-immobi

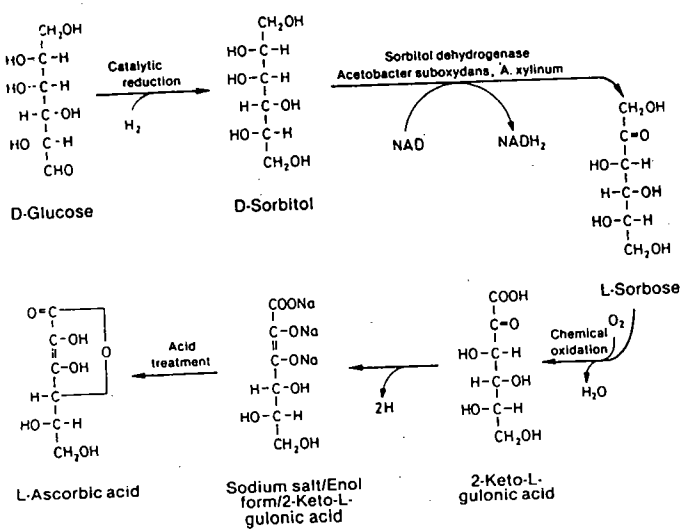


Figure 15.6 Microbial dehydration of D-sorbitol to L-sorbose in the production of L-ascorbic acid (Reichstein-Grüssner synthesis)

lized cells are used. In the overall process approximately 1 kg of L-ascorbic acid is produced from 2 kg of glucose.

In addition to the Reichstein-Grüssner synthesis shown in Figure 15.6, a two-step fermentation process has been developed and carried to commercial production. As shown in Figure 15.7, the first step involves the oxidation of glucose by an *Erwinia* species to 2,5-diketo-D-gluconic acid (2,5-DKG), via D-gluconic acid and 2-keto-D-gluconic acid. During a 26 hour incubation, 328 g/l calcium 2,5-DKG is formed with a 94% efficiency. The second step, a reduction of 2,5-DKG to 2-keto-1-gulonic acid, is catalyzed by a *Corynebacterium* sp. In this process, after the *Corynebacterium* has grown for 16 hours, it is fed with a sterilized *Erwinia* culture. After 66 hours incubation, 106 g of calcium 2-keto-L-gulonate is formed, with an efficiency of 92%. The latter product is easily transformed chemically into L-ascorbic acid and the overall balance, based on glucose consumed, is 86%.

Scientists at the Genentech Company have succeeded in cloning and expressing the gene for the 2,5-DKG reductase of *Corynebacterium* into *Erwinia herbicola*, opening up the possibility of a single-step process from glucose to 2-keto-L-gulonic acid. However, the hybrid strain produced exhibited a low tolerance to high concentrations of glucose and a yield of only 0.6 g/l of 2-keto-L-gulonic acid was obtained from 20 g/l glucose after 57 hours incubation.

Dihydroxyacetone from glycerol

The microbial conversion of glycerol to dihydroxyacetone (used in suntan lotions and cosmetics), is of some significance (Figure 15.8). Various acetic acid bacteria convert 10% glycerol in a suitable nutrient solution (0.5% yeast extract, 0.5% KH_2PO_4, 2% $CaCO_3$) at 28°C and at a pH below 6.0. Conversion time is 72–96 hours but can be reduced to 33 hours with *Gluconobacter melanogenus* IFO 3293 by using O_2-enriched air (oxygen partial pressure 0.05 bar). Growth is not affected, but the amount of dihydroxyacetone produced is raised from 12.2 g/g biomass in normally aerated cultures to 35.8 g/g in cultures with improved oxygen supply.

Prostaglandins

Prostaglandins are unsaturated C_{20} fatty acids that function as tissue hormones. They are of increasing medical significance because of their varied physiological activities. Currently marketed are PGE_2 as a contraceptive, PEG_2 for the alleviation of pain of childbirth, PEG_1 for the treatment of congenital heart failure, and a methyl derivative of PEG_1 for the treatment of digestive diseases. The prostaglandins PGE_1, PGE_2, PGF_1, and PGF_2 can be produced from unsaturated fatty acids (particularly arachidonic acid) (Figure 15.9) by microbial transformation with fungi. The isolation of compounds with im-

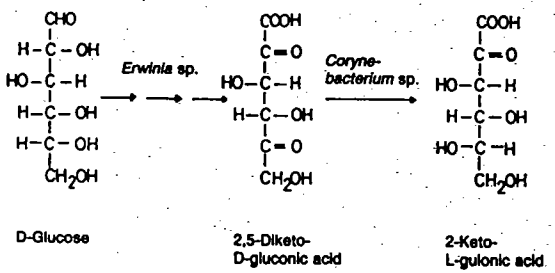


Figure 15.7 Two-stage fermentation for the production of 2-keto-L-gulonic acid from D-glucose

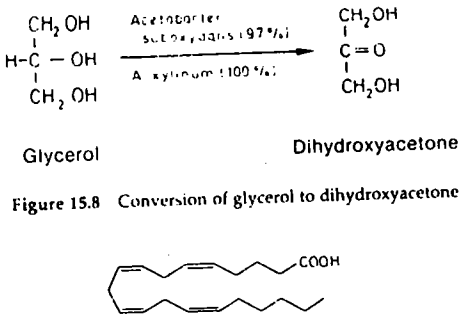


Figure 15.8 Conversion of glycerol to dihydroxyacetone

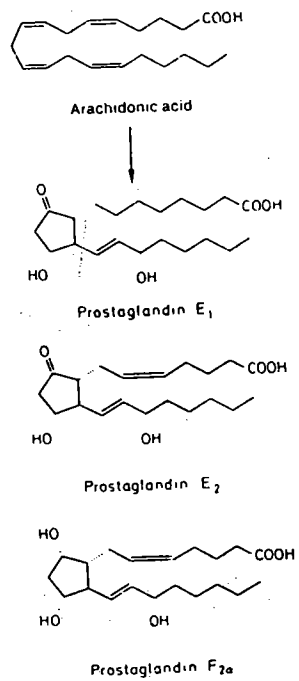


Figure 15.9 Biotransformation of arachidonic acid to prostaglandins

proved effectiveness can be expected in light of new reports on successful biotransformations of this molecule.

15.7 TRANSFORMATION OF ANTIBIOTICS

In addition to screening procedures, studies in recent years have also been directed at the production of new and improved antibiotics by the microbial transformation of existing compounds. The objective here is the development of modified antibiotics with improved effectiveness, reduced toxicity, wider antimicrobial spectrum, improved oral absorption, decreased development of resistance, or lower allergic effects. In most cases, any transformation step causes a partial or complete inactivation of the antibiotic. Thus, many biotransformations must be attempted and their products assayed to find those rare cases where the modified product has desirable improved properties.

Several typical examples of the many possible reactions are given here.

Indirect transformation The addition of inhibitors or modified precursors to the medium may result in the synthesis of altered antibiotics via **controlled biosynthesis**. For instance, in the presence of cis-4-methylproline, *Streptomyces parvulus* produces two new actinomycins (K_{1c} and K_{2c}) in which proline is replaced by this proline analog. New compounds have been found when mutants blocked in the synthesis of a particular antibiotic were used. For instance, ribostamycin (Figure 15.10) accumulates as an intermediate product of neomycin biosynthesis with a mutant of the neomycin producer *S. fradiae*. A few improved antibiotics have been isolated after **mutational synthesis** (see Section 3.6), of which only 5-*epi*-sisomicin has proved of sufficient utility to undergo clinical trials. Several genetic techniques, mutagenesis, intra- and interspecific recombination by protoplast fusion, and recombinant DNA technology, have been used to isolate strains capable of producing modified versions of rifamycins, aminoglycosides, and tylosin.

Direct transformation Acylation reactions have been described for various antibiotics. Inactive compounds develop in most cases but in the case of lankacidin-C-14-butyrate, a bioconversion product formed from lankacidin C and methylbutyrate by *Bacillus megaterium* IFO 12108, improved antimicrobial activity with lower toxicity

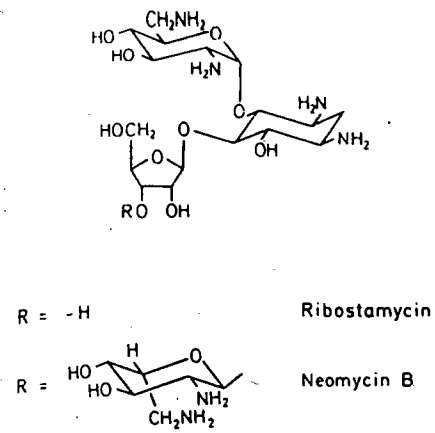
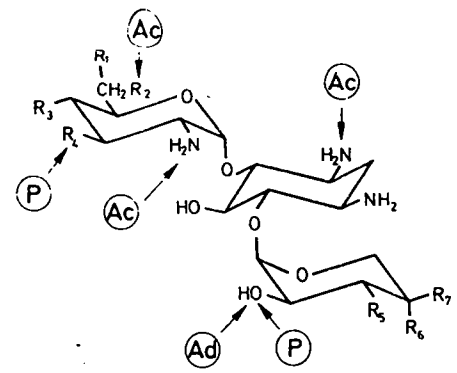


Figure 15.10 Ribostamycin formation by a mutant of *Streptomyces fradiae* blocked in neomycin production

was obtained. Deacylation reactions have been described in particular for the macrolide antibiotics. The biologically less active deacylated products can then be used for the production of semisynthetic compounds. Phosphorylation via different phosphotransferases and adenylylation via adenyltransferases take place chiefly with aminoglycosides and lead to inactivation. Acetylation (Ac), phosphorylation (P), and adenylylation (Ad) are reactions that are primarily responsible for the development of bacterial resistance to aminoglycosides. The example of gentamicin in Figure 15.11 shows the target at which the three modifying enzymes act.

Hydrolysis reactions are especially significant in β-lactam antibiotics (Figure 15.12). Hydrolysis involves the splitting of the lactone ring of penicillins and cephalosporins by β-lactamases, leading to inactivation of the antibiotics. Bacterial resistance to β-lactam antibiotics is mainly caused by this reaction. On the other hand, the enzymatic splitting of penicillin by penicillin acylase into 6-aminopenicillanic acid (Section 11.9) is of great economic significance.



Gentamicin	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Gentamicin A	H	OH	OH	OH	$NHCH_3$	H	OH
Gentamicin A_2	H	OH	OH	OH	OH	OH	H
Gentamicin X_2	H	OH	OH	OH	$NHCH_3$	OH	CH_3
Gentamicin C_{1a}	H	NH_2	H	H	$NHCH_3$	OH	CH_3
Gentamicin C_2	CH_3	NH_2	H	H	$NHCH_3$	OH	CH_3
Gentamicin C_1	CH_3	$NHCH_3$	H	H	$NHCH_3$	OH	CH_3

Figure 15.11 Structure of gentamicins with indications of the site of action of the inactivation enzymes

Hydroxylation is another frequent microbial transformation reaction in antibiotics (example in Figure 15.13).

15.8 TRANSFORMATION OF PESTICIDES

Until now, we have been discussing microbial transformations that lead to the production of commercially useful compounds. We now change our perspective and discuss some transformations that cause the *destruction* of a major class of commercially useful compounds, the pesticides. Agents for plant disease and pest control are necessary for the survival of the world's population. Presently one-third of the world's harvest is lost because of damaging organisms. It is estimated that another one-third would be lost without the use of chemical control agents. Contagious diseases such as malaria, chagas disease,

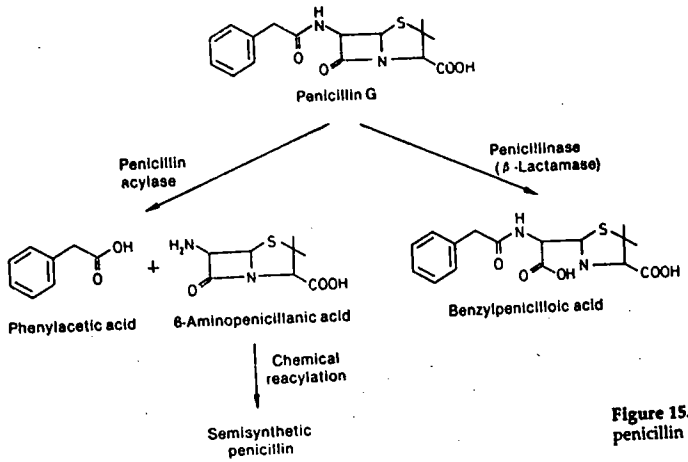


Figure 15.12 Microbial transformation of penicillin G

typhus, cholera, and spotted fever have been reduced in severity and in some regions completely eliminated through the control of disease-carrying insect vectors. High stability (persistence) of the compounds used is vital for these vector-control programs, but this stability has a negative effect on the environment.

This problem of environmental persistence is apparent with chlorinated hydrocarbon insecticides such as DDT, lindane, and dieldrin. A remarkable success in infectious disease control can be attributed to DDT, but due to its resistance to decomposition, the compound accumulates in microorganisms and thus enters into the food chain. This development, along with the restricted use of DDT since the early 1970's, has led to research for new control methods and toxicologically and environmentally safe preparations.

In this context, microbial transformation is of interest not for the production of new active agents, but for the greatest possible detoxification of the environment. This involves enzymatic conversions of so-called *xenobiotics*, substrates which do not normally occur in nature, such as halogenated hydrocarbons, aromatic nitro-compounds, and sulfonic acid derivatives. Many of the enzymes involved in these transformations must be induced and different organisms are frequently involved in the breakdown of a compound. Depending on chemical structure, some compounds cannot be easily converted; thus persistence times in the soil range from a few days to several years.

Removal of xenobiotics from ecosystems can be accomplished through various mechanisms.

Metabolism Xenobiotics can serve as substrates for microbial growth and energy production. Complete breakdown of some substances to CO_2 and H_2O has been described. Figure 15.14 shows the example of the herbicide dalapon, a chlorinated fatty acid, which is converted by *Arthrobacter* sp. into pyruvate after oxidative dehalogenation.

Cometabolism In cometabolism, microorganisms do not obtain energy from the transformation reaction and require another substrate for growth. Hence, cometabolism normally causes a mere modification of molecules, which may result in either a decrease or an increase in toxicity. A further breakdown can be achieved through the combined action of different organisms.

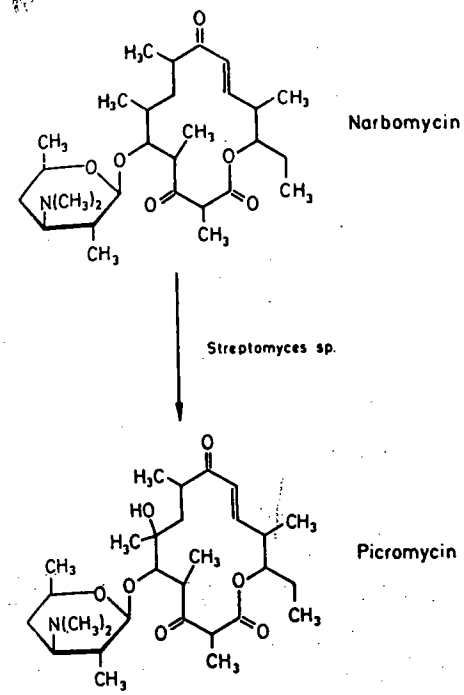


Figure 15.13 Hydroxylation of narbomycin

Some examples of cometabolism are given in Table 15.5.

Dehalogenation or oxidative dehalogenation reactions (Figure 15.15) are important cometabolism reactions which may make pesticide molecules accessible for further breakdown. Some compounds such as chlordecone, a hexachlorocyclopentadiene derivative with excellent insecticide and acaricide effects, are not easily attacked by microorganisms because of their complicated structure and high degree of halogenation.

Conjugate formation Linkage of xenobiotics or decomposition products with naturally occurring compounds such as amino acids or carbohydrates results only in a temporary detoxification; the toxic compound can be released again at any time. Figure 15.16 shows the conversion of a dithiocarbamate fungicide.

Table 15.5 Cometabolism of pesticides

Substrate	Conversion product	Microorganism
Chlorbenzilate (ethyl-4,4'-dichlorobenzilate)	4,4'-Dichlorbenzophenone	*Rhodotorula gracilis*
Chloroneb (1,4-dichloro-2,5-dimethoxybenzene)	2,5-Dichlor-4-methoxyphenol	*Fusarium* sp.
DDT	p,p'-Dichlordiphenylmethane	*Aerobacter aerogenes*
p,p'-Dichlorodiphenylmethane	p-Chlorphenylacetate	*Hydrogenomonas* sp.
2,4,5-Trichlorophenoxyacetate	3,5-Dichlorcatechol	*Brevibacterium* sp.
3,5-Dichlorcatechol	3,5-Dichlor-2-hydroxymuconic-acid semialdehyde	*Achromobacter* sp.

