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Recent Advances and Trends in Antibiotics Fermentation Technology

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It is well known that the era of antibiotics began with the finding of penicillin by Sir Alexander Fleming in 1928. Following the discovery of penicillin, many antibiotics (*e.g.* gramicidin, bacitracin, polymyxin, streptomycin, tetracyclines, etc.) were discovered. The characteristics and pharmacology of penicillin have been studied extensively and necessary technology has been developed for production of penicillin in laboratory, pilot plant, and large scale fermentation plants during 1940~1950 in England and USA. Since that time novel antibiotics produced by various microrganisms have been discovered and studied. Mass production of several antibiotics have been achieved, following their antimicrobial activity test and scale-up through laboratory and pilot plant stages.

Antibiotics produced by fermentation and widely used for medical and clinical purpose have reached about 90 items and these are produced by fermentation industries all over the world. There are currently more than 145 companies using fermentation processes for production of fine chemicals and therapeutic substances (*e.g.* antibiotics). Annual production of penicillin 1975 alone was 75 million pounds. Refer to Table I for the lists of antibiotics that are widely used for medical and clinical purposes and of the major fermentation industries.

In Korea, antibiotic fermentation industries have just started budding out now. Tetracycline derivatives (Pfizer, CKD), Kanamycin (Dongmyung) are produced by fermentation processes. Penicillin, erythromycin, gentamicin, rifamycin, and a few others are being planned for production within a few years by major pharmaceutical and chemical firms in Korea.

Reviewing past and present activities in the antibiotic fermentation industries in Korea suggests that the prospects for antibiotics will be very bright.

STATE OF THE ART

Almost all antibiotics are considered to be secondary metabolites of cells under certain conditions. There are several important aspects to be considered carefully in the antibiotic fermentation processes. They involve strain problems, physiology, cellular environments (*e.g.* nutrient, precursor, temperature, pH, O_2-respiration, pressure, etc.), and fermentor design.

Table I—Fermentation antibiotic product, 1977 (Perlman[32])

Products	Some producers
Adriamycin	42
Amphomycin	77
Amphotericin B	122
Avoparcin	7
Azalomycin F	113
Bacitracin	11, 13, 39, 60, 77, 88, 92, 93, 106, 121
Bambermycin	55
Bicyclomycin	48
Blasticidin S	64
Bleomycin	86
Cactinomycin	41
Candicidin B	39, 77, 92
Candin	77
Capreomycin	37
Cephalosporins	8, 20, 23, 30, 42, 46, 48, 50, 63, 72, 74, 88, 96, 99, 130, 134
Chromomycin A_3	130
Colistin	13, 14, 39, 68, 71, 105
Cycloheximide	64, 138
Cycloserine	5, 60
Dactinomycin	81, 103
Daunorubicin	42
Destomycin	78
Enduracidin	130
Erythromycin	1, 6, 8, 12, 22, 23, 30, 31, 37, 44, 63, 74, 93, 96, 99, 109, 132, 138
Fortimicins	71
Fumagillin	1, 25
Fungimycin	77
Fusidic acid	73
Gentamicin	23, 25, 116
Gramicidin A	77, 92, 139
Gramicidin J(S)	78, 87
Griseofulvin	23, 50, 61, 73, 86, 130
Hygromycin B	18, 74, 130
Josamycin	114, 144
Kanamycins	6, 20, 23, 38, 78, 86, 96
Kasugamycin	14, 58, 78, 114
Kitasatamcin	134
Lasalocid	56
Lincomycin	138
Lividomycin	67, 105
Macarbomycin	78
Mepartricin	120
Midecamycin	78
Mikamycins	14, 65
Mithramycin	93
Mitomycin C	20, 71
Mocimycin	49
Monensin	74
Myxin	56
Neomycins	10, 11, 18, 23, 42, 77, 86, 92, 93, 105, 109, 122, 130, 138
Novobiocin	78, 105, 138
Nystatin	7, 23, 25, 96, 105, 122
Oleandomycin	93
Oligomycin	103
Paromomycins	91
Penicillin G	3, 4, 5, 6, 10, 14, 15, 17, 20, 23, 41, 45, 49, 50, 54, 55, 62, 74, 78, 81, 88, 93, 96, 105, 119, 122, 126, 134, 143
Penicillin V	1, 3, 4, 5, 14, 17, 18, 20, 37, 45, 49, 50, 55, 74, 78, 88, 93, 105, 122, 124, 143
Penicillins (semi-synthetic)	3, 8, 10, 14, 15, 17, 20, 23, 30, 41, 45, 48, 49, 62, 63, 73, 78, 81, 93, 96, 101, 122, 130, 134, 143
Pentamycin	87
Pimaricin	49
Polymyxins	39, 88, 93,
Polyoxins	64, 114
Pristinamycins	105
Quebemycin	71
Ribostamycin	78
Rifamycins	12, 46, 52
Sagamicin	71
Salinomycin	64
Siccanin	113
Siomycin	118
Sisomicin	41, 116
Spectinomycin	1, 64, 138