(Bollag, 1974)

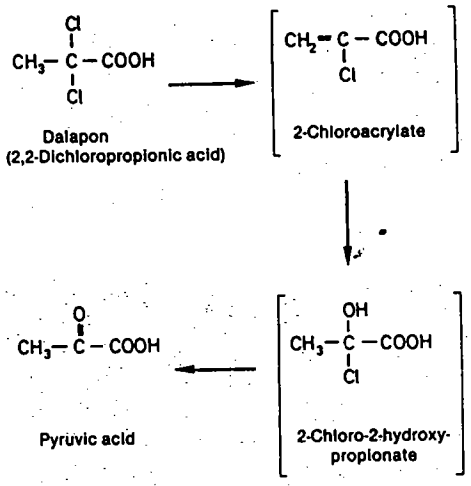


Figure 15.14 Microbial breakdown of dalapon

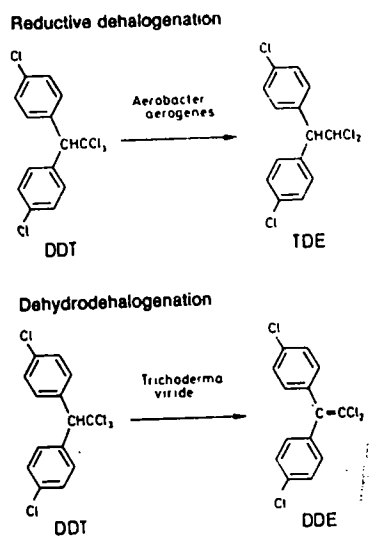


Figure 15.15 Transformation of the chlorinated hydrocarbon DDT (DDT=2,2-Bis-[4-chlor-phenyl]-1,1,1-trichlor-ethane; TDE=2,2-Bis-[4-chlor-phenyl]-1,1-dichlor-ethane; DDE=1,1-dichlor-2,2-Bis-[4-chlor-phenyl]-ethylene)

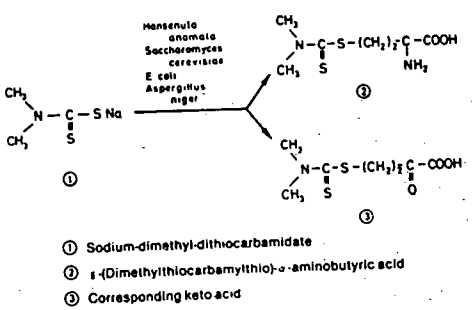


Figure 15.16 Transformation of a thiocarbamate fungicide

Accumulation of xenobiotics When microorganisms absorb xenobiotics, only temporary detoxification of the environment occurs. It has been found that marine microorganisms and plankton absorb DDT and concentrate it by a factor of 100. These microorganisms are eaten by marine animals and the DDT is then stored in fat tissue, leading to an even greater concentration factor. The end result is an accumulation of the compound to high levels as it passes up the food chain.

Intensive research of recent years has shown a whole series of aerobic and anaerobic biodegradative reactions that can occur with environmentally significant compounds. Further progress can be anticipated through the following:

- The search for microorganisms capable of breaking down compounds of interest, by use of strong selective pressure in the chemostat for enrichment culture. If particular enzyme systems are being sought, special genetic probes can be used to quickly screen and identify a wide variety of natural isolates.
- The use of recombinant DNA technology to construct microorganisms that contain the complete biochemical sequence for the breakdown of a particular chemical. It should be possible to combine in one organism that processes that occur now only in co-metabolizing organisms. For example, a hybrid *Pseudomonas* has been constructed capable of breaking down chlorosalicylic acid.
- Immobilized cells should tolerate higher concentrations of xenobiotics and should also carry out the desired biochemical reactions more rapidly. This approach has already been used to develop a continuous process for the biodegradation of phenolic compounds.

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16

Single-cell protein (SCP)

16.1 INTRODUCTION

The term "single-cell protein" (SCP) was coined at Massachusetts Institute of Technology in 1966 and is used today to refer to microbial biomass used as food and feed additives. Either the isolated cell protein or the total cell material may be called SCP.

The **human food chain** is diagrammed in Figure 16.1. Animals and plants have always provided the main food sources, but microorganisms are also eaten in small quantities in such products as cheese, vinegar, mushrooms, koji, yeast, and blue-green algae (the species *Spirulina* is eaten in parts of Africa).

In view of the insufficient world food supply and the high protein content of microbial cells (Table 16.1), the use of biomass produced in the fermenter would be an ideal supplement to conventional food supply. Single-cell protein is of great nutritional value because of its high protein, vitamin, and lipid content and the general presence of a complete array of all essential amino acids. In many countries, however, there are psychological barriers to the use of microorganisms as a major food source. The following points must also be considered with regard to safety:

- The high nucleic acid content (4–6% in algae, 10–16% in bacteria, 6–10% in yeast, and 2.5–6% in fungi) can be hazardous to health.
- Toxic or carcinogenic substances adsorbed from the microbial growth substrate (odd-numbered and branched-chain hydrocarbons, polycyclic aromatic compounds) may be present. There is also the possibility that microorganisms may produce highly toxic substances (aflatoxins are produced by some fungi, for example.).
- Slow digestion of microbial cells in the digestive tract may cause indigestion and allergic reactions.

The following substrates are presently being studied for SCP production: alkanes, methane,

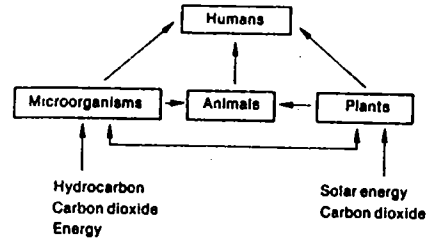

Figure 16.1 Food chain of humans

methanol, cellulose, carbohydrates, and waste materials.

Cost of production will also be a deciding factor in determining the ultimate place of SCP in the human or animal diet. It is clear that SCP must compete with natural protein sources such as soy meal or fish meal. The production costs will be strongly influenced by the nature of the raw starting material used. If a starting material is used that must otherwise be disposed of as a waste product, for instance, sulfite-waste liquor from the paper industry, then the raw material cost is actually negative. However, if a useful material such as natural gas is used, the cost of the starting material will play a major role in the final cost of the SCP. Another factor is that the price of SCP varies depending on how it is used. For instance, SCP for humans is about 10 times more expensive than SCP for animal feed because a more highly refined product must be used. Further, in less industrialized countries the price structure is more critical than in more i dustrialized countries.

16.2 PRODUCTION OF SINGLE-CELL PROTEIN FROM ALKANES

The use of longer-chained alkanes will be di cussed in this section, and gaseous methane, tl simplest alkane, will be discussed in the next se tion. Alkanes can be catabolized by many yeas and by some fungi (for instance, fungi of the o ders *Mucorales* and *Moniliales*) and by some ba teria (partially through cooxidation). The follov ing yeast species have been intensively studie for SCP production: *Candida tropicalis*, *Candi oleophila*, and *Saccharomycopsis lipolytica*.

The disadvantage of the use of alkanes is th they are not easily soluble. During growth in bi reactors with impellers or in airlift fermenter large alkane drops are formed which are 1–10 μm in size and which remain suspended. Cor sidering the low water solubility of alkanes (so ubility 10^{-4}–10^{-9} v/v), the observed high growt rates of microorganisms on alkanes cannot b explained merely by transport of alkane dis solved in water; other mechanisms of uptak must be present. It seems likely that the cell form emulsifying substances which convert th insoluble alkanes into droplets of 0.01–0.5 μm Alkane molecules can then reach the cytoplasmi membrane through the cell wall via passive dif fusion. Cells growing on alkanes are enriched i lipids and it seems likely that these lipids play role in the transfer of alkanes through the cel membrane.

Table 16.1 Composition of crude single-cell protein (percent)

	Alkane yeast	Methanol bacterium	Protein isolate	Fungus	Alga	Soy meal	Milk powder
Raw protein	60.0	83	80	42	70	45.0	34.0
Fat	9.0	7.4	8–10	13	5	1.8	1.0
Nucleic acid	5.0	15	1–2	9.7	4		
Mineral salts	6.0	8.6	8–12	6.6	7	6.0	8.0
Amino acids	54	65				40	
Moisture	4.5	2.8	4.0	13.0	6	12.0	5.0

The values for soy meal and milk powder are given for comparison.

Catabolism of longer-chained alkanes

The first step in the utilization of alkanes is the introduction of molecular oxygen into the molecule. There are two pathways for oxygen introduction: terminal oxidation and subterminal oxidation (Figure 16.2). In **terminal oxidation**, the corresponding monocarboxylic acid is produced via the intermediate stages of the primary alcohol and aldehyde. After this terminal oxidation, breakdown generally proceeds to acetyl-CoA units by means of β-oxidation. Terminal oxidation is the chief pathway of metabolism for bacteria and yeast. In some cases, terminal oxidation at both ends of the molecule occurs by means of ω-oxidation, leading to the production of the corresponding dicarboxylic acid, which is further broken down into acetate units and succinate by means of β-oxidation. The enzymatic mechanisms of terminal oxidation are not yet fully understood.

In **subterminal oxidation**, the appropriate ketone is first produced via a secondary alcohol. This can either happen in the C_2 position or in the interior of the molecule, e.g. at C_3, C_4, C_5 or C_6. Internal oxidation refers to subterminal oxidation which does not take place in position C_2. Organisms show specificity in which carbon atom is attacked in the subterminal oxidation process. Further catabolism of the secondary alcohol is variable. α-Oxidation with subsequent decarboxylation and β-oxidation has been found in *Candida*. It is also possible for the molecule to be converted to an ester which is then split with an esterase. The acid and the alcohol, which are products of the esterase action, are then metabolized further.

Large-scale processes using yeast

Two petroleum products have been used as starting material:

- *Gas oil*, also known as fuel oil or diesel oil, is a fraction derived from crude oil and contains 10–25% C_{15}–C_{30} alkanes.
- C_{10}–C_{13} alkanes or C_{13}–C_{17} alkanes are separated from gas oil with molecular sieves.

British Petroleum Co. has developed processes using both of these substrates.

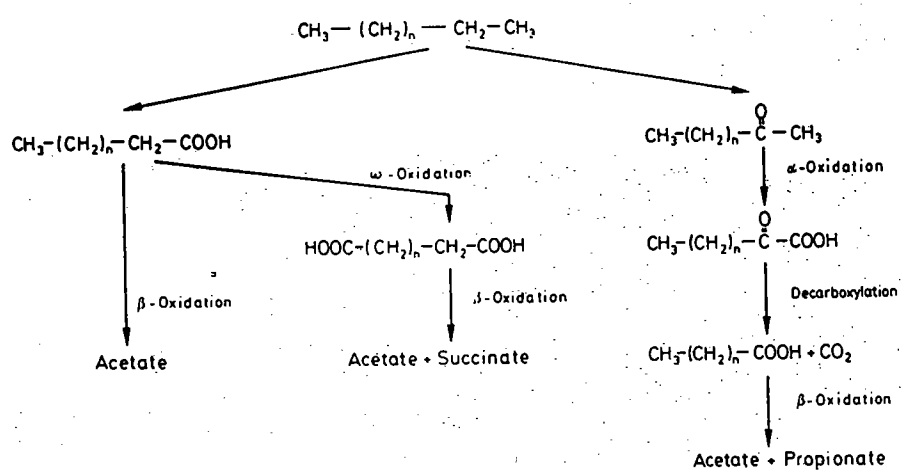

Figure 16.2 Alkane oxidation in Candida

Gas-oil process *Candida tropicalis* was tested in the gas-oil process in a nonsterile, continuous system (16,000 tons/year) in Cap Lavera, France from 1973–1975. In Grangemouth (U.K.), *Saccharomycopsis lipolytica* (previously called *Candida lipolytica*) was grown aseptically with n-alkanes in a 300 m³ bioreactor in a system which produced 4000 tons SCP/year over a period of several years. A complete factory of 100,000 tons/year with three 1000 m³ fermenters was built in Sardinia for the alkane procedure, but was subsequently not put into operation for political reasons.

The gas-oil fermentation was run in an airlift bioreactor with an increased aeration rate. Since alkanes make up only a small proportion of gas oil, insoluble gas oil had to be fed repeatedly to the growth medium. This resulted in poor mixing, poor oxygen transfer, and low yields.

Alkane process In comparison to glucose, *Candida tropicalis* grows much more poorly on alkanes (μ_{max} = 0.28 as compared with 0.62 on glucose), but the yield is better (0.98 g cells/g alkane versus 0.51 g cells/g glucose) and the oxygen uptake rate is similar (14 μM O_2/g·h on alkane versus 11 μM O_2/g·h on glucose).

16.3 BACTERIA WHICH UTILIZE METHANE

There is an excess of methane, the chief component of natural gas, in some parts of the world, making this a desirable energy source for SCP production. Methane can be obtained as a very pure gas. However, in contrast to higher hydrocarbons, methane cannot easily be liquefied, making long-distance transport difficult and expensive. Also, considerable security measures must be taken when handling methane, due to the risk of explosion.

Methane-oxidizing bacteria are classified among the obligate methylotrophs. This group grows only on C_1 substrates (methane, methanol, methylamine, formaldehyde, or formate). Yeasts which assimilate methane have not yet been isolated, and thus far only relatively few methane-utilizing bacteria have been identified. Among the bacteria are *Methylomonas methanica*, *Methylococcus capsulatus*, *Methylovibrio soehngenii*, *Methanomonas margaritae*, and some unclassified organisms.

The enzyme methane oxygenase oxidizes methane to methanol, which is further channeled into the primary metabolism (see Section 16.4).

$$CH_4 + O_2 + XH_2 \rightarrow CH_3OH + H_2O + X$$

Methanol accumulates as a result of the oxidation process and inhibits the growth of bacteria. Primary metabolites, such as amino acids, sugars, or acetate, can also inhibit growth and methane oxidation.

Using *Methylococcus capsulatus*, 0.4 g/l dry weight was obtained with a yield of 1.00–1.03 g dry weight/g methane.

Since methanol is much easier to handle and can be produced chemically from methane, methanol is the preferred starting material for all systems using C_1 substrate (see below).

16.4 METHANOL FERMENTATIONS

Methanol was at one time the most important substrate for single-cell protein production and extensive research on methanol-utilizing organisms was carried out. Although cost factors currently rule out methanol as a starting material, it still has many advantages as a substrate for SCP production and changing economics could easily bring it back.

Methanol may be obtained from synthesis gas, natural gas, methane, oil, or coal. Wood could theoretically also be used as a starting material for methanol production. Bacteria, yeasts, and fungi may all be considered for the production of SCP from methanol (Table 16.2). Besides the obligate methylotrophic bacteria which only grow on C_1 compounds, facultative methylotrophic bacteria, yeasts, and fungi which metabolize longer-chained hydrocarbons as well are

Table 16.2 Microorganisms that grow on methanol

1. Obligate methylotrophic bacteria	
Methylobacter	*Methylocystis*
Methylococcus	*Methylosinus*
Methylomonas	
2. Facultative methylotrophic organisms	
a. Bacteria	
Arthrobacter	*Protaminobacter*
Bacillus	*Pseudomonas*
Hyphomicrobium	*Rhodopseudomonas*
Klebsiella	*Streptomyces*
Micrococcus	*Vibrio*
b. Yeast	
Candida boidinii	*Pichia haplophila*
Candida parapsilosis	*Pichia lindnerii*
Hansenula capsulata	*Pichia pastoris*
Hansenula henricii	*Torulopsis glabrata*
Hansenula minuta	*Torulopsis methanolovescens*
Hansenula nonfermentans	*Torulopsis methanosorbosa*
Hansenula wickerhamii	*Torulopsis molischiana*
	Torulopsis memodendra
c. Fungi	
Gliocladium delinquescens	
Paecilomyces varioti	
Trichoderma lignorum	

(Sahm, 1979)

included in this list. In contrast to many bacteria, yeasts are unable to use any C_1 compounds other than methanol.

In **methanol fermentation** for SCP production, bacteria rather than yeasts are employed in essentially all existing production processes for the following reasons: rapid growth (Table 16.3), higher protein content, better yields, and simpler culture medium requirements.

Methanol oxidation

Methanol is oxidized to CO_2 by bacteria via the following intermediate steps:

$$CH_3OH \rightarrow HCHO \rightarrow HCOOH \rightarrow CO_2$$

The first step to formaldehyde requires an inducible nonspecific methanol dehydrogenase.

Yeasts oxidize methanol by means of a nonspecific, FAD-containing, inducible methanol oxidase. Methanol can also be oxidized by means of H_2O_2 with the peroxidase activity of an inducible catalase, the hydrogen peroxide being produced by methanol oxidase. Methanol oxidation to formaldehyde results in no energy gain for the yeast.

The next step is formaldehyde oxidation, which in bacteria can be carried out by several enzymes:

- Conversion of formaldehyde to formate with reduced glutathione (GSH) by means of an NAD-dependent formaldehyde dehydrogenase, a reaction which also occurs in yeast.

$$HCHO + NAD + H_2O \xrightarrow{GSH} HCOOH + NADH_2$$

- A dichlorophenol-indophenol (DCPIP)-dependent formaldehyde dehydrogenase.
- An unspecific methanol dehydrogenase.

Two to three ATP per mole of substrate are obtained at this oxidation level.

The last step, the oxidation of formate, is common to all the methanol-utilizing microorganisms. Formate oxidation involves a NAD-dependent formate dehydrogenase and yields 3 ATP per mole of substrate:

$$HCOOH + NAD \rightarrow CO_2 + NADH_2$$

Table 16.3 Maximal specific growth rates (μ_{max}) of various methanol utilizers

Microorganism	$\mu_{max}(h^{-1})$
Pseudomonas rosea (ICI)	0.38–0.50
Protaminobacter ruber	0.10
Pseudomonas extorquens	0.18
Methylomonas methanolica	0.53
Pseudomonas B45	0.198
Kloeckera sp. 2201	0.075
Hansenula polymorpha	0.22
Torulopsis glabrata	0.11

(Braunegg, 1975)

In some obligate methanol-utilizing bacteria (for example, *Methylomonas* M 15), formaldehyde dehydrogenase and formate dehydrogenase are not found. Instead, these organisms oxidize formaldehyde by means of the phosphogluconate pathway (Figure 16.3).