Products	Some producers
Streptomycins	6, 10, 23, 49, 50, 78, 81, 88, 93, 126, 143
Tetracyclines:	
Chlortetracycline	7, 9, 23, 36, 42, 54, 62, 76, 99, 102, 118, 120, 130
Demeclocycline	7, 30, 96, 99, 105, 130
Oxytetracycline	9, 18, 23, 25, 30, 44, 49, 61, 63, 69, 70, 72, 93, 97, 99, 102, 121, 132
Tetracycline	3, 7, 9, 10, 12, 14, 20, 22, 23, 26, 30, 31, 36, 42, 43, 44, 52, 54, 55, 58, 59, 62, 63, 75, 88, 93, 94, 99, 101, 102, 109, 120, 121, 122, 130, 132, 138
Tetranactin	27
Thiopeptin	48, 64
Thiostrepton	122
Tobramycin	18, 74
Trichomycin	48
Tylosin	37, 74
Tyrothricin	17, 77, 92, 139
Tyrocidine	77
Uromycin	103
Validamycin	130
Vancomycin	74
Variotin	73, 86
Viomycin	134
Virginiamycin	104

Index to Fermentation Companies:

1. Abbott Laboratories, North Chicago, Illinois
2. Ajinomoto Company, Tokyo, Japan
3. Aktiebolaget Astra, Sodertalje, Sweden
4. Aktiebolaget Fermenta, Strangas, Sweden
5. Aktiebologet KABI, Stockholm, Sweden
6. Alembic Chemical Works Co., Ltd., Baroda, India
7. American Cyanamid, Wayne, New Jersey
8. Anheuser-Busch, Inc., St. Louis, Missouri
9. Ankerfarm S.P.A., Milano, Italy
10. Antibioticos S.A., Madrid, Spain
11. Apothekernes Laboratorium fur Specialpraeparater A/S, Oslo, Norway
12. Archifar A.P.A., Milano, Italy
13. Asahi Chemical Industry, Tokyo, Japan
14. Banyu Pharmaceutical Company, Tokyo, Japan
15. Beecham Pharmaceutical Company Ltd., Surrey, England
16. Joh.A. Benckhiser GmbH, Ludwigshafen/Rhein, West Germany
17. Biochemie GmbH, Kundl/Tirol, Austria
18. Biogal, Debrecon, Hungary
19. C.H. Boehringer Sohn, Ingelheim/Rhein, West Germay
20. Bristol-Myers Company, Syracuse, New York
21. Carlo Erba S.P.A., Milano, Italy
22. Chemibiotic Ltd., Inishannon, Ireland
23. China National Chemicals Import and Export Corporation, Peking, Peoples Republic of China
24. Chinese Petroleum Corporation, Taipei, Republic of China
25. Chinoin, Budapest, Hungary
26. Chong-Kun-Dang Corporation, Seoul, Korea
27. Chugai Pharmaceutical Company, Tokyo. Japan
28. Citrique Belge, Tienen, Belgium
29. Clinton Corn Processing Company, Clinton, Iowa
30. Companhia Industrial Produtora de Antibiotics S.A.R.L. (CIPAN) Lisboa, Portugal
31. Compania Espanola de la Pencillina y A ntibioticos S.A., Arenjulz, Spain
32. CPC International Inc., Argo, Illinois
33. Croda Bowmans Chemicals Ltd., Cheshire, England
34. Dairyland Food Laboratorie Inc., Waukesha, Wisconsin
35. Dawe's Laboratories Inc., Chicago Heights, Illinois
36. Diaspa S.P.A., Coranna, Italy
37. Dista Products Ltd., Liverpool, England
38. Dong-Myung Industrial Company, Ltd., Seoul, Korea

39. Dumex Ltd., Copenhagen, Denmark
40. Eurolysine Company, Paris, France
41. Farbenfabriken Bayer AG, Wuppertal, West Germany
42. Farmitalia S.P.A., Milano, Italy
43. Fermentfarma S.P.A., Milano, Italy
44. Fermic S.A. de S.V., Ixapalape, Mexico
45. Fermion Oy, Tapiola, Finland
46. Fervet S.P.A. (division of CIBA-Geigy Ltd.) Torre Annuziata, Italy
47. Finnish State Alchol Monopoly, Helrinki, Finland
48. Fujisawa Pharmaceutical Company, Osaka, Japan
49. Gist-Brocades, Delft, Holland
50. Glaxo Laboratories Ltd., Greenford, England
51. Grain Processing Corporation, Muscatine, Iowa
52. Gruppo Lepetit S.P.A., Milano, Italy
53. Hailsun Chemical Company, Ltd., Taipei, Republic of China
54. Hindustan antibiotics Ltd., Pimpri, India
55. Hoechst AG, Frankfurt Hoechst, West Germany
56. Hoffmann-LaRoche Inc., Nutley, New Jersey
57. F. Hoffmann-LaRoche and Company, Basle, Switzerland
58. Hokko Kagaku Kogyo Company, Tokyo, Japan
59. ICN-Chimica S.P.A., Milano, Italy
60. IMC Chemicals Group Inc., Terre Haute, Indiana
61. Imperial Industries Ltd., Manchester, England
62. I.S.F. S.P.A., Rome, Italy
63. Istituto Biochimico Italiano, Milano, Italy
64. Kaken Chemical Company, Tokyo, Japan
65. Kanegafuchi Chemical Industries, Osaka, Japan
66. Knoll GmbH, Ludwigshafen, West Germany
67. Kowa Company, Nagoya, Japan
68. Koyaku Antibiotics Research Company Ltd., Tokyo, Japan
69. Krakow Pharmaceutical Works ("Polfa"), Krakow, Poland
70. KRKA Pharmaceutical and Chemical Works, Novo Mesto, Yugoslavia
71. Kyowa Hakko Kogyo Company, Tokyo, Japan
72. Lark S.P.A., Milano, Italy
73. Leo Pharmaceutical Products, Ballerup, Denmark
74. Eli Lilly and Company, Indianapolis, Indiana
75. Linson Ltd., Dublin, Ireland
76. Lohmann and Company AG, Cuxhaven, West Germany
77. H. Lundbeck and Company, Valby, Demark
78. Meiji Seika Kaisha Ltd., Tokyo, Japan
79. Meito Sangyo Company Ltd., Tokyo, Japan
80. E. Merck, Darmstadt, West Germany
81. Merck and Company, Inc., Rahway, New Jersey
82. Mi-Won, Seoul, South Korea
83. Miles Laboratories, Inc., Elkhart, Indiana
84. Misubishi Chemical Industries, Tokyo, Japan
85. Nagase and Company Ltd., Tokyo, Japan
86. Nihon Kayakau Company, Tokyo, Japan
87. Nikken Chemicals Company Ltd., Tokyo, Japan
88. NOVO Industri A/S, Bagsvaerd, Denmark
89. Orsan S.A., Paris, France
90. Pao Yeh Chemical Company, Ltd., Taipei, Republic of China
91. Parke, Davis and Company, Detroit, Michigan
92. S.B. Penick and Company, Lyndhurst, New Jersey
93. Pfizer, Inc., New York, New York
94. Pharmachim Antibiotic Works, Razgrad, Bularia
95. Pharmacosmos, Valby, Denmark
96. Pierrel S.P.A., Milano, Italy
97. Pliva Pharmaceutical and Chemical Works, Zagreb, Yugoslavia
98. Premier Malt Products, Inc., Milwaukee, Wisconsin
99. Proter S.P.A., Milano, Italy
100. Publicker Industries, Inc., Philadelphi a,