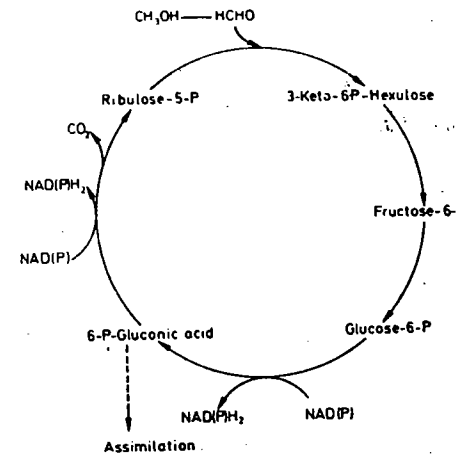

Figure 16.3 Phosphogluconate pathway

Carbon assimilation by methanol-oxidizing organisms

Bacteria growing on methanol must produce C_3 molecules which then feed into primary metabolism as pyruvate. Three distinct pathways for one-carbon assimilation have been recognized:

- CO_2 may be taken up in photosynthetic organisms via the **ribulose diphosphate (Calvin) cycle.**
- Formaldehyde may be condensed with ribulose-5-P in the **ribulose monophosphate cycle (Quayle cycle).** The enzyme hexulose phosphate synthase (HUPS) carries out this reaction (Figure 16.4). After isomerization of 3-keto-6-P-hexulose to fructose-6-P, either dihydroxyacetone-P is produced through glycolysis or pyruvate is produced through the Entner-Doudoroff pathway. Ribulose-5-P is regenerated in the ribulose monophospha cycle through transketolase and transaldola reactions. HUPS, the key enzyme of the c cle, is partly constitutive, partly inducible methanol, and is repressed by formaldehyc
- In the **serine pathway**, condensation of fc

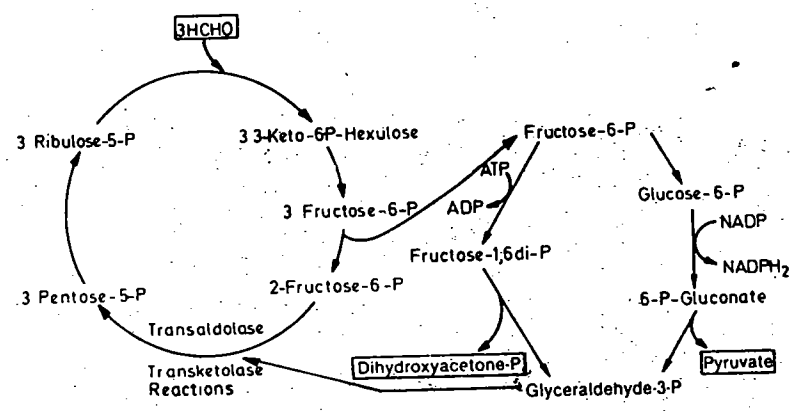

Figure 16.4 Ribulose monophosphate (Quayle) cycle

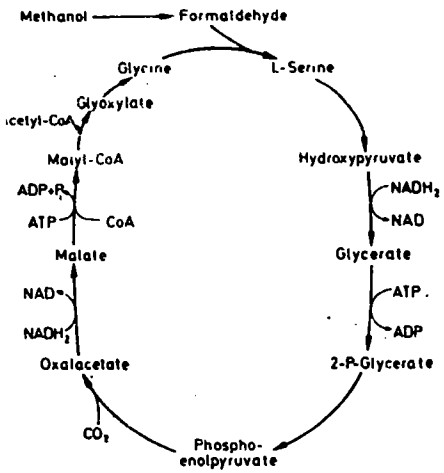

Figure 16.5 Formaldehyde fixation via the serine pathway

maldehyde and glycine takes place through the action of serine transhydroxymethylase (Figure 16.5).

A pathway very similar to the ribulose monophosphate cycle, the dihydroxyacetone cycle, has been found in yeasts (Figure 16.6).

Table 16.4 shows theoretical yield coefficients for bacteria and yeasts that use the various one-carbon pathways. It is clear from this table that bacteria using the ribulose monophosphate pathway are the best producers; therefore, all commercial processes are conducted with this group.

Production processes

Imperial Chemical Industries (ICI) was the first company to develop a continuous methanol fermentation for the commercial production of SCP. They studied the effect of O_2, CO_2, and methanol concentration on productivity and the effect of the pressure differential between the bottom and the surface of the bioreactor. The "ICI Pressure Cycle Fermenter", a combination of air lift and loop reactor, is illustrated in Figure 16.7. This 37 m³, 30 m-high pilot fermenter consists of 3 units: air lift column (I), down-flow tube with heat removal (II), and gas release space (III). The pilot

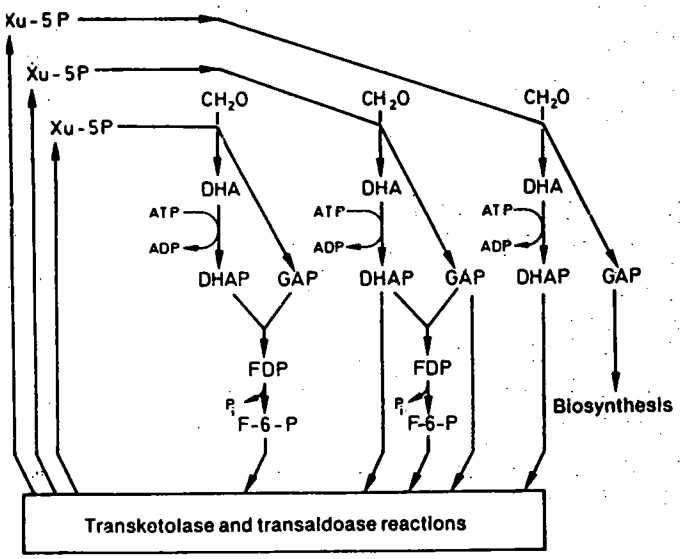

Figure 16.6 Formaldehyde fixation in yeast via the dihydroxyacetone pathway

Table 16.4 Yield coefficients of microorganisms growing on methanol

Microorganism	Pathway	Y, (g cell dry weight/ g methanol)
a. Bacteria		
Pseudomonas C	RMP	0.54
P. methylotrophus	RMP	0.53
Methylomonas methalonica	RMP	0.49
Pseudomonas AM 1	SER	0.30
Pseudomonas M 27	SER	0.41
Pseudomonas rosea	SER	0.41
b. Yeasts		
Candida boidinii	DA	0.32
Hansenula polymorpha	DA	0.38

RMP Ribulose monophosphate cycle, SER Serine pathway, DA Dihydroxyactone cycle
(Sahm, 1979)

plant system, which had a capacity of 1000 tons/year, operated at pH 6.5–6.9 and 34–37°C. The organism used for industrial SCP production is the methanol oxidizer *Pseudomonas methylotrophus*, which was isolated by ICI and then significantly improved through genetic and physiological development (Table 16.5).

In the product recovery process, partial cell lysis is first achieved via heat and acid treatment and the nutrient solution is then clarified by decanting. The water is then recycled back into the

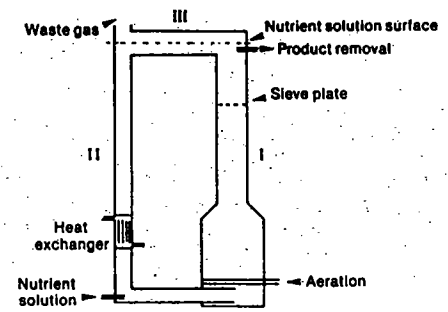

Figure 16.7 ICI pressure cycle fermenter

fermenter and the cells are spray-dried. Based on the results of this pilot study, ICI invested £40 million in 1979 to install a continuous culture system with a capacity of 50,000–70,000 tons/year, which began operation in 1980. In this fermenter, which had a volume of 1000 m³, cultivation of the inoculum could be carried out right in the fermenter.

Although the original chemostat process was methanol-limited, it was later operated as a nitrogen-limited system. With the bacterial strain originally used, NH_4-assimilation occurred via two inefficient enzyme systems:

Glutamine-Ketoacid Transaminase (GOGAT)

$$\alpha\text{-Ketoglurate} + NAD(P)H + \text{Glutamine} \rightarrow 2\ \text{Glutamate} + NAD(P)$$

Glutamine synthetase

$$\text{Glutamate} + NH_3 + ATP \rightarrow \text{Glutamine} + ADP + P_i$$

The more effective glutamate dehydrogenase system from *Escherichia coli* was therefore transferred to a GOGAT⁻ mutant of *Pseudomonas methylotrophus* by means of genetic engineering, and such strains, which presumably grow more rapidly due to improved nitrogen assimilation, were placed in commercial production. However, the yield increase amounted to only 3%.

Because of difficulties in removing the cells from the liquid, the completely automatic large-scale process for SCP was never placed in full production due to economic reasons. For instance, in 1984 the price of soy meal was around $125–190 per ton, whereas the SCP from ICI (going under the trade name Pruteen) was being sold at $600 per ton!

Table 16.5 Results of the optimization of the ICI fermentation process for growing *Pseudomonas methylotrophus* on methanol

Raw protein	83% → 85%
Pure protein	59% → 64%
Cell dry weight	4 → 30 g/l
μ_{max}	0.38 h⁻¹ → 0.5 h⁻¹
Y	→ 0.5 g/g

(Gow et al. 1975)

16.5 SINGLE-CELL PROTEIN FROM WOOD

At the present time cellulose from natural sources and waste wood is still an attractive starting material for single-cell protein production as well as a potential source for production of fermentation ethanol. Although there is an abundance of cellulose on earth, it is usually mixed with substances such as lignin, hemicellulose, starch, protein, and salts (Table 16.6). Therefore, cellulose sources must be pretreated physically and chemically in order to break down the cellulose into fermentable sugars. Pretreatment may either be enzymatic (cellulases) or chemical (acid hydrolysis). We discuss here the enzymatic processes for cellulose hydrolysis. Great advances are being made in the breakdown of lignin, and ligninase enzymes can now be produced by submerged fermentation.

Physiology

Only extracellular cellulases can be used commercially. Such cellulases are excreted by both bacteria (*Cellulomonas* and actinomycetes) and fungi (*Trichoderma, Penicillium, Thermoascus, Sporotrichum*, and *Humicola*). For pilot plant studies, the following fungi have been used: *Trichoderma reesei (T. viride), T. koningii*, and *Sporotrichum pulverulentum*.

Table 16.6 Composition of cellulosic substrates

	Cellulose %	Hemi-cellulose %	Lignin %
Wood (Angiosperms)	40–55	24–40	18–25
Wood (Gymnosperms)	45–50	25–35	25–35
Grasses	25–40	25–50	10–30
Leaves	15–20	80–85	—
Newspaper	40–55	25–40	18–30
Waste from paper manufacture	60–80	20–30	2–10

Cellulase is an enzyme complex consisting of at least three enzymes:

- Endo-β-1,4-glucanase, also called endocellulase, carboxymethyl cellulase (CMCase), or C_x cellulase.
- Exo-β-1,4-glucanase, also called cellobiohydrolase, avicelase, or C_1 cellulase. C_1 cellulase comprises 80% of the cellulase complex during fermentation with *T. viride*.
- β-1,4-glucosidase, or cellobiase. The extracellular concentration of these cellobiose-splitting enzymes is low, with the bulk of the enzyme, in *T. viride*, being intracellular.

Current status of cellulase research

Enzyme production has been optimized but cellulase yields are still low. The effects on *Trichoderma viride* of pH control, inoculum age, and culture medium additives have been tested in a basal medium containing KH_2PO_4 0.2%, $(NH_4)_2SO_4$ 0.14%, urea 0.03%, $MgSO_4 \cdot 7\ H_2O$ 0.03%, $CaCl_2$ 0.03%, $FeSO_4 \cdot 7\ H_2O$ 5.0 mg/l, $MnSO_4 \cdot H_2O$ 1.6 mg/l, $ZnSO_4$ 1.4 mg/l and $CoCl_2$ 2.0 mg/l. Maximal yields were obtained at 30°C after 140 hours' growth. As seen in Figure 16.8, yields are dependent on the cellulose concentration. Purification of the enzyme was carried out using ion exchangers.

Based on pilot plant experiments, computations can be made for the production of cellulase and for sugar production from cellulose with cellulases. Production costs have been quoted at \$0.011/l for crude culture broth and \$0.11/l for purified enzyme solution. Depending on the process and the extent of cellulose breakdown, the production cost to make fermentable sugar from cellulose ranges from \$0.037–0.489/kg sugar. The higher price corresponds to the world market price of sucrose in 1980. Obviously, it would not be feasible to use the higher-priced sugar equivalents as substrate for the production of SCP which itself entails a considerable production cost. Before SCP can be economically produced from cellulose, cellulase yields must show a drastic increase, less expensive pre-saccharification wood treatment methods must be developed, and energy-saving measures must be implemented throughout the process.

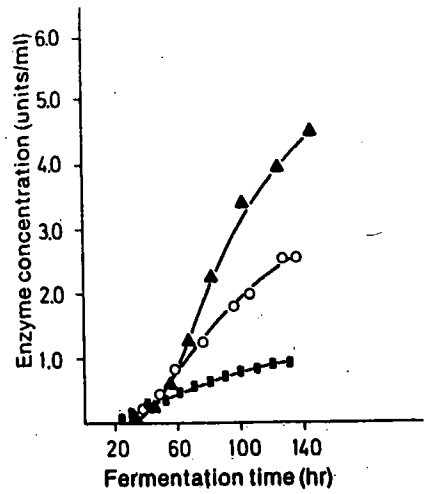

Figure 16.8 Effect of cellulose concentration on cellulase production in *Trichoderma viride* QM 9414. Squares, 0.94% cellulose; circles, 2.55% cellulose; triangles, 5.04% cellulose (Nystrom and DiLuca, 1978)

An alternate possibility is the direct fermentation of pretreated lignocellulose-containing materials with mixed cultures. The resulting biomass and the unmetabolized substrate can be used directly as animal feed.

16.6 SCP FROM CARBOHYDRATES

Large-scale cultivation of yeasts on molasses is widely used in the manufacture of baker's yeast, and in smaller-scale for food yeast and for the production of wine yeasts. In addition, several large-scale yeast processes employ whey (whose principal sugar is lactose) as a starting material.

A promising product is a mycoprotein which was introduced on the market in England in 1986. The fungus *Fusarium graminearum* is grown completely continuously on glucose with a dilution rate of 0.1 h^{-1}. Mineral salts (Na-, K-, Mg-, Mn-, Cu-, Ca-, Co-ions) and biotin are added, with ammonium ions serving as the nitrogen source. The pH is regulated at 6.0 and the incubation temperature is 30°C. From 1 kg of glucose 1 kg wet weight of fungal mycelium is obtained, with a protein content of 136 g. The yield is 3—3.5 kg/$m^3 \cdot h$. The culture taken directly from the fermenter is heated to 64°C to inactivate proteases and to activate endgenous RNases. After 30 min heating, the ribosomal RNA content is reduced from 9 to 1.1% and the breakdown products (principally 5' nucleotides) diffuse from the cells. The dried protein is eventually formed into structures that resemble pork, chicken, or beef meat and an appropriate artificial flavor added. For test marketing, ICI has used fermenters of 2 × 1.3 m^3 size, with scale-up in pilot size of 30 m^3. The final product is intended to be produced in the same fermenters that ICI used for Pruteen production (see Section 16.4).

16.7 SCP FROM SEWAGE

Domestic sewage is at present not suitable for large-scale SCP production but may be more important for methane production in the future (Section 17.5). Thus far, industrial waste water from cellulose processing, coffee production, starch production, and food processing have been used for SCP production. Sulfite waste liquors from paper and cellulose production have also been extensively used to produce SCP for the following reasons: Consistent availability of large quantities, low investment and production costs, and availability of organisms capable of high growth rates on the substrate.

Appropriate microorganisms for growth on sulfite waste liquor are *Candida utilis, C. tropicalis, Chaetomium cellulolyticum*, and *Paecilomyces varioti*.

A continuous process was used in a Finnish plant with two 360 m^3 fermenters. The SCP yields were 2.7–2.8 kg/$m^3 \cdot h$ using *Paecilomyces* at dilution rates of 0.2 h^{-1}. With a solution containing 32 g/l reducing sugar, 55% of the sug

was converted into biomass. This installation is no longer in operation.

In Czechoslovakia a SCP factory producing 25,000 tons per year is operating using the effluent from a paper-manufacturing facility. The so-called System Paskov uses *Candida utilis* in 3 × 800 m³ fermenters, operated continuously with a capacity of 3 × 1.5 tons/h. Product recovery involves two concentration steps in a separator (to 18% and 25%), followed by drying. In this installation, SCP production is an ancillary result of waste-water stabilization and purification.

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17

Newer approaches to sewage treatment

17.1 INTRODUCTION

In urbanized countries, vast amounts of industrial and domestic sewage are treated biologically. At present, sewage is treated primarily by the classic, aerobic, activated sludge process with surface aeration (Figure 17.1) or with oxygen supplied by forced air. In another type of aerobic treatment process (the trickling filter), a bed of stones or sand is used and the waste water is allowed to trickle down over the supporting medium, the organisms growing attached to the surfaces of the filter bed and oxidizing the organic compounds in the waste material. In another process, anaerobic digestion is used (Figure 17.2), occasionally directly on raw sewage or more frequently on the solid material (sludge) obtained from sedimentation in the aerobic treatment processes.

Aerobic systems have the following disadvantages: open construction which restricts process control, uncontrolled populations of organisms, environmental pollution due to odor emission, and fog formation.

The developments in sewage treatment over the past century have been carried out primarily in an empirical fashion, but aerobic and anaerobic sewage treatment processes are being increasingly studied scientifically, since it is felt that optimization will lead to the greatest success in improving these processes. Although studies on aerobic processes have been primarily directed to an understanding of carbon metabolism, in the future it is anticipated that research will be directed at sulfur, nitrogen, and phosphate metabolism, since elimination of these elements from sewage will become increasingly important. Further, accumulations of heavy metals in the stabilized sludge are being increasingly recognized to be of serious concern.