Pennsylvania
101. Quimasa S.A., Sao Paulo, Brazil
102. Rachelle Laboratories Inc., Long Beach, California
103. Reanal, Budapest, Hungary
104. Recherche et Industrie Thērapeutigue, Genval, Belgium
105. Rhone-Poulenc S.A., Paris, France
106. G. Richter, Budapest, Hungary
107. Rohm GmbH, Darmstadt, West Germany
108. Rohm and Hass, Philadelphia, Pennsylvania
109. Roussel-UCLAF, Romainville, France
110. Sandoe-Wander AG, Basle, Switzerland
111. Sandoz, Inc., Hanover, New Jersey
112. San Fu Chemical Company Ltd., Taipei, Republic of China
113. Sankyo Company Ltd., Tokyo, Japan
114. Sanraku Ocean Company Ltd., Tokyo, Japan
115. Schering AG, West Berlin, West Germany
116. Schering Corporation, Bloomfield, New Jersey
117. G.D. Searle and Company, Skokie, Illinois
118. Shionogi and Company Ltd., Osaka, Japan
119. Sociedade Produtora de Leveduras Seleccionadas, Mastosinhus, Portugal
120. Societa Prodotti Antibiotici (SPA), Milano, Italy
121. Societé Chimique Pointet Girard, Velleneuve la Garenne, France
122. E.R. Squibb and Sons, Inc., Princeton, New Jersey
123. Standard Brands, Inc., New York, New York
124. Stauffer Chemical Company, Westport, Connecticut
125. John and E. Sturge Ltd., Birmingham, England
126. Surbhai Chemicals Ltd., Baroda, India
127. Tai Nan Fermentation Industrial Company Ltd., Taipei, Republic of China
128. Taiwan Sugar Corporation, Taipei, Republic of China
129. Taiwan Tobacco and Wine Monopoly Bureau, Taipei, Republic of China
130. Takeda Chemical Industries, Osaka, Japan
131. Tanabe Seiyaku Company Ltd., Osaka, Japan
132. Tarchomin Pharmaceutical Works ('Polfa'), Warsaw, Poland
133. Tate and Lyle Ltd., Yorkshire, England
134. Toyo Jozo Company Ltd., Tokyo, Japan
135. Tsin Foods Company, Taipei, Republic of China
136. Tung Hai Industrial Fermentation Company Ltd., Taipei, Republic of China
137. Universal Foods Corporation, Milwaukee, Wisconsin
138. The Upjohn Company, Kalamazoo, Michigan
139. Wallerstein Laboratories, Inc., Morton Grove, Illinois
140. Wei Chuan Foods Corporation, Taipei, Republic of China
141. Wei Wang Industrial Fermentation Company Ltd., Taipei, Republic of China
142. The World Champion Company Ltd., Taipei, Republic of China
143. Wyeth Laboratories, Philadelphia, Pennsylvania
144. Yamanouchi Pharmaceutical Company, Tokyo, Japan
145. Yamasa Shoyu Company Ltd., Chosi, Japan

1. Strain Problems

In general, parent strains are less potent in producing the antibiotics. Many researchers have devoted their endeavors to find an improved strain and maintain it well. The rewarding effort of this kind in this area is well-exemplified for the penicillin and the streptomycin producing microorganisms. See Fig. 1 and 2[1]. Increases in broth potencies of penicillin G from 1941 to 1969 are shown in Fig. 1. Each point represents the highest yield quoted in the literature during each year. Since this type of information is usually proprietary in

nature, it would be expected that a curve drawn from industrial yields would have a greater slope, but its shape would probably be similar. Although the steep part of the curve (1941~1946) is mainly due to advances in the technological development of submerged fermentation, it can be seen that between 1946 and 1969 there was a slower, but still exponential, increase in penicillin yields resulted from strain improvements by mutation. A similar situation can be seen in Fig. 2, which shows the advances in potencies of streptomycin.

2. Physiology

Microorganisms have evolved over the years, developing better and better mechanisms to prevent overproduction of their metabolites there-by achieving the cellular economy. However, our desire is to make the microorganisms to carry out complete conversion of nutrient into antibiotic product with as little nutrient as possible going into the microbial protoplasm or biomass. All microorganisms have to possess regulatory control mechanisms in order to survive very often in the adverse environment. Very efficient organisms are tightly controlled. Our genetic and environmental manipulations often can eliminate, circumvent, or by-pass the residual control mechanisms and results in an increased fermentation yields. In order to work toward this purpose, the cell physiology must be studied and understood (*e.g.* microbial metabolism, pathways, induction, catabolite regulation, feed-back regulation, etc.).

Almost all antibiotics are secondary metabolites produced by a limited number of organisms. They have no general function in life processes although they may be important to the particular antibiotic producing organism. They are usually produced as mixtures of a closely related chemical family, *e.g.*, there are at least three neomycins, five mitomycins, 10 bacitracins, 10 polymyxins, over 10 natural penicillins, and more than 20 actinomycins etc. The ability of an organism to produce secondary metabolite products is easily lost by mutation ("strain degeneration"). The subject of secondary metabolism has been well treated by Weinberg[2].

Derepression of enzymes forming secondary metabolites—Another characteristic of secondary metabolites is that they are usually not produced during the phase of rapid growth (trophophase) but are formed during a subsequent stage known as the idiophase[3]. This phenomenon was first observed during the early development of the penicillin fermentation and has now been found to be a characteristic of many antibiotics fermentations. (Table II)

The factor that triggers secondary product formation at the end of trophophase is unknown. Perhaps it is an internal inducer which accumulates at high cell densities or production is inhibited during trophophase because of catabolite regulation resulting from rapid sugar utilization. Whatever the mechanism is, there is no doubt that at the end of trophophase marked changes occur in the enzymatic composition of the cells and enzymes specially related to formation of secondary products suddenly appear. This derepression of enzyme synthesis is clearly observed in the streptomycin fermentation. Here, a key enzyme of streptidine biosynthesis, amidinotransferase, makes its appearance when the fermentation is 30-hr old

and increases in specific activity for the next 10~30 hrs.

The importance of this enzyme to streptomycin synthesis is shown by the fact that it is present only in streptomycin forming microorganisms. Since appearance of the enzyme can be prevented by chloramphenicol, *de novo* protein synthesis after trophophase is involved. Another enzyme, streptidine kinase, is also formed only during idiophase. In the penicillin fermentation, two enzymes have been observed to increase markedly after trophophase; One activates phenylacetic acid (the side chain of benzyl penicillin) while the other, penicillin acyltransferase, attaches the activated (CoA-ester) phenylacetate to the penicillin nucleus, 6-aminopenicillanic acid (6-APA). The enzyme was not found in fungi which do not produce penicillin and its level was highest in superior penicillin producing strains. In other antibiotic synthesis, similar phenomena were observed and known. (*e.g.* actinomycin, gramicidin S, bacitracin, and patulin fermentations).