A further critical problem is the phenomenon of **bulking** in sludge, which inhibits the settling process in the clarification basins. Bulking is due to the development of filamentous microorga-

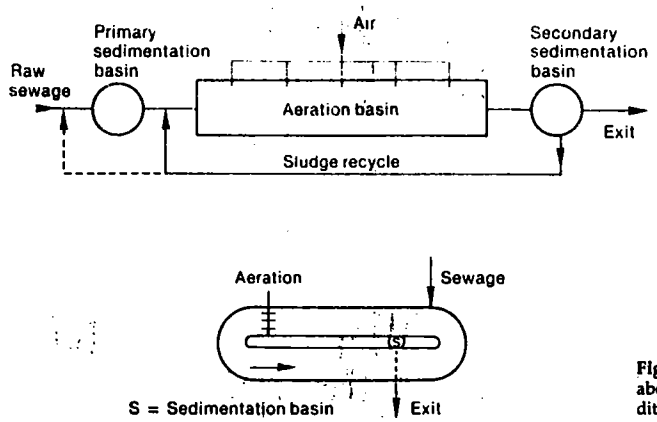

Figure 17.1 Activated sludge process: above, aeration basin; below, oxidation ditch

nisms that do not settle well. Although bulking can be controlled in some cases by introduction of flotation processes, in domestic sewage this technical solution is too expensive. Therefore, a microbiological solution to the bulking problem must be found so that a satisfactory clear effluent can be delivered from the final settling basin.

One of the most encouraging developments over the past several decades has been the remarkable decrease in organic material in industrial effluents. This has resulted from extensive application of research and development efforts and considerable expenditures for new treatment installations.

In this chapter four new processes will be discussed which have the potential for increasing the efficiency of sewage treatment.

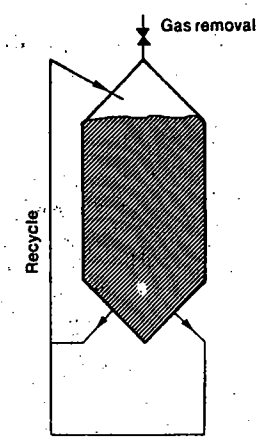

Figure 17.2 Sludge digester

17.2 STARTER CULTURES FOR TREATMENT PROCESSES

Conventional sewage treatment involves the use of microorganisms which develop naturally within the sewage treatment system, no attempt being made to optimize the organisms involved. An approach which may have some potential for increasing the efficiency of the sewage treatment process is to inoculate the system wih microorganisms which have been specially selected for the particular sewage-treatment process. In analogy with their use in food fermentations, such organisms might be called "starter cultures". Although starter cultures might find use in the treatment of domestic sewage, it seems more likely that they will find use in the treatment of special or unusual industrial wastes or in the treatment of accidental spills of industrial chemicals. Such wastes often cannot be channeled into ordinary treatment plants because their toxicity and lack of biodegradability cause significant damage to the nonadapted organisms. Another way in which such starter cultures might be used is in shortening the start-up time that is generally required after a sewage treatment plant is shut down for one or another reason. Before the system can become fully operative once again, the optimal bacterial culture mixture must be re-established; this usually requires a week-long enrichment process. A starter culture could be expected to shorten this start-up time. In the United States, starter cultures resembling those of the dairy industry have been developed and are suitable for a variety of special applications in sewage treatment, such as tank cleaning, pipeline cleaning, start-up of city purification plants, and breakdown of special substances contained in sewage.

Bacteria from cold habitats have been isolated which degrade alkanes and aromatic compounds at 0–15°C in saline habitats; these could be useful in the degradation of oil spills in the ocean. Mixed cultures which metabolize DDT, polychlorinated diphenols, and phenols or which possess high protease, lipase, or cellulase activity are also on the market. If special operating conditions are desired, such as growth at pH≤5.0 or ≥9.0, these requirements can also be met with selected enrichment cultures.

A patented process has been developed with a strain of *Pseudomonas putida* containing plasmids which code for the breakdown of octane, xylene, metaxylene, and camphor. Starter cultures have also been used to deodorize animal excrements.

In 1978, the starter-culture industry grossed $2–4 million in the United States, but the potential total value is estimated at $200 million. Twenty manufacturers produce single strains or mixed cultures under sterile conditions in vessels up to 10 m³ capacity. The culture conditions used are determined by how the cultures are to be used, in order to ensure that the cells are fully induced. Research in this field is still in its infancy and scientific studies on the breakdown of individual compounds are underway, but practic applications are not yet widespread.

17.3 AEROBIC SEWAGE TREATMENT—AIRLIFT PROCESS

A disadvantage of customary aerobic sewag treatment by the activated sludge system is th low efficiency of oxygen transfer and the larg amount of space required by the installation. Be cause these installations are open to the atmos phere, another problem is the odor which the emit. Tower reactors have been developed i which aeration and oxygen utilization are im proved and the efficiency of the overall proces increased. Both tube reactors and airlift fermen ters have been used. These installations are fa vorable economically because of 30% less spac required, 20% lower investment costs, and 20 less energy costs.

The British chemical company ICI uses a tu bular loop reactor which is embedded 100 meter into the ground. Two German companies, Baye (Biotower) and Uhde/Hoechst (Bio-high Reac tor) have constructed bioreactors 30 m in height In these systems, the circular settling basins ar located around the top rim of the bioreactor (Fig ure 17.3). The dimensions of this type of acti vated sludge fermenter allow a considerably bet ter oxygen-transfer efficiency. Table 17.1 show performance data from various systems of th

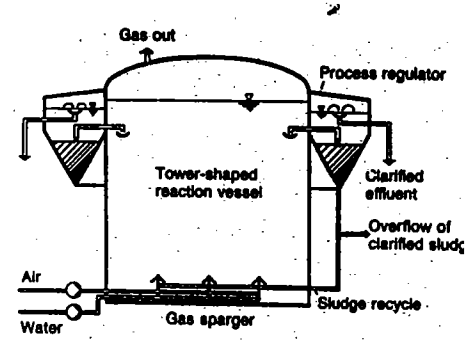

Figure 17.3 Bayer Biotower

Table 17.1 Data from an activated sludge system (Uhde/Hoechst)

Water depth (m)	4	8	20
O_2-Saturation (g O_2/m³)	13	17	28
Oxygen yield (%)	8–15	15 –30	40–60
Air required (Nm³/kg O_2)	42–23	23 –11	9– 6
Energy-specific O_2 input (kg O_2/kWh)	2– 2.5	2.5– 3	3– 3.5
Bioreactor diameter (m)	18	13	8
Reactor surface area for 1000 m³ volume (m²)	250	125	50

Table 17.2 Parameters for a Bayer Biotower in continuous operation

Process	Parameters
Pretreatment	Screening, neutralization, primary sedimentation
Bioreactors	4 parallel, rubber-lined tanks (26 × 30 m) with 13,600 m³ total volume
Aeration	22,000 m³/h
Sewage	
From industry	90,000 m³/d with 95 tons/d BOD
From domestic sources	70,000 m³/d with 14 tons/d BOD
Residence time	14.5 h
Treatment capacity	105 tons/d BOD
Oxygen requirements	120 tons/d O_2

Uhde/Hoechst type and Table 17.2 shows data for the Bayer system.

The Hoechst system has baffles to facilitate mixing. In the Bayer Biotower process, two-component nozzles have been developed as injectors by means of which the kinetic energy of the liquid stream is utilized to disperse air in small gas bubbles in a manner similar to a water spray pump (Figure 17.4). These injectors are installed in groups of four (injector clusters) above the bottom of the tower reactor (1 injector/1–2 m² bottom surface).

17.4 AERATION WITH PURE OXYGEN

Aeration of activated sludge treatment facilities with pure oxygen has been tried in both the

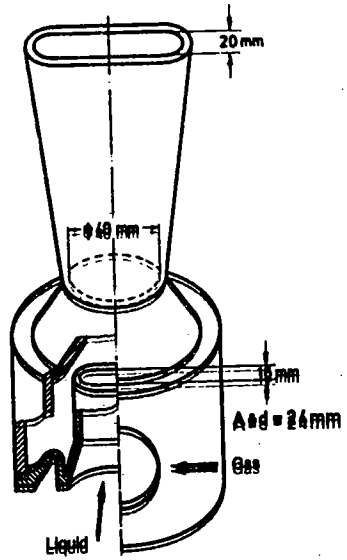

Figure 17.4 Bayer injector (Zlokarnik, 1985)

United States and the Federal Republic of Germany. The objective has been to increase the efficiency of oxygen transfer, since the loading capacity of a conventional purification plant and the size of the microbial population are limited by the low solubility of dissolved oxygen.

Pure oxygen has 4.8 times the partial pressure of oxygen from the air, so that aeration with pure oxygen markedly increases the oxygen content of the activated sludge system. Dissolved oxygen in such a system is 90–95% utilized, and the off gas (1% of that from conventional aeration) can subsequently be treated chemically or thermally in the closed system in order to prevent unpleasant odors.

High loading capacity in the oxygen process allows smaller aeration tanks and smaller settling basins. Due to the smaller amount of sludge produced, the costs of facilities for further treatment are also reduced. On the negative side is the higher capital investment, the more complicated

installation, the necessity for careful control of the process parameters, and the need for highly trained personnel.

In both domestic and industrial installations systems are in use that employ pure oxygen. These systems are especially used in the paper, chemical, and food industries.

The Unox system is one such example. The first aeration stage consists of 2 channels, each of which is connected in a cascade arrangement to three bioreactors (Figure 17.5) with capacities of 1500, 750 and 730 m³. In the first bioreactor (activated sludge basin), pure oxygen from an air-separation plant is forced in via injectors. In this stage, 90–93% of the organic, decomposable material is eliminated. The CO_2 produced is collected along with the residual oxygen and treated at 1000°C. In the second treatment stage (settling basin), the accumulating sludge is pumped back into the activated sludge basin. The waste water flows through this second treatment stage to remove material which is not easily broken down (Figure 17.6). After a third treatment stage, the clarified waste water proceeds to the receiving stream. Table 17.3 gives performance characteristics using the Unox process.

17.5 METHANE PRODUCTION

Methane, or natural gas, is a microbial product of the anaerobic decomposition of organic matter. Methanogenesis is a widely used process in organic waste disposal, primarily because the methane gas, being insoluble, is readily removed from the treatment system. Since it is a fuel, the methane produced in the treatment system can also be used as a source of energy. In some agricultural situations, the methane formed is a significant energy source (biogas) and the main goal of the treatment process is the maximization of

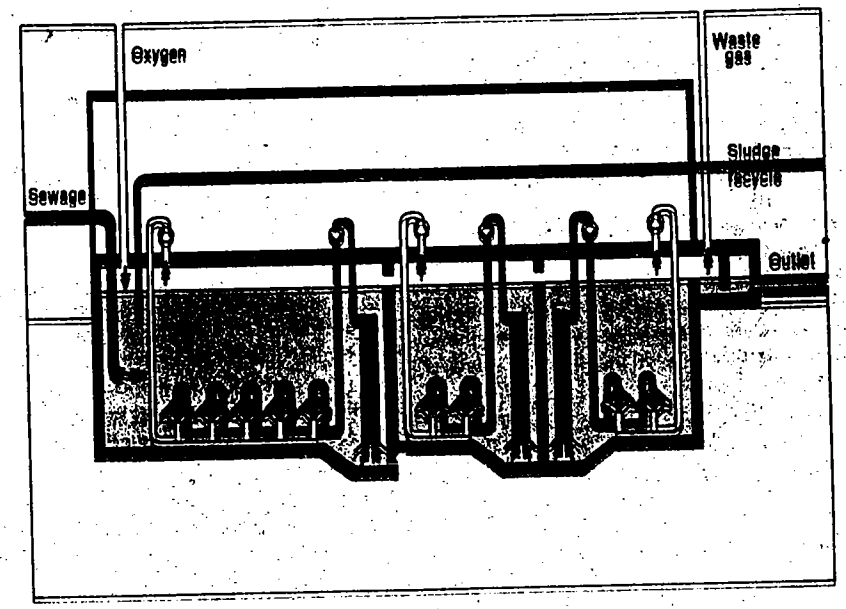

Figure 17.5 Diagram of an activated sludge system using pure oxygen

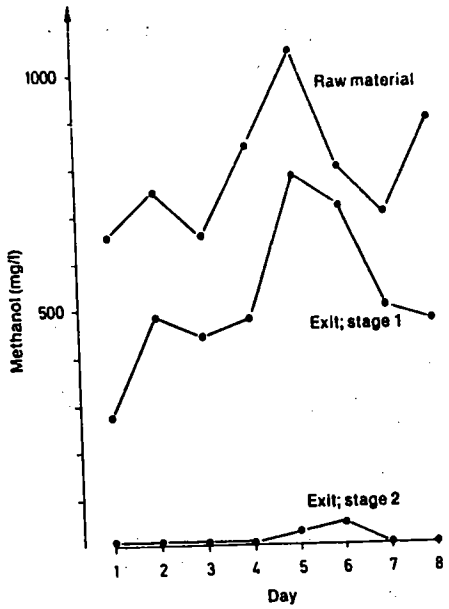

Figure 17.6 Methanol breakdown in a two-stage purification plant using pure oxygen (Mack, 1973)

methane yields. Anaerobic installations for sewage treatment are called **digestors**, because the primary process taking place is the digestion of the organic material to soluble and gaseous constituents. In most waste treatment systems, however, even though the methane may be used as a fuel, the main goal has not been the production of methane as an energy source but rather the stabilization of the sludge so that it can be safely disposed of in the environment. In such systems, little attention has been given to the optimization of the methane production process. In one study of 275 sewage treatment systems in western Europe, only a small fraction of the methane produced was distributed to other users, although 75% of the gas produced was used as an energy source within the treatment plants themselves.

In comparison to aerobic processes, anaerobic process have some significant advantages. Energy expenditure for aeration is not required, saving considerably on operating costs. The organic waste is converted almost quantitatively (>90%) to methane and CO_2. Biomass formation is low, so that the cost of sludge disposal is lower. Also, odor problems are decreased since the process is carried out in closed reactors. Finally, the methane produced can be utilized as an energy source.

However, the anaerobic process is not suitable for the more dilute liquid wastes that are primarily treated by the aerobic activated sludge processes.

Table 17.3 Data from a Unox purification plant with pure oxygen

Amount of sewage	20,000 m³/day
Chemical oxygen demand (COD)-breakdown capacity	61 tons/day
Biological oxygen demand $(BOD)_5$-breakdown capacity	34 tons/day
Oxygen input	
Activation stage 1	51 tons/day
Activation stage 2	7 tons/day
Volume	
Activation stage 1	6200 m³
Activation stage 2	3200 m³
Intermediate purification:	4400 m³
Final purification	4800 m³
Time required	
Activation stage 1	7.5 h
Activation stage 2	3.8 h
Intermediate purification:	5.3 h
Final purification	5.8 h
Breakdown activity	
Activation stage 1	max. 80% COD max. 93% BOD_5
Activation stage 2	max. 7% COD, total 87% max. 5% BOD_5, total 98%
Sludge load	
Activation stage 1	0.7 kg BOD_5/kg dry weight·d
Activation stage 2	0.2 kg BOD_5/kg dry weight·d
Space requirement	
Activation stage 1	5.6 kg BOD_5/m³·d
Activation stage 2	0.8 kg BOD_5/m³·d
Excess sludge	25% of the BOD_5 load

(Lemke and Mack, 1978)

The methanogenic bacteria constitute a unique group of organisms which are the final and key link in the breakdown of organic matter in the anaerobic food chain. The methanogenic bacteria are able to utilize only a restricted group of substrates for the production of methane, including: acetate, methanol, formate, and $H_2 + CO_2$. In waste disposal systems, about 75% of the methane is derived from acetate and most of the rest from $H_2 + CO_2$. The starting materials of the anaerobic decomposition process are complex polymeric materials such as cellulose, starch, fats, and proteins, none of which the methanogenic bacteria are able to use. The methanogenic bacteria are consequently dependent upon other anaerobic fermentative organisms for the initial breakdown of the substrates and the production of acetate and $H_2 + CO_2$.

Three groups of microorganisms participate in the anaerobic process. The first group breaks down the original organic material (starches, fats, proteins) into organic acids (propionic acid, butyric acid, acetic acid, lactic acid, and valeric acid), alcohol, H_2, and CO_2. Since one of the constituents of the initial load, lignin, is not broken down anaerobically, any lignocelluloses in the starting material are metabolized slowly. The group of bacteria involved in this initial breakdown of organic matter include obligate anaerobes such as the clostridia and facultative anaerobics such as the streptococci and enteric bacteria.

The second group of bacteria convert the longer-chain fatty acids (for instance, propionic and butyric acids) and alcohols to acetic acid, H_2, and CO_2. This conversion is endergonic at pH 7 and can only occur if coupled with exergonic reactions. Thus, the reaction only occurs in mixed cultures. For example, ethanol is converted to acetic acid and H_2 by one bacterium and in a second reaction a methane bacterium utilizes the H_2, thus pulling the reaction:

$$CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2H_2$$
$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$$

The third group of organisms are the extremely anaerobic methanogens. As noted, only a limited range of substrates are used by these bacteria. Most methanogens will use H_2 as energy source and CO_2 as carbon source and electron acceptor. A few species of morphologically diverse groups (cocci, sarcinae, and spirillae) also metabolize formic acid, methanol, or methylamine. The most important organic substrate of methanogens is acetic acid, which is fermented in a reaction that is only weakly favorable energetically:

$$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$$
$$\Delta G^0 = -37 \text{ kJ}$$

Because of this, these bacteria grow very slowly and are the most critical organisms in the mixed culture of the anaerobic digestor.

Because of the low biomass formed and the low energy yield, the rate of the digestion process is low, the average residence time in the digestor being greater than 20 days. Immobilization of microorganisms in a fixed-bed reactor can be used to increase greatly the biomass concentration. Solutions with very high organic loads (chemical oxygen demand >3 g O_2 consumption/l) can be treated with such fixed-bed reactors within a few hours. Some studies have been carried out using porous sintered glass as carrier material for fixed-bed reactors. In a 1000 l reactor the biochemical oxygen demand (BOD) was reduced from 6.4 kg/m³ to 1.3 kg/m³ in 4.8 hours. In another installation for treating high-concentration wastes from a fermentation plant, a two-stage process was used, the first reactor being used for acid production, the second for methanogenesis. Both reactors were 380 m³ in volume and sand was used as the carrier. Waste volumes of 150–200 m³/h were treated within 1–1.5 hours with a removal efficiency of 30 kg COD/m³·day.

In the United States, some studies have been done to produce methane from the biomass of the water hyacinth, a plant which causes considerable problems by excess growth in canals and streams in the warmer parts of the country. Under optimal conditions, about 2 tons dry mass

per day and per hectar could be treated, yielding theoretically 800 m³ biogas.

When optimizing methanogenesis, it is important to note that the rate is affected by the nature of the substrate as well as by the temperature (Table 17.4).

With mixed sludges, as much as 600 l biogas per kg dry organic matter have been produced. The gas consists of about 60–70% methane, about 25–30% CO_2, and the rest H_2 and N_2. Developing countries represent the most promising areas for further development of biogas production, since energy is often in short supply and waste disposal can often not be done by the elaborate processes used in developed countries. In such cases, the efficiency of the process is less critical than the energy produced; biogas production in developing countries can often be accomplished in simple installations with little requirement for complicated engineering skills. Biogas can also be used as a motor fuel in those regions where petroleum sources are expensive or in short supply.

Table 17.4 Biogas production in relation to temperature

Substrate	Temperature °C	% Dry weight in input	Time h	l Methane/ g dry weight
Sludge	35–40	2.1	23	0.488*
	50	2.1	9	0.488*
	60	2.1	12.5	0.488*
Sludge	30	3	33–50	0.371
	50	3	20	0.368
Waste and sludge	35	3	30	0.288*
	50	3	10	0.369*
	60	3	4	0.455*
Cow manure	55	2	3	0.16
			9	0.19
	55	6	3	0.16
			9	0.19
	55	10	3	0.10
			9	0.19

*=Biogas
(Kandler, 1979)

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18

Leaching

18.1 INTRODUCTION

Microbial leaching is the process by which metals are dissolved from ore-bearing rocks using microorganisms. At present a number of ores cannot be economically processed with chemical methods due to their low metal content. In addition, large quantities of low-grade ores are produced during the separation of higher-grade ores and are generally discarded in waste heaps. Throughout the world there are vast quantities of such low-grade copper ores that cannot be profitably purified by conventional chemical methods, but that could be processed by microbial leaching. There are also significant quantities of nickel, lead, and zinc ores which could be leached.

Leaching was first discovered as a process occurring in pumps and pipelines installed in mine pits containing acidic water. It was subsequently developed for the recovery of metal from low-grade ores. For many metals, there are now leaching methods which permit extraction from metal sulfides or other ores. The metals are converted to water-soluble metal sulfates with the aid of biochemical oxidation processes.

In commercial applications, copper and uranium have been widely produced through the use of microorganisms. However, there have been difficulties in extrapolating the results from laboratory and pilot-plant studies into practical field conditions. In addition, problems may arise when the large-scale leaching process of a waste dump is improperly managed. Leach fluids containing large quantities of metals and having extremely low pH values (pH 3) can seep from such dumps into nearby natural water supplies and ground waters, causing enormous and lasting damage.

18.2 ORGANISMS FOR LEACHING

The two most commonly used organisms in microbial leaching are *Thiobacillus thiooxidans* and *Thiobacillus ferrooxidans*. A number of others may also be used including: *Thiobacillus concre-*

tivorus, Pseudomonas fluorescens, P. putida, Achromobacter, Bacillus licheniformis, B. cereus, B. luteus, B. polymyxa, B. megaterium, and several thermophilic bacteria including *Thiobacillus thermophilica, Thermothrix thioparus, Thiobacillus* TH1, and *Sulfolobus acidocaldarius*. The heterotrophic organisms listed have not as yet actually been used, but it seems likely that processes will be developed by which metals are extracted from ores with microbially produced organic acids via chelate and salt formation. Because of their more rapid growth rate, the thermophilic bacteria may significantly accelerate the leaching process.

18.3 CHEMISTRY OF MICROBIAL LEACHING

Thiobacillus ferrooxidans is the organism that has been most extensively studied. It is a Gram-negative rod-shaped bacterium which is 0.5–0.8 $\mu m \times 1.0$–$2.0\ \mu m$ in size. An autotrophic aerobe, it can obtain carbon for biosynthesis solely from CO_2 fixation, and obtains its energy from the oxidation of Fe^{2+} to Fe^{3+} or from the oxidation of elemental sulfur and reduced sulfur compounds to sulfate.

$$4\ FeSO_4 + 2\ H_2SO_4 + O_2 \rightarrow 2\ Fe_2(SO_4)_3 + 2\ H_2O \quad [1]$$

$$2\ S^0 + 3\ O_2 + 2\ H_2O \rightarrow 2\ H_2SO_4 \quad [2]$$

$$2\ FeS_2 + 7\ O_2 + 2\ H_2O \rightarrow 2\ FeSO_4 + 2H_2SO_4 \quad [3]$$

The oxidation of insoluble sulfur to sulfuric acid, which is also performed by *Thiobacillus thiooxidans*, occurs in the periplasmic space. According to equation 3, iron is dissolved through **"direct bacterial leaching"**.

In addition to this leaching process performed only by microorganisms, there is another process, "indirect, bacterially supported leaching" which takes place slowly in the absence of microbes. The oxidation of pyrite can be used as an example. Pyrite is a common rock mineral that is found in association with many ores. The following equation describes the initial oxidation of pyrite by ferric ions:

$$FeS_2 + Fe_2(SO_4)_3 \rightarrow 3\ FeSO_4 + 2\ S^0 \quad [4$$

The sulfur which is formed via this process is reoxidized as shown in equation 2.

Examination of leaching dumps always shows the presence of mixtures of *T. thiooxidan* and *T. ferrooxidans*. In pilot-plant reactors (50 li ter), leaching can be performed continuously i a cascade series with recycling of the cells an leachate.

Yields such as those in other areas of micro biology can be attained in the laboratory unde optimal conditions (temperature control, O_2 an CO_2 adjustment, maintenance of pH around 2 3 and Eh around -300 mV) with very finel ground ores in a tower (percolator), or better ye in fermenters under optimal conditions. How ever, in field experiments, these conditions an yields cannot be realized due to the high cost.

18.4 COMMERCIAL PROCESSES

Three methods have practical application (Figu 18.1):

- **Slope leaching.** Finely ground ores (up 100,00 tons) are dumped in large piles dow a mountainside and continuously sprinkl with water containing *Thiobacillus*. The wat is collected at the bottom and reused aft metal extraction and possible regeneration the bacteria in an oxidation pool.
- **Heap leaching.** The ore is arranged in lar heaps and treated as in slope leaching.
- **In-situ leaching.** Water containing *Thiob cillus* is pumped through drilled passages unextracted ore which remains in its origin location in the earth. In most cases, t permeability of the rock must be first i creased by subsurface blasting of the ro The acidic water seeps through the rock a

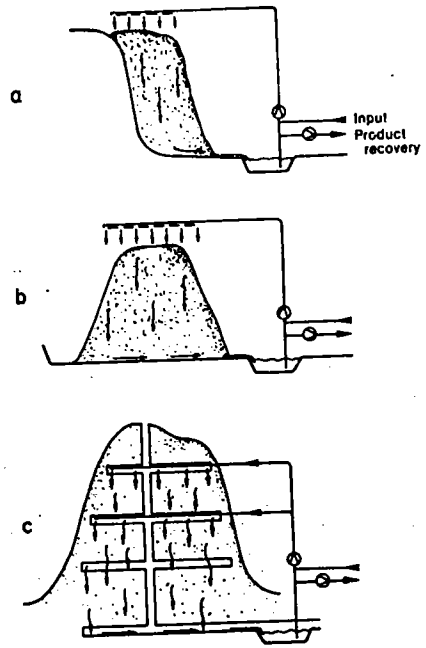

Figure 18.1 Diagram of (a) slope, (b) heap, and (c) in-situ leaching

collects in the bottommost cavity from which it is pumped, the minerals extracted, and the water reused after regeneration of bacteria.

Copper leaching

If chalcocite, chalcopyrite, or covellite are used for the production of copper, several metals are usually found together. For example, chalcopyrite contains 26% copper, 25.9% iron, 2.5% zinc, and 33% sulphur. Chalcopyrite is oxidized as follows:

$$2\ CuFeS_2 + 8\ 1/2\ O_2 + H_2SO_4 \rightarrow$$

$$2\ CuSO_4 + Fe_2(SO_4)_3 + H_2O \qquad [5]$$

Covellite is oxidized to copper sulfate:

$$CuS + 2\ O_2 \rightarrow CuSO_4 \qquad [6]$$

Copper leaching plants have been in wide use throughout the world for many years, generally operated as simple heap leaching processes but sometimes as combinations of heap and in-situ leaching. The leaching solution (sulfate/Fe^{3+} solution) carries the microbial nutrients in and the dissolved copper out. The solution is sprinkled over the heap and percolates through the rock pile to the lower level where the copper-rich liquid is collected. The copper-containing solution (up to 0.6g/l) is removed, the copper is precipitated, and the water is reused after readjusting the pH to 2.

Countries in which microbial leaching of copper has been widely used include the United States, Australia, Canada, Mexico, South Africa, Portugal, Spain, and Japan. About 5% of the world copper production is obtained via microbial leaching. A single installation in the United States has produced up to 200 tons of copper per day.

Uranium leaching

Although less uranium than copper is obtained by microbial leaching, the uranium process is more significant economically. Because a thousand tons of uranium ore must be handled to obtain one ton of uranium, in-situ microbial leaching is gaining greater acceptance, since it eliminates the expense of moving such vast amounts of material.

In the uranium leaching process, insoluble tetravalent uranium is oxidized with a hot H_2SO_4/Fe^{3+} solution to soluble hexavalent uranium sulfate.

$$UO_2 + Fe_2(SO_4)_3 \rightarrow UO_2SO_4 + 2\ FeSO_4 \qquad [7]$$

This is an indirect leaching process since the microbial attack is not on the uranium ore directly but on the iron oxidant. Ferric sulfate and sulfuric acid can be produced by *T. ferrooxidans* from the pyrite within the uranium ore.

$$2\ FeS_2 + H_2O + 7.5\ O_2 \rightarrow Fe_2(SO_4)_3 + H_2SO_4 \qquad [8]$$

The pyrite reaction is used for the initial production of the Fe^{3+} leach solution. Pilot plants operate with surface reactors similar to the trickling filters used in sewage.

Optimal uranium leaching conditions are pH 1.5–3.5, 35°C and 0.2% CO_2 in the incoming air. Some thermophilic strains are known which have a temperature optimum of 45–50°C.

In commercial processes, the dissolved uranium is extracted from the leach liquor with organic solvents such as tributylphosphate and the uranium is subsequently precipitated from the organic phase. Adsorption of the uranyl ions with ion exchangers is another possibility. The organic solvents which remain in the water system after extraction may be toxic and hence cause problems when the microbiological system is reused.

In-situ leaching has the disadvantage that the permeability of the rock may be low and the drilled passages may not always allow an adequate supply of nutrients and oxygen to enter deeply into the ore. In such situations the heap system is often still used commercially for leaching of uranium.

Areas where uranium leaching has been carried out include the United States, Canada, and South Africa.

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19

Extracellular polysaccharides

Polysaccharides are used commercially to produce gels, and to thicken and stabilize foods, medicines, and industrial products. They are also used as polymers in the fluids used to force oil to the surface in the tertiary oil recovery process. Although polysaccharides of plant origin (such as starch, alginate, or agar) have been used for many years, microbial polysaccharides have become widely used over the past several decades. Both intracellular polymers such as polybutyric acid and poly-β-hydroxybutyric acid/polyhydroxyvaleric acid copolymer, and extracellular polysaccharides are produced commercially. Around 20 different microbial polysaccharides with market potential have been described, but the largest part of the market is held by xanthan, with production of about 10,000 tons per year. However, microbial polysaccharides are really only a small part of the polysaccharide market as shown by the fact that xanthan holds only about 4% of the market. An important aspect of a polysaccharide, if it is to have market potential, is its rheological properties. Microbial polysaccharides vary greatly in such rheological properties as pseudoplasticity, thixotrophy, and viscoelasticity.

Table 19.1 shows the most important microbial exopolysaccharides produced commercially. In this list, alginate has thus far been produced commercially only from seaweed, but in the future *Azotobacter* may be used. Alginate and xanthan are anionic polysaccharides composed of uronic acid residues.

The **biosynthesis of heteropolysaccharides** is comparable to that of bacterial cell wall components. Proceeding from glucose, the appropriate sugar nucleotide is produced via glucose phosphate, followed by transformation of the glucose into another sugar. At the sugar nucleotide level, further transformations can take place, such as UDP-mannose → UDP-mannuronic acid. The transport of monosaccharides through the membrane and out of the cell takes place after coupling to a C-55 isoprenoid alcohol phosphate. Relatively little is known about the manner in which the polysaccharide polymerase on the out-

Table 19.1 Microbial polysaccharides with commercial uses

Polysaccharide	Structure	Organism
Xanthan	– Glc 1 $\xrightarrow{\beta}$ 4 Glc $\uparrow^{3}_{1}\alpha$ Mann 6-OAc $\uparrow^{2}_{1}\beta$ GlcA $\uparrow^{4}_{1}\beta$ Mann 4,6-OPyruvate	*Xanthomonas campestris*
Alginate	– 4 D–MannA 1 $\xrightarrow{\beta}$ 4D –MannA 1 –; – 4 L– GulA 1 $\xrightarrow{\alpha}$ 4 L – GluA	*Pseudomonas aeruginosa, Azotobacter vinelandii*
Curdlan	– Glc 1 $\xrightarrow{\beta}$ 3 Glc –	*Alcaligenes*
Sclero-glucan	– Glc 1 $\xrightarrow{\beta}$ 3 Glc – $\uparrow^{6}_{1}\beta$ Glc	*Sclerotium glucanicum, S. delphinii, S. rolfsii*
Pullulan	→ 6 (Glc 1 $\xrightarrow{\alpha}$ 4 Glc 1 $\xrightarrow{\alpha}$ 4 Glc) 1 →	*Aureobasidium pullulans*
Dextran	– Glc 1 $\xrightarrow{\alpha}$ 6 Glc – several 1 → 2, 1 → 3 and 1 → 4 compounds	*Acetobacter* sp., *Leuconostoc mesenteroides, Leuconostoc dextranicum, Streptococcus mutans*

Glc = Glucose, Mann = Mannose, GlcA = Glucuronic acid, MannA = Mannuronic acid, GulA = Guluronic acid, Ac = Acetate

side of the cell membrane acts to bring about chain lengthening and termination and the influence on the development of secondary and tertiary structure. An additional factor influencing the structure of the resulting polysaccharide is degradation by microbial enzymes. It is known for alginate and pullulan, for instance, that at the end of the fermentation enzymes are excreted which degrade these polymers and hence reduce the viscosity of the final product.

Various factors regulate the rate of exopolysaccharide biosynthesis. Molecular oxygen (>90% saturation) is necessary not only for primary energy metabolism but also for the oxidation of sugar to the corresponding alcohol and for reoxidizing reduced pyridine nucleotides. A carbon/nitrogen ratio of around 10:1 is generally most favorable for optimal yield. For some polysaccharide-forming organisms, *Xanthomonas, Pseudomonas, Azotobacter,* and *Aureobasidium,* growth and product formation can be described quantitatively by the Leudeking-Piret equation:

$$\frac{dP}{dt} = nX + m\frac{dX}{dt}$$

Especially in large-scale fermentations, the marked increase in viscosity which occurs as the polysaccharide concentration increases causes the stirring rate to decrease, resulting in a slowing of the mixing time. This leads to a reduction in mass transfer efficiency and hence to the formation of a polymer of heterogeneous quality.

Isolation of the polymer from the fermentation broth involves precipitation by treatment with an organic solvent, a salt, or an acid. The producing bacteria become entrapped in the precipitating polymer and are carried out of the broth also.

Xanthan

Xanthan was the first heteropolysaccharide to b commercially available. It is produced by *Xan thomonas campestris,* and was developed in th late 1950's and marketed in 1964. The molecula weight of this polymer is around 2–15 × 1(Daltons. As shown in Figure 19.1, the basic re peating unit of xanthan consists of a pentasac charide containing glucose, mannose, glucuron acid, acetate, and pyruvate. The number of py ruvate units in the molecule and the molecula weight determines the viscosity of the resultir xanthan gum.

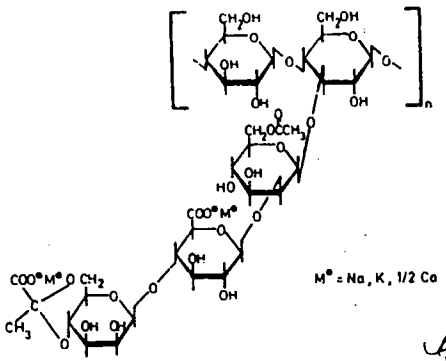

Figure 19.1 Repeating unit of a xanthan gum

For commercial production, the nutrient solution consists of 4–5% carbohydrate (glucose, sucrose, corn starch hydrolysate), 0.05–0.1% nitrogen (yeast extract, peptone, ammonium nitrate, or urea) and salts, with the pH controlled at 7.0. During the 2-day batch culture, an increase in viscosity begins during the log phase and continues into the resting phase. Large-scale fermentations can have an apparent viscosity up to 20–30,000 centipoise. However, viscosities greater than 10,000 cP may cause technical problems. The efficiency of production of xanthan in weight per weight of carbohydrate used is 70–80% and the yield is 25–30 g/l. Product recovery is carried out by precipitation of the polysaccharide with isopropanol or methanol, a step which also kills the culture. The precipitated xanthan is then dried and ground. Production in continous fermentation has been successfully tested; the effect of the limiting nutrient and the dilution rate on xanthan yield is shown in Table 19.2.

In one actual installation capable of producing 2000 tons of xanthan per year (40 hour fermentation time, yield of 0.7 kg xanthan/kg glucose, 2.5% xanthan final concentration), calculations showed that the production cost per m^3 fermenter volume was markedly lower with the continuous process.

Table 19.2 Influence of limiting nutrient and dilution rate on yield of xanthan

Limitation	Medium	Dilution rate (h^{-1})	Yield (%)
Nitrogen	Complex	0.023	71
Nitrogen	Complex	0.054	82
Sulfur	Synthetic	0.035	60
Sulfur	Synthetic	0.050	32
Phosphate	Synthetic	0.030	40
Magnesium	Synthetic	0.050	28

Yield based on added glucose
Data of Sutherland (1983)

Alginate

Pseudomonas aeruginosa and *Azotobacter vinelandii* produce alginate from two uronic acids, mannuronic acid and guluronic acid, in proportions of 4:1 to 20:1. Sodium alginate is a commonly used agent for the immobilization of microorganisms (see Chapter 11) and also finds use as an ion-exchange agent.

Curdlan

Alcaligenes faecalis var. *myxogenes* produces two β-1,3 glucans, curdlan and succinoglucan. Succinoglucan contains glucose, about 10% succinic acid, and galactose, whereas curdlan is a simple glucose polymer. Yields of curdlan of 40 g/l are obtained on a defined medium containing 8% glucose in the course of the 80-hour fermentation. Because curdlan is water insoluble, the viscosity does not increase during the fermentation; it only begins to form a gel at temperatures above 54°C. There is an increase in viscosity with succinoglucan, however, so it must be produced from a 4% glucose medium, with an efficiency of production of 35%.

Scleroglucan

Sclerotium glucanicum, *S. delphinii*, *S. rolfsii* and *Helotium* sp. produce scleroglucan, a polysaccharide with glucose units connected primarily in β-1,3- with occasional β-6,1-glycosidic bonds. The organisms grow on hydrolyzed lignocellulose substrates. Scleroglucan is used in food production and is under development for use in tertiary petroleum recovery.

Figure 19.2 shows the kinetics of the production of scleroglucan with *Sclerotium rolfsii* growing on glucose under nitrate limitation. Production (fermentation, purification and possibly enzymatic splitting) of scleroglucans has been shown to be an economically feasible process, provided the conditions of production are optimized.

Pullulan

Pullulan, a glucan with α-1,4- and a few α-1,6-glycosidic bonds, is produced by the fungus *Aureobasidium pullulans* from a 5% glucose solution with 70% efficiency within 5 days. The enzyme pullulanase, produced by bacteria, was discussed in Section 11.2. The physiology of pullulan formation has been intensively studied; Figure 19.3 shows the fermentation kinetics of pullulan production.

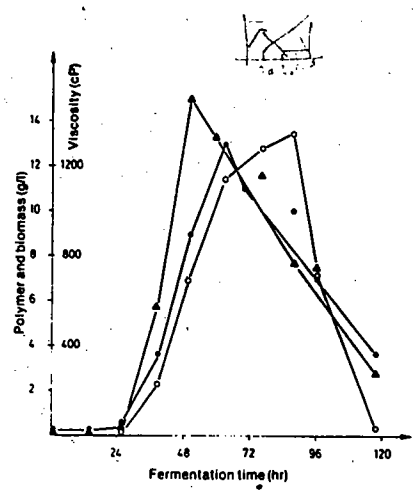

Figure 19.2 Scleroglucan production by *Sclerotium rolfsii* ATCC 15206 during nitrate limitation. ● —— ● biomass; ▲ —— ▲ scleroglucan; O —— O viscosity (Griffith and Compere, 1978).

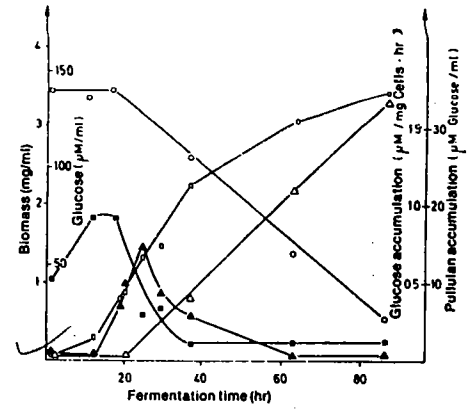

Figure 19.3 Pullulan production by *Aureobasidium pullulans* in a medium with 140mM glucose and 4.5 mM ammonium sulfate. □ —— □ cell dry weight (mg/ml); O —— O extracellular glucose (μM/ml); △ —— △ pullulan (μM anhydroglucose/ml); ■ —— ■ ^{14}C-accumulation rate as a measure of glucose assimilation rate (μM/mg cells·h); ▲ —— ▲ pullulan formation rate (μM anhydroglucose/mg cells·h) (Catley, 1973).

Dextran

Dextrans are important as blood plasma extenders and are also used in food production. They are a complex group with molecular weights from 15,000–500,000. Most occur as glucans with 1,6-glycosidic bonds, but some dextrans also have 1,2-, 1,3-, or 1,4-glycosidic bonds.

The polysaccharide dextran is produced by the enzyme dextransucrase, a transglucosidase which acts on sucrose (glucose-fructoside), polymerizing the glucose units into dextran and liberating free fructose. This enzyme is produced by many strains of the lactic acid bacterium *Leuconostoc mesenteroides*. In contrast to other exopolysaccharides, dextran is produced extracellularly in the medium. Commercial dextran production can be carried out in a batch process with *L. mesenteroides* with a medium containing inorganic phosphate and an organic nitrogen source. The crude dextran (MW 500,000) obtained through alcohol precipitation is treated

with acid for hydrolysis. The desired dextrans (MW 40,000 to 60,000) are subsequently fractionated by ethanol precipitation and dried. The batch process has been further developed into a strictly enzymatic process, in which the cells are separated from their medium, which now contains the enzyme, after fermentation. The extracellular dextransucrase is able to carry out the transformation of sucrose into dextran in the cell-free nutrient solution at pH 5.0–5.2 and a temperature of 25–30°C. The fructose remaining in the solution can be recovered.

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20 Other fermentation processes and future prospects

In this chapter we discuss several fermentations that do not fit into the categories established in the previous chapters, including several which are not now of commercial use but may become significant in the future.

Gibberellins

The gibberellins are one class among the five known classes of phytohormones. Gibberellins are used as growth hormones and have found application with barley to improve grain quality in malt production. Sales of plant growth regulators are estimated at $40 million for 1980 in American agriculture, with gross world sales at $118 million.

The gibberellins are derived from gibberellan (Figure 20.1) and are given arbitrary consecutive numbers according to when they were discovered. From the first gibberellin discovery in 1938, many more have been described; by 1975 the number was up to GA_{45}. The most important are GA_3 (also called gibberellic acid) and GA_7. Other gibberellins are also active, e.g. GA_4 from *Sphaceloma manihoticola*. Plants produce only a few gibberellins (GA_1, GA_3, GA_4, and GA_5), and the majority of the rest are produced by fungi. Biosynthesis takes place via isoprenoid units.

Fusarium moniliforme, the imperfect stage of the fungus *Gibberella fujikuroi*, is used for commercial production. This fungus produces the antibiotic bikaverin simultaneously with its production of gibberellins. Six physiologically distinct phases have been characterized for the production process (Figure 20.2).

1. Lag phase
2. Growth phase (24–36 h) without nitrogen limitation, low production of gibberellin
3. Glycine limitation, little cell reproduction, slow gibberellin production
4. Glycine absent, remaining glucose catabolized, strong gibberellin production
5. Glucose content zero, slow accumulation of gibberellins
6. Lysis of cells, increase in pH

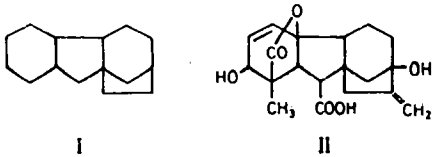


Figure 20.1 Structure of gibberellan (I) and gibberellic acid (II)

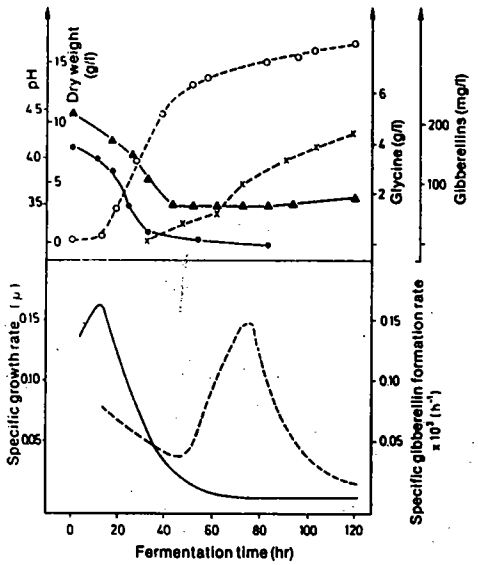


Figure 20.2 Batch fermentation with glycine limitation for the production of gibberellins by *Gibberella fujikuroi*. Upper graph: ▲—▲ pH; ●—● glycine; O—O mycelium dry weight; X---X gibberellin concentration; Lower graph: - - - specific gibberellin formation rate; —— specific growth rate (Bu'Lock et al., 1974)

A low nitrogen content (<0.2%) and a mixture of different carbon sources are two important conditions for gibberellin production. Yields in production-scale fermenters after 120–150 hours are 1–2 g/l of total gibberellin at 25°C, with the aeration rate at 0.5 vvm. In addition to direct fermentation in submerged culture, some studies have been carried out on the use of surface cultures as well as submerged fed-batch cultures. Continuous processes have been described, but have not become widely used. Table 20.1 shows that in continous culture, gibberellins are formed only at very low dilution rates.

Zearalenone

Zearalenone (Figure 20.3) and its derivatives have estrogenic activity. The various zearalenone derivatives differ by the presence of a keto or hydroxyl group on the 6′ carbon and by the presence or absence of a double bond in the macrolide lactone ring. These substances are formed by *Gibberella zea*, the sexual stage of the fungus *Fusarium roseum graminearum*. Production scale today is carried out in a submerged process in 80 m³ fermenters. The yields at 32°C over a 21-day incubation period are 30 g/l. Zearalenones are used as anabolic growth-stimulating compounds in swine and sheep production.

Table 20.1 Gibberellin formation by *Gibberella fujikuroi* under glycine limitation

Dilution rate h^{-1}	0.12	0.09	0.07	0.05	0.02
Mycelium dry weight g/l	7.6	11.6	14.4	15.6	22.5
Glycine content g/l	1.25	0.90	0.49	0.31	0.12
Glucose content g/l	100	92	81	63	53
Gibberellin mg/g dry weight	0	0	0	0.45	2.05

(Bu'Lock et al., 1974)

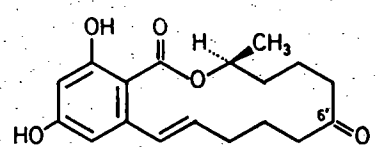


Figure 20.3 Zearalenone

Triglycerides and fatty acids

Fats and oils derived from plant sources find wide use in foods and for industrial applications. Techniques have been developed for the microbial production of fats and oils, but such processes have not been cost-effective for the most significant materials. The fats and oils for food uses mostly contain the longer-chain fatty acids (C_{16}–C_{18}), but uses for those with other chain lengths also exist. Because of the high prices for the fats with shorter-chain fatty acids (C_8 and C_{10}) and for a few of the longer-chain materials, considerable research is underway to produce glycerides with such fatty acids. The proportion of sterols produced in such fermentations must be low (<0.5%) and the fatty acid contents of the cells must be greater than 40%. Only yeasts and filamentous fungi apppear suitable for such processes. In England a process has been developed using species of the fungus genus *Mucor*. Using glucose as the starting material, an oil containing 7% γ-linolenic acid has been produced in 220 m³ fermenters. γ-Linolenic acid, a C_{18} unsaturated fatty acid with three double bonds (C_6, C_9, and C_{12}) is a starting material in prostaglandin biosynthesis and is also used in dietary foods.

Another approach to the production of desired oils is by the use of specific lipases which will catalyze the exchange of a fatty acid ester of lower value for one of higher value. This approach is currently undergoing research for large-scale production.

Enzyme inhibitors

It has been known for a long time that animals and plants produce enzyme inhibitors. Microorganisms have been studied for enzyme-inhibitor production in Japan since 1965 and worldwide beginning more recently. By 1974, 50 microbial enzyme inhibitors were known. Initially, protease inhibitors were sought, which were usually peptides themselves and were developed for varying applications, including possible use as antitumor agents or as immunosuppressives. Table 20.2 gives examples of several microbially produced protease inhibitors.

Table 20.2 Protease inhibitors

Enzyme inhibitor	Enzyme	Microorganism	Application
Pepstatins	Acid proteases e.g. pepsin	*Streptomyces testaceus*, *S. argenteolus*	Ulcers
Antipain	Papain	*S. yokosukanensis* *S. michiganensis*	Reduces fever
Chemostatin	Chymotrypsin	*S. hygroscopicus* *S. lavendulae*	Reduces fever
Elastatinal	Elastase	*S. griseoruber*	Pancreatitis
Leupeptin	Trypsin Plasmin		Reduces fever
Bestatin	Amino peptidases	*S. olivoreticuli*	Inhibits cancer
Subtilisin inhibitor	Subtilisin	*S. albogriseolus*	

Figure 20.4 shows the peptide structure of the substance antipain, an inhibitor of papain. In contrast to the relatively simple structure of antipain, the inhibitor of the enzyme subtilisin contains 113 amino acids.

A second group of substances that have been isolated are inhibitors of amylase and other glycoside hydrolases. These substances are important in medicine because they inhibit the breakdown of carbohydrates in the digestive tract. Included here are inhibitors of α-glucosidases, β-glucosidases, cellulases, and dextranases. α-Glu-

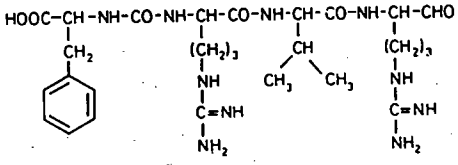


Figure 20.4 Antipain

cosidase inhibitors are widely distributed (Table 20.3). Chemically, such inhibitors are pseudo-oligosaccharides, monosaccharides, cyclopeptides, and polypeptides. Figure 20.5 shows the general formula of a group of α-glucosidase inhibitors which contain a central molecule of an unsaturated cyclitol plus 4,6-dideoxy-4-amino-D-glucopyranose with α-1,4-glucopyranose. This

Table 20.3 α-Glucosidase inhibitors produced by microorganisms

Microorganism	Type of compound	Specificity
Actinomycetes	Proteins	1,2 plus microbial α-amylases
	Pseudo-oligosaccharides	
Streptomyces flavochromogenes	Pseudo-oligosaccharides	1,2,3
S. diastaticus	Pseudo-oligosaccharides	3 plus α-amylases, microbial α-amylases
S. fradiae	Acid polypeptides	Human α-amylases
Bacilli, *S. lavendulae*	Nojirimycin, 1-deoxynojirimycin	2,3 plus β-glucosidases from plants and microorganisms
S. calidus	Glycopeptide	1 plus intestinal maltase and sucrase
S. violaceoruber	Polypeptide	1
Aspergillus niger	Glycopeptide	*Aspergillus* glucoamylase
Cladosporium herbarum	Glycopeptide	1
Cladosporium cladosporioides	Acid glycopeptide	Human α-amylases

1 = pancreatic amylase; 2 = intestinal amylase and disaccharase; 3 = *Rhizopus* glucoamylase.
(Müller, 1986)

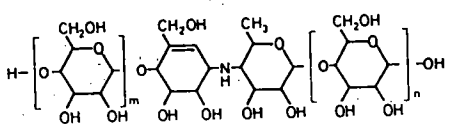


Figure 20.5 α-Glucosidase inhibitor complex. (m+n=1–8)

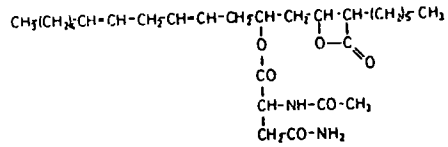


Figure 20.6 Esterastin

group of acarboses are produced by species of the genus *Actinoplanes* using a medium containing maltose and yeast extract. For purification, the products are complexed to an ion-exchange resin and then eluted for further purification.

Nojirimycin (see Figure 13.19) is an example of a substance which has anti-β-glucosidase activity.

Enzyme inhibitors of adrenalin biosynthesis (dopastin, fusaric acid, isoflavone, phenolpicolinic acid, oudenone, and oosponol) have been intensively studied because of their blood-pressure regulating effect. The structure of esterastin, an esterase inhibitor (Figure 20.6) is unusual because it contains a β-lactone ring. β-Lactamase inhibitors such as clavulanic acid are valuable because they overcome bacterial penicillin resistance. Because antibiotics which are active against microbial cell wall synthesis are generally less toxic to humans, penicillinase inhibitors are being sought for possible use in penicillin therapy. Another area of application for enzyme inhibitors concerns the inhibitors of chitin synthesis, such as the polyoxins, which have found limited use as insecticides.

Microbial insecticides

The production of insecticides from microbiological sources is of considerable interest. The emphasis here is not on the production of insecticide-active chemicals, but on the use of microbes which are themselves insect pathogens. In such applications, the living microorganism must be dispersed into the environment. Five specific requirements must be met before living preparations can be used as insecticides:

1. Large-scale production must be feasible
2. Viability must be maintained
3. The organism must not be toxic or pathogenic to other animals and plants
4. The organism must be less expensive than chemical agents.
5. No development of resistance by the target insects.

Bacteria, viruses, fungi, or protozoa can be used as insecticides. They are produced commercially either in large-scale fermentation or in insect hosts.

Five protozoa, (*Nosema locustae, N. algerae, N. pyrausta, Vairimorpha necatrix,* and *Mattesia trogodermae*) have been tested thus far, but have not yet found practical application.

Over 400 fungi are known which attack insects. They are relatively nonspecific in host range but are also less effective than other agents. Those fungi currently under study are summarized in Table 20.4. Many of these fungi can be cultured well in open dishes in semi-solid medium such as rice seeds. Within 15–20 days at 26–29°C, about 2 × 10⁹ conidia per gram are produced.

Over 650 insect viruses have been described. Such viruses offer the best possibilities for practical use as insecticides and several viruses are already being marketed. One disadvantage of viruses for large-scale production is that they must be cultured on living insects, making scale-up difficult.

Three bacteria (*Bacillus popilliae, B. moritae,* and *B. thuringiensis*) are commercially produced. The first of these can only be cultivated in living insects but the latter two can be grown in submerged culture. *B. thuringiensis* is widely cultivated in North America and Europe, with amounts of 2300 tons per year, and is also produced in the USSR and China. About 40 different insects that cause agricultural or forest diseases are treated with *B. thuringiensis*. In addition, control of certain insects which are carriers of human diseases (for example, *Anopheles* sp., malaria; *Simulium damnosum*; river blindness) is also a pos-

Table 20.4 Fungi pathogenic against insects

Fungus	Production technique	Status	Countries
Aschersonia sp.	Submerged	Pilot plant	USSR, Holland, UK
Beauveria bassiana	Submerged/ 2 stage	Approved for market	USSR
	Semi-solid medium/ 2 stage	Small scale	USA
Conidiobolus obscurus	Submerged	Pilot plant	France, UK, USA
Culcinomyces clavosporus	Submerged	Small scale	Australia
Entomophthora grylli	In insects	Small scale	USA
Erynia neoaphidis	Submerged In insects	Small scale	UK
Hirsutella thompsonii	Semi-solid	Pilot plant	USA
Lagenidium giganteum	Submerged	Small scale	USA
Metarhizium anisopliae	Semi-solid	Approved for market	Brazil
Nomurea rileyi	Solid	Small scale	USA
Verticillium lecanii	Submerged	Approved for market	UK
Zoophthora radicans	Submerged	Laboratory studies	USA

(Quinlan and Lisanski, 1983)

sibility. The active ingredient of *B. thuringien*
is a polypeptide toxin (γ-endotoxin) which is pr
duced during the bacterial sporulation proce
The cultivation procedure is therefore design
to obtained a high percentage of sporulation a
toxin accumulation. The fermentation is carri
out in a medium containing starch, corn-ste
liquor, casein, and yeast extract. Sporulation
curs after 25–30 hours and the γ-endotoxin
cumulates as parasporal crystalline inclusi
that amount to around 30% of the cell mass. T
toxin is quite selective in its action and is
ministered by being incorporated into a suita
insect food. Another toxin, the β-exotoxin, i
nucleoside which is produced during the gro
phase. It is a nonspecific toxin which rapidly
caterpillars. Between 10–15 minutes after in
tion of the β-exotoxin, the animals quite feec
and die within 3–5 days.

losporin A

cyclosporins are a group of oligopeptides luced by the fungus *Tolypocladium inflatum* ch have antifungal, anti-inflammatory, and unosuppressive properties. The immunopressive effect is due to the inhibition of the vation of the T cell response. Cyclosporin A n eleven-membered cyclic peptide which is d because of its immunosuppressive proper- to prevent rejection reactions during organ isplantation. The cyclosporins are produced 15-day submerged fermentation from a me- m containing 4% glucose, 1% peptone, and neral salts.

chips

quite different application of biotechnology in- lves the combination of electronics, biochem- ry, and genetics in the development of the con- pt of the **biocomputer**. Microprocessors of the ure may consist of chips in which monolayers protein are the functional material. Studies th polylysine have given some success. These ips have over 100,000-fold more switches than nventional microprocessors. The appropriate oteins may be produced from microorganisms onomically by the use of genetic engineering chniques.

lavoring substances

/e discussed the production of flavor-enhancers Chapters 9 and 10. Substances with inherent avor properties are also produced by microoranisms and are of great significance in the manfacture of food and drink. Moreover, microbial nzymes used in food can alter the substrate and hus produce flavoring substances. Intensive tudies are being conducted on the use of mirobes in food production and on the genetics nd biochemistry of aroma production. Among arious alcoholic drinks alone, 400 different subtances have been identified; of these 118 were esters, 80 aliphatic and aromatic acids, 41 carbonyl compounds, and 38 aliphatic and aromatic alcohols. The aroma components and the percentage distribution vary depending on the strain and substrate solutions.

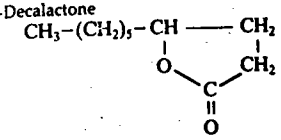
In addition to the flavor enhancers already discussed (sodium glutamate, Chapter 9, and 5′-nucleotides, Chapter 10) a number of specific flavoring agents can be produced by fermentation. An example is the production of complex flavoring ingredients suitable for cheese production. The most important flavoring substances in cheese are methyl ketones (2-heptanone, 2-nonanone, 2-pentanone) and secondary alcohols with 5–11 carbon atoms. Such flavoring substances can also be produced in separate fermentation processes and then used as supplements to increase the flavor of prepared foods such as salad dressings and cheese pastries. As an example, the fermentation of *Penicillium roqueforti*, the fungus involved in Roquefort cheese production, can be described. Milk, salt, and milk fat are added for the fermentation which is carried out at 24–26°C for three days. During this period, the milk fat is converted by the fungal lipases into methyl ketones and secondary alcohols.

$$\text{Fats} \xrightarrow{\text{Lipases}} \text{Fatty acids} \longrightarrow \beta\text{-Keto acids}$$
$$\longrightarrow \text{Methylketones} \longrightarrow \text{Secondary alcohols}$$

After growth, the sterilized culture broth contains 4% fat, 5% protein, 8% carbohydrates, 5% salts, and 78% water; this final fermentation product has 6–20 times more cheese aroma than does the cheese itself.

A number of specific microbial products that have distinctive odors are summarized in Table 20.5. In addition, biotransformation of terpenes to terpenols have been used to produce flavor ingredients. For instance, *Penicillium digitatum* is able to convert the terpene R(+)limonene to α-terpineol, a substance with a lilac odor that is used in perfumes.

Table 20.5 Microbial compounds with specific odors

Compound	Structure	Type of odor
Diacetyl	$CH_3-CO-CO-CH_3$	Butter flavor (buttermilk)
Acetaldehyde	CH_3-CHO	Oranges and yogurt
γ-Decalactone		Peach
Esters		Fruit
Pyrazine		Roast and nut

Biofilters

Biofilters have been used since about 1970 for the removal of odors from air streams arising from sewage treatment plants, animal feed lots, paper mills, tobacco, chocolate and coffee roasting plants. Highly odoriferous off-gasses are purified and moistened by passing through beds containing compost, peat, pine needles, moss, or plastic bodies. Microorganisms growing on the beds are responsible for the removal of much of the odor. During the time which the gas passes through the bed (generally only a few seconds), the odors are absorbed and metabolized to biomass and CO_2. Biofilters themselves only have a compost-like odor. Biofilters may also be used in the future to purify the air from large-scale fermentation installations.

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Index

Absidia, 215
Absolute filter, 112
Acarbose, 120, 338
Acetaldehyde, 131, 341
Acetaldehyde dehydrogenase, 144
Acetamidocinnamate amidohydrolase, 155
Acetic acid, 48, 61, 129, 134
  amino acid production from, 154
  biosynthesis, 129–30, 144–45
  as carbon source, 74, 92
  glutamic acid production from, 159, 161, 163–64
  lysine production from, 168–69
  methane production from, 323
  production:
    product recovery, 147
    submerged process, 144–47
    surface process, 143–45
  riboflavin production from, 225
Acetobacter, 144, 234, 331
Acetobacter aceti, 144
Acetobacter pasteurianus, 144
Acetobacter peroxidans, 144
Acetobacter suboxydans, 135, 143, 298
Acetone, 48, 52, 61
  biosynthesis, 129–30
  production, 130–31
Acetylation reaction, 294
Acetyl-CoA carboxylase, 161, 263–64, 283
3-Acetylpyridine, 28
Acholeplasma laidlawii, 7
Achromobacter, 207, 303, 327
Achromobacter lipolyticum, 208
Achromobacter liquidum, 170
Achromobacter obae, 155, 165
Acidophile, 6
Acid protease, 203, 205
Aclacinomycin, 231
Aconitase, 136
cis-Aconitic acid decarboxylase, 148
Acrasiomycete, 50
Acremonium chrysogenum, 240
Acridine orange, 16, 18
Acriflavine, 18
Actilyse, *see* Tissue plasminogen activator
Actinomyces levoris, 52
Actinomycete, 230
  recombination, 31–32
Actinomycin, 37–39, 52, 230–31, 244–45, 263, 300
Actinoplanes, 6, 338
Actinoplanes missouriensis, 200
Actinorhodin, 32, 48, 51–52, 230
Activated charcoal, 214
Activated sludge basin, 321
Activated sludge process, 317–20
N-Acylation reaction, 290
Acyltransferase, 38
Adaptive repair, 14–15, 17
Adenine, 28, 176
Adenine nucleotide, 186–87
Adenosine, 176, 186–87
Adenosine deaminase, 186
S-Adenosylhomocysteine, 187
S-Adenosylhomocysteine hydrolase, 187
S-Adenosylmethionine, 187
Adenyl cyclase, 186–87
Adenyl deaminase, 180
Adenylsuccinate lyase, 176, 178
Adenylsuccinate synthetase, 178
Adriamycin, 51, 230–31, 267
Adsorption chromatography, 120
Aeration, 85, 105
Aeration basin, 318
Aeration rate, 88
Aerobacter, 135, 209
Aerobacter aerogenes, 92, 164, 198, 200, 303
Affinity chromatography, 120
Agar plug method, 28
Agriculture, 54–55
Agrobacterium, 44, 198
Agrobacterium radiobacter, 234
Agrobacterium tumefaciens, 54–55
Agroclavine, 275, 278, 280–81
AICA, 185
AICAR, 184–85
AICARP, 177, 185
AICARP formyltransferase, 185
Air, sterilization, 100–3
Airlift process, 76, 319–20
L-Alanine, 152–56, 291
D,L-Alanine, 151–54, 156
Alanine racemase, 244
Alarm system, 107
Albomycin, 247
Albumin, serum, 49, 55
Alcaligenes, 206, 209, 331
Alcaligenes faecalis, 332
Alcohol dehydrogenase, 125, 144, 189
Alcohol oxidase, 216
Alginate, 330–32
Aliphatic antibiotic, 230
Alkaline phosphatase, 180–81
Alkaline protease, 203–4
Alkaloid, 39
  ergot, *see* Ergot alkaloid
  indole, 275
n-Alkane, 69
  amino acid production from, 154
  as carbon source, 61, 92
  catabolism, 308
  citric acid production from, 137, 141–42
  glutamic acid production from, 164
  lysine production from, 168–69
  organic acid production from, 135
  riboflavin production from, 225
  single-cell protein production from, 306–9
  vitamin B_{12} production from, 223
Alkylating agent, 17
O^6-Alkylguanine, 17
O^4-Alkylthymine, 17
Allosteric effect, 35
Allosteric site, 35, 37
Alternaria solani, 269
Aluminum oxide, 214
Amberlite, 120
Amidinotransferase, 38
Amikacin, 251
Amino acid, 2, 10, 27, 48, 51, 71, 105, 112, 150–73, 298
  biosynthesis, 36, 157
  production, 151–56, 215
    chemical synthesis, 153
    process control, 157–58
    product recovery, 158

from protein hydrolysate, 151
strains, 156–57
acemic mixture, 153
ases, 151–52
nino acid antibiotic, 230, 232, 243–49
nino acid dehydrogenase, 153, 291
nino acylase, 153, 214
minoadipic acid pathway, 165
Aminobenzoic acid, 28
Aminobutyric acid, 28
L-α-Aminocaprolactam, 165
-Aminocaprolactam hydrolase, 155
-Aminocaprolactam racemase, 155, 165
-Aminocephalosporanic acid, 210, 241
-(β-Aminoethyl)-L-cysteine, 154, 165, 169
Aminoglycoside antibiotic, 39, 71, 105, 230, 232, 250–51, 301
biosynthesis, 253–55
mode of action, 251–53
production:
methods, 255–56
strains, 253–55
structure, 253
6-Aminopenicillanic acid, 208–10, 234–36, 301
p-Aminophenylalanine, 154, 171
2-Aminopurine, 18
p-Aminotyrosine, 154
Ammonia, 163, 168
gaseous, 62
Ammonium salt, 62
Amoxycillin, 243
5'-AMP, 176, 178, 180
AMP deaminase, 178, 180
Amphibolic pathway, 71
Amphomycin, 247
Amphotericin B, 39, 260
Ampullariella regularis, 268
Amylase, 7, 105, 112, 137, 189–98
saccharogenic, 191–92
starch-liquefying, 191–92
α-Amylase, 127, 139, 191–98, 211–12, 214
bacterial, 192–96
fungal, 195
uses, 193
β-Amylase, 191, 195, 198, 214
Amylase inhibitor, 337
Amyloglucosidase, 189
Anaerobe, 6
Anaerobic digestion, 317, 322–24
Anaplerotic reaction, 136
Androgen, 292
1,4-Androstadiene-3,17-dione, 295–97
Androstatriendione, 297
Androstendione, 297
Animal feed, 220, 231–32, 246, 250
lysine content, 151
Animal growth promoter, 231–32
Ansa chain, 262
Ansamitocin, 262
Ansamycin, 230, 262
Anthracene synthase, 263
Anthracycline, 5, 230–31, 267
Anthranilate synthase, 172–73
Anthranilic acid, 170–71
Antibiotic, 1–2, 6, 51, 69, 71, 112–13, 121
biosynthesis, controlled, 300
classification, 230
economic significance, 232
hybrid, 52–53
increases in production, 10
microbial groups producing, 230
microbial transformation, 300–3
modified, 51–53
mutational synthesis, 300
in producer strain, 230
production:
strains, 10, 51
levels, 25, 28–29, 32
purification, 120
research on, 232–33
screening for, 6–7
uses, 231–33
Antibiotic resistance, 21, 27, 44, 46, 48, 231, 233, 301
Antifoam agent, 76–77, 88, 115, 141
Antimetabolite resistance, 27–28, 37, 165
Antimycin A_1, 52
Antipain, 337
Antithrombin III, 55
Antitumor agent, 7, 51, 203, 231, 249, 267, 271
AP endonuclease, 13
Apramycin, 52, 252
β-D-Arabinofuranosyladenine, 176
Arabinosyladenine, 187
Arachidonic acid, 300
L-Arginine, 28, 152, 154
Arginine hydroxamate, 154
Aroma component, 340–41
Aromatic antibiotic, 230, 268–69
Arrhenius equation, 96–97
Arthrobacter, 158, 192, 200, 223, 302, 310
Arthrobacter hyalinus, 223
Arthrobacter paraffineus, 135, 154, 164, 187
Arthrobacter simplex, 294–96
Artificial sweetener, 2, 151
Arylamine synthetase, 38
Aschersonia, 339
Ascorbic acid, 216, 219, 298–99
Ascorbic acid oxidase, 216
Ashbya gossypii, 223–25
Asparaginase, 189, 203, 283
L-Asparagine, 152–53
Aspartame, 2, 151, 156
Aspartase, 155, 214
Aspartate decarboxylase, 155
Aspartate phenylalanine transaminase, 155
L-Aspartic acid, 150–52, 154–56, 191, 291
Aspartic semialdehyde dehydrogenase, 166
Aspartokinase, 35, 166–68
Aspergillus, 29, 33, 136, 156, 192–93, 206–7, 233, 274
Aspergillus awamori, 49, 195
Aspergillus clavatus, 135
Aspergillus flavus, 148, 203
Aspergillus fumigatus, 34
Aspergillus itaconicus, 148
Aspergillus nidulans, 29, 31, 34, 66, 234
Aspergillus niger, 49, 66, 85, 89, 139–41, 143, 192, 195–98, 203, 206–8, 210, 338
Aspergillus ochraceus, 208, 296
Aspergillus oryzae, 49, 148, 153, 180, 192, 195, 198, 203–4, 210
Aspergillus rugulosus, 34
Aspergillus sojae, 203–4
Aspergillus terreus, 148–49
Aspergillus wentii, 135, 206
Astaxanthine, 226
Asymmetric control, 178
ATC oxygenase, 265
ATP, 35, 40, 176, 186, 216
Atrial natriuretic factor, 55
Attenuation, 36
Aureobasidium, 331
Aureobasidium pullulans, 331, 333
Autolysis, 118
Autoregulation, 40–41
Auxotrophic mutant, 27–29, 32, 36, 156, 165
Avermectin, 261
Avidin, 42
Avilamycin, 250
8-Azaguanine, 175, 182, 185–86
4-Azaleucine, 28
Azaserine, 186, 243
6-Azauridine, 176
8-Azaxanthine, 28, 186
Azide, 171
Azotobacter, 330–31
Azotobacter vinelandii, 331–32
Azthreonam, 151, 243
Azureomycin, 7

Bacillus, 6, 33, 48, 182, 193, 198, 210, 230–31, 245, 310
Bacillus acidocaldarius, 192
Bacillus amyloliquefaciens, 54, 191–92, 203
Bacillus amylosolvens, 194
Bacillus brevis, 39, 247
Bacillus caldolyticus, 192, 212
Bacillus cereus, 116, 191, 195, 204, 327
Bacillus circulans, 53, 252, 255–56
Bacillus coagulans, 191–92, 200–1
Bacillus firmus, 203
Bacillus licheniformis, 31, 39, 185, 192, 203–4, 246–49, 327
Bacillus luteus, 327
Bacillus megaterium, 153, 185, 203–4, 221, 300, 327

Bacillus mesentericus, 206
Bacillus moritae, 339
Bacillus natto, 192
Bacillus polymyxa, 52, 192, 195, 198, 206, 247, 327
Bacillus popilliae, 339
Bacillus pumilus, 31, 185, 203, 222
Bacillus sphaericus, 155
Bacillus stearothermophilus, 96, 98, 158, 192
Bacillus subtilis, 7, 29, 31, 34, 45, 47–48, 170–71, 178, 181–86, 191–94, 198, 204, 206
Bacillus subtilis-ituriensiens, 247
Bacillus thuringiensis, 55, 339
Bacilysin, 245
Bacitracin, 39, 120, 230, 246–49
Bacitracin synthetase, 247–48
Bacteria, recombination, 30–31
Bacteriocin, 44
Bacto-soytone, 200
Baffle, 76, 83
Baffle centrifuge, 116
Bafilomycin, 261
Bagasse, 127
Baker's yeast, 95
Ball mill, 118
Base analog, 16, 18
Base excision repair, 13
Base substitution, 11
Basic research, 53–54
Batch fermentation, 65–67, 70, 103
Batch sterilization, 98–99
Bayer Biotower, 319–20
Beauveria bassiana, 339
Beer, 70
Bentonite, 214
Benzoic acid, 164
Benzoquinone antibiotic, 230–31
Benzylalcohol dehydrogenase, 212
Benzylpenicillin, 234, 236
Benzylthiocyanate, 265
Bestatin, 337
Betaine, 222
β-rays, 16
Bgll, 21
Bialophos, 245–46
Bikaverin, 335
Bingham-plastic solution, 84–85
Biochemical assay, 216–17
Biochemistry, antibiotics as tools in, 231–32
Biochip, 340
Biocomputer, 340
Bioconversion, *see* Microbial transformation
Biofilter, 341
Biogas, 321–23
Biogel, 119
Biomass concentration, 66
Bioreactor, *see also* Fermenter
construction types, 74–76
homogeneously mixed, 68
for immobilized enzymes or cells, 78–81
stirred, 76–78, 80, 88
Biosensor, 106
Biosynthetic pathway:
branched, 35, 37
unbranched, 35
Biotin, 42, 159, 161, 163, 168
Biotransformation, *see* Microbial transformation
Bisulfite, 21
Blade-wheel reactor, 76
Blakeslea trispora, 225, 227–28
Blasticidin S, 232, 267
Bleomycin, 230–31, 249
Bluensomycin, 252
Bordetella, 209
Botrytis, 269
Bovine growth factor, 55
Brevibacterium, 33, 48, 51, 116, 155, 158–59, 164–65, 182, 303
Brevibacterium ammoniagenes, 182–84, 186–87, 215
Brevibacterium divaricatum, 163–64
Brevibacterium flavum, 154, 156, 163–64, 167, 169, 171, 185
Brevibacterium imperiale, 200
Brevibacterium lactofermentum, 34, 154, 156–57, 165
Brevibacterium liquifaciens, 186
5-Bromouracil, 16, 18–19
Bubble column, 88
Bulking, sludge, 317–18
Butadiene, 129
2,3-Butandiol, 61
Butanol, 48, 51, 61
biosynthesis, 129–30
production, 130–31
Butirosin, 39, 53, 252, 255–56
Butyribacterium rettgeri, 221
Butyric acid, 48, 129–30
Butyryl phosphoadenosine, 41

Calcium phosphate gel, 214
Calvin cycle, 311
Campesterol, 296
Camphor degradation, 44, 319
Canavanine, 28
Candicidin, 37, 39–40, 230, 260–61
Candida, 33, 135, 192, 206–7, 308
Candida boidinii, 116, 186, 310, 313
Candida cylindraceae, 208
Candida flareri, 223
Candida guilliermondii, 135
Candida lipolytica, see *Saccharomycopsis lipolytica*
Candida oleophila, 307
Candida parapsilosis, 310
Candida periculosa, 165
Candida tropicalis, 34, 307, 309, 315
Candida utilis, 92, 170, 179, 315–16
Candidin, 260
Candihexin, 38–39
Capillary bundle filtration, 114–16
Capreomycin, 94, 246–47
Caramelization, 99
N-Carbamoyl-L-tryptophan hydrolase, 170
Carbapenem, 243
Carbohydrate, single-cell protein production from, 307, 315
Carbohydrate antibiotic, 230, 249–56
Carbomycin A, 257
1-Carbon assimilation, 311
Carbon catabolite repression, 38
Carbon dioxide, 86
effect on fermentation, 92–93
measurement, 106
Carbon dioxide electrode, 106–7
Carbon source, 38, 60–61
Carboxymethyl cellulose, 214
β-Carotene, 219, 225
biosynthesis, 226–27
economic significance, 226
occurrence, 226
production, 227–28
structure, 227
Cartridge filter system, 102
Casein hydrolysate, 200
Cassava, 125–27
Cassette filtration system, 114–16
Catabolite activator protein, 38
Catabolite repression, 38–39, 59, 67, 194, 200
Catalase, 189, 216, 310
CDP-choline, 176, 187
Cell concentration, 66, 68–69
Cell formation rate, 69
Cell-free extract, 291
Cell stabilization, 210–17
Cellulase, 32, 314–15, 319
Cellulase inhibitor, 337–38
Cellulomonas, 209, 314
Cellulose:
as carbon source, 61
decomposition, 69
organic feedstocks from, 124
single-cell protein production from, 307
Cellulose triacetate, 215
Centrifugation, 115–17
Cephalosporin, 33, 38, 51, 234
assay, 216
biosynthesis, 241–42
production:
methods, 242
strains, 242
regulation, 241–42
semisynthetic, 156, 210, 240–41
structure, 240
Cephalosporin C, 31, 39, 52, 210, 230, 240–42
Cephalosporin N, 52
Cephalosporin P, 52
Cephalosporin acylase, 210
Cephalosporinase, 216
Cephalosporium, 33, 192, 208
Cephalosporium acremonium, 29–33, 49–52, 234, 240–42
Cephamycin, 37, 39, 240–41
Chaetomium cellulolyticum, 315

Chalcopyrite, 328
Chanoclavin, 275, 278, 281
Chanoclavin-I-cyclase, 38, 282
Chelate-forming antibiotic, 230, 247-49
Chemical modification of products, 4
Chemical sterilization, 98
Chemostat, 68
Chemostatin, 337
Chitinase, 32, 268
Chitin synthase, 268
Chloramphenicol, 32, 38-39, 44, 229-30, 268
Chlorbenzilate, 303
α-Chlorcaprolactam, 154, 169
Chlordecone, 303
Chlorobenzoate degradation, 44
Chloroneb, 303
p-Chlorophenylalanine, 284
Chlorsalicylic acid, 304
Chlortetracycline, 10, 32, 37, 39, 51-52, 231, 262-67
Cholesterol, 216, 295-97
Cholesterol oxidase, 189, 216
Chorismate mutase, 172
Chromatography, 118-21
Chromobacter violaceum, 234
Chromomycin A_3, 231
Chromopeptide antibiotic, 230-31, 244
Chromosome mutation, 11, 16
Chymase, *see* Rennin
Chymosin, *see* Rennin
Cider, 144
Citrate synthase, 136, 283
Citric acid, 71, 76, 112, 134-35, 148
  biosynthesis, 136-37
  production:
    media, 137-38
    process, 138-39
    product recovery, 142
    strains, 135-36
    submerged process, 138, 140-42
    surface process, 138-40
    yield, 137, 140
  uses, 135
Citrinaxanthine, 226
Citrobacter, 216
Citromyces pfefferianus, 135
Cladosporium cladosporioides, 338
Cladosporium herbarum, 338
Claviceps, 29, 33, 277
Claviceps fusiformis, 275, 280-81
Claviceps gigantea, 275
Claviceps paspali, 275, 280
Claviceps purpurea, 274-75, 279-80, 283-84
  developmental cycle, 275-76
Clavine, 274, 276
Clavulanic acid, 234, 243, 338
Clindamycin, 250
Cloning, 4, 41, 47-50, 284
  in eucaryotes, 49
  in filamentous fungi, 49
  in gram-negative bacteria, 47-48
  in gram-positive bacteria, 48-49
  isolation of DNA sequences, 41-47
  in acrasiomycetes, 50
Clostridium, 52
Clostridium acetobutylicum, 129-30, 223
Clostridium butylicum, 129
5'-CMP, 180
Cobalamin, *see* Vitamin B_{12}
Coenzyme A, 187
Coenzyme B_{12}, 220
Colistin, 29
Colony hybridization, 47
Column chromatography, 118-19
Combination electrode, 106
Cometabolism, 286, 302-3
Complementary DNA, 42-43
Computer, 107-8
Comutation, 20
Condensation reaction, 286, 289-90
Conidiobolus obscurus, 339
Coniothrium hellbori, 85
Conjugation, 30-31, 34
Contact dryer, 121
Continuous fermentation, 2, 67-70, 72-73, 103
Continuous gas phase, 76
Continuous sterilization, 99-102
Convection dryer, 121
Cooling system, 73
Copper leaching, 326, 328
Co-repressor, 36, 40
Coriolus, 206
Corn starch, 125-27, 197
Corn steep liquor, 62, 131, 200, 225
Cortisol, 292, 295
Cortisone, 292, 294-95
Corynebacterium, 33, 48, 51, 135, 165, 182, 206, 209, 223, 299
Corynebacterium acetoacidophilum, 154
Corynebacterium alkanolyticum, 164
Corynebacterium glutamicum, 103, 150, 154, 156, 158-59, 162-73, 183
Corynebacterium guanofaciens, 185
Corynebacterium hydrocarboclastus, 164
Corynebacterium murisepticum, 186
Corynebacterium petrophilum, 182, 185
Corynebacterium simplex, 295
Cosmid, 44, 51
Cost effectiveness, 9, 72, 115
Cosynthesis, 52
Covellite, 328
Critical oxygen concentration, 89-90, 94
Cross-flow filtration, 114, 116
Crossing over, 29-30
Cryptococcus laurentii, 155, 165
Crystallization, 112, 121
Culcinomyces clavosporus, 339
Culture media, 59-63
  optimally balanced, 59
  sterilization, 60, 96-103, 105
Curamycin, 250
Curdlan, 331-32
Curvularia lunata, 294-95
Cyanocobalamin, 220
Cyclic AMP, 38, 40, 176, 186-87
Cyclic peptide antibiotic, 245-47
Cycloalkane antibiotic, 230
Cycloheximide, 38-39, 52, 230, 232
Cyclol-synthetase complex, 279
Cyclonic reactor, 80
Cycloserine, 230, 243-44
Cyclosporin A, 340
Cylindrocarpon radicicola, 295-96
L-Cysteine, 151-53, 155-56, 163
Cysteine desulfhydrase, 155
L-Cystine, 153

Dactinomycin, 244
DAHP synthetase, 172-73
Dalapon, 302-3
Dapiramine, 268
Data:
  acquisition, 107
  analysis, 107-8
Daunomycin, 51, 231, 267
Daunorubicin, *see* Daunomycin
DDT, 302-4, 319
Death phase, 65, 67
Death rate, 96-97
γ-Decalactone, 341
Decane degradation, 44
Decanter, 116-17
Decoyinine, 154, 186
Dehalogenation reaction, 303-4
1-Dehydration, 295
Dehydration reaction, 287, 291
C-1(2)-Dehydrogenase, 297
1-Dehydrogenation, 295
3,4-Dehydroproline, 28
1-Dehydrotestololactone, 295-96
Deletion, 11, 15, 17-18, 20-21
6-Demethyl tetracycline, 52
5-Deoxybutirosamine, 53
Deoxycholic acid, 292
Deoxycortisol, 294-95
2-Deoxystreptamine, 253, 255
2-Deoxystreptidine, 53
Depsipeptide antibiotic, 230, 244-45
Depth filter, 101-2, 112
Deregulated mutant, 59
Design criteria, 96
Destomycin, 252
Dextran, 2, 331, 333-34
Dextranase inhibitor, 337-38
Dextransucrase, 333
Dextrin, 61, 191, 195
Diacetyl, 341
Dialysis, 81
Diaminobutyric acid, 246
Diaminohydroxyazelain acid, 246
meso-Diaminopimelate decarboxylase, 166
Diaminopimelate epimerase, 166
Diaminopimelic acid pathway, 164-66
Diaminopropionic acid, 246
2,6-Diaminopurine, 28
Diatomaceous earth, 113
Diauxy, 67
Diazomethane, 16, 18

Dibekacin, 251
Dicarboxylic acid cycle, 283
3,5-Dichlorcatechol, 303
p,p'-Dichlorodiphenylmethane, 303
Di-(2-chloroethyl)-sulfide, 16-17
2,4-Dichlorophenoxyacetic acid degradation, 44
Dictyostelium discoideum, 50
2,4-Dideoxystreptamine, 53
Dieldrin, 302
Diepoxybutane, 16-17
Diethylsulfate, 16-17
Dihydrodipicolinate reductase, 166
Dihydrodipicolinate synthase, 165-67
Dihydroelymoclavine, 275
Dihydroergotamine, 275
Dihydropenicillin F, 209
Dihydrostreptomycin, 29, 251-52
Dihydroxyacetone, from glycerol, 299-300
Dihydroxyacetone cycle, 312-13
L-Dihydroxy phenylalanine, 156
2,5-Diketo-D-gluconic acid, 299
Dilatant solution, 84
Dimethylallylpyrophosphate, 278
4-Dimethylallyl tryptophan, 278
4-Dimethylallyl tryptophan synthase, 38, 40, 282
Dioscorea composita, 295
Diosgenin, 294-95
Diploidization, 51
Disintegration of microorganisms, 117-20
Distiller's solubles, 200, 225
DNA:
  alkylation, 11-15, 17-18
  crosslinks, 11-12, 15-17
  deamination, 11-13, 17, 21
  double-strand breaks, 11, 16
  foreign:
    incorporation into plasmid, 45
    insertion into host, 45
  isolation of sequences for cloning, 41-47
  nonreplicating, 16-17
  repair mechanisms, 11-15
  single-strand breaks, 11, 16
  synthetic, 42
DNA endonuclease, 12
DNA exonuclease V, 14
DNA gyrase, 269
DNA polymerase, 11-13, 18
L-DOPA, 155-56
Dopastin, 338
Doubling time, 69
Dowex, 120
Downstream processing, *see* Product recovery
Doxorubicin, *see* Adriamycin
Dry ice, 131
Drying of product, 112, 121
Dry malt extract, 60
Dunaliella salina, 132, 225
Duplication, 11, 20
Dynamic method, determination of oxygen uptake rate, 90-91
Dynamic viscosity, 84

Edein A, 246
Effector, 35, 40
Ejector aerator, 88
Elastatinal, 337
Electrofusion, 32-33
Elymoclavine, 275, 278, 281
Embden-Meyerhof-Parnas pathway, 136, 159
Emericellopsis, 29, 240
Emericellopsis salmosynnemata, 31
Emericellopsis terricola, 31
Endoenzyme, 191
Endo-β-1,4-glucanase, 314
Endomyces, 85-86
Endomycopsis, 143
Endonuclease, 180-81
Endothia, 206
Endothia parasitica, 206
End product, 35
End product regulation, 38
Enduracidin, 232, 247
Energy charge, 35
Energy metabolism, 70-71
Enrichment procedure, 6
Enterobacter cloacae, 171
Entner-Doudoroff pathway, 311
Entomophthora, 206
Entomophthora grylli, 339
Environmental protection, 55
Enzyme, 105, 112
  breakdown, 35
  carrier-bound, 211, 213-14, 291
  constitutive, 36-37
  cross-linked, 211-13
  encapsulated, 204, 211, 214-15
  immobilized, *see* Immobilized enzyme
  modification, 35
  plate assay, 28
  purification, 121
  regulation of activity, 35
  regulation of synthesis, 36
  stabilization, 210-17
  uses, 189-90
Enzyme electrode, 216
Enzyme inhibitor, 337-38
Enzyme/thermistor, 216
Epidermal growth factor, 55
Epidermophyton floccosum, 208
2-Epistreptamine, 53
Epithienamycin, 234, 243
Epoxidation reaction, 287
Eremothecium ashbyii, 223-24
Ergoblasine, 274
Ergocornine, 275-77, 280-81, 284
Ergocristine, 275-77, 280-82, 284
Ergocryptine, 275-77, 280-82, 284
Ergometrine, 275-76, 278, 280-81
Ergopeptam, 276, 279
Ergopeptin, 276-79
Ergosine, 275, 277, 279-81, 284
Ergosterol, 219
Ergot, 274
Ergot alkaloid, 38, 40, 274-84
  biosynthesis, 277-79
  occurrence, 274
  production:
    chemical synthesis, 279
    extraction from sclerotia, 279-80
    fermentation, 280-81
    regulation, 281-83
    semicontinuous with immobilized mycelium, 281
    strains, 283-84
  structure, 275-77
  uses, 274-75
Ergotamine, 274-75, 277, 279, 281, 284
Ergotism, 274
Ergotoxine, 275, 281, 284
Erwinia, 198, 209, 299
Erwinia aroideae, 203
Erwinia carotovora, 203
Erwinia herbicola, 155, 299
Erynia neoaphidis, 339
Erythromycin, 10, 32, 51, 53, 92, 108, 230, 256-59
Escherichia, 192, 209
Escherichia coli, 2, 35-36, 44-48, 51, 96, 154-56, 164-65, 170, 172-73, 185-86, 203, 206
  concentration, 115
  conjugation, 31
  critical oxygen concentration, 90
  DNA repair, 14-15
  filtration, 116
  foreign protein production, 42
  growth rate, 66
  lysine biosynthesis, 165-67
  mutagenesis, 17-20
  oxygen requirement, 92
  penicillin acylase, 103, 209
  protoplast, 33
  specific oxygen requirement, 89
Escherichia freudii, 200
Escherichia intermedia, 200
Essential amino acid, 151, 164
Esterase inhibitor, 338
Esterastin, 338
Estrogen, 292
Ethanol, 112
  acetic acid production from, 144-46
  assay, 216
  biosynthesis, 125-26
  as carbon source, 61, 74, 92
  from cellulose, 61
  citric acid production from, 137
  glutamic acid production from, 164
  lysine production from, 168-69
  production, 2, 70, 124-28, 216
  from starch, 61
  vitamin B_{12} production from, 223
Ethidium bromide, 21
Ethionine, 28, 154
Ethylmethanesulfonate, 16-17
Eucaryote:

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