Table II—Some antibiotics produced in idiophase

Actinomycin	Hadacidin	Prodigiosin
Bacitracin	Mycobacillin	Tyrocidine
Chlortetracycline	Novobiocin	Streptomycin
Erythromycin	Penicillin	
Gramicidins	Polymyxin	

The most common method used to obtain high-yielding mutants is that of treating a microbial population with a mutagenic agent (physical and/or chemical) until a certain "desired" strain is obtained, plating out the survivors and testing each resulting colony or a randomly selected group of colonies for product formation in flask experiments. Another common procedure is to select the obvious morphological mutants (including those with changes in color) for testing in flasks. It is said that as yields of secondary products reach the 10~15 g/liter range, new methods of selection are needed. The exponential nature of strain selection (Fig. 1) requires that a new mutant of *P. chrysogenum* in 1969 had to produce 1,500 mg/l more penicillin than its parent to show 15% superiority, whereas in 1946 only 100 mg/l were required. Studies on morphological mutations in the organisms producing cycloheximide, nystatin[5] and tetracyclines[6] indicate qualitative connection between clonal morphology and production of antibiotics. Maybe, it is a fortune to obtain a superior mutant strain, but selection methods known would make it possible to meet the chance.

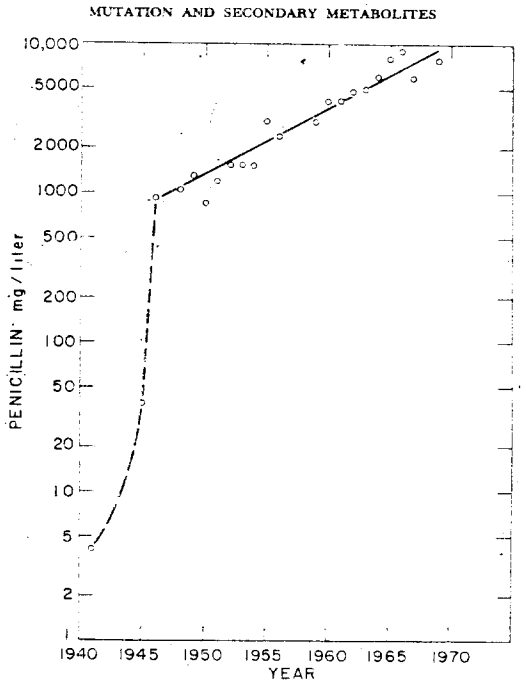


Fig. 1—Maximum literature values for ability to produce penicillin from 1941 to 1969 (Demain,[1])

The mutagenic methods commonly used to obtain a superior antibiotic producing strains

are following:

(a) Physical methods: Irradiation of high energy light (*e.g.* UV, x-ray, γ-ray, etc.)

(b) Chemical methods: Use of reagents producing alterations in deoxyribonucleic acid (DNA)

(1) Analogs of purines and pyrimidines (*e.g.* 5-bromouracil, aminopurine)

(2) Nitrous acid, oxidative deamination

(3) Alkylating agents (*e.g.* ethyl ethane-sulfonates, ethyl methanesulfonates, etc.)

(4) Intercalating agent (*e.g.* acridine, etc.)

Catabolite regulation.—Catabolite regulation was actually observed in antibiotic fermentations years before the general significance of the phenomenon was appreciated. During the early days of penicillin development in the 1940 s, it was found that the rapidly used glucose was an extremely poor substances for penicillin production. Lactose, on the other hand, was slowly used but supported excellent penicillin yields[7]. In the classic chemically defined medium (Jarvis and Johnson)[7] containing a mixture of glucose and lactose, the glucose is rapidly used during trophophase. Upon glucose exhaustion, *Penicillium chrysogenum* is derepressed for lactose utilization and the idiophase begins. During the slow utilization of lactose, the antibiotic is produced in the absence of growth. Lactose is not a specific precursor for penicillin synthesis; its value lies in slow utilization. Today slow glucose feeding or sucrose fed-batch sytems[8] has replaced lactose in the penicillin fermentation. Apparently limiting concentration of glucose keeps catabolites at a low level. A similar situation is seen in the cephalosporin C fermentation, where the chemically defined medium contains the rapidly assimilable glucose for trophophase and the slowly utilized sucrose for the idiophase production of siomycin by washed mycelial suspensions of *Streptomyces sioyaensis* is inhibited by glucose or acetate[9].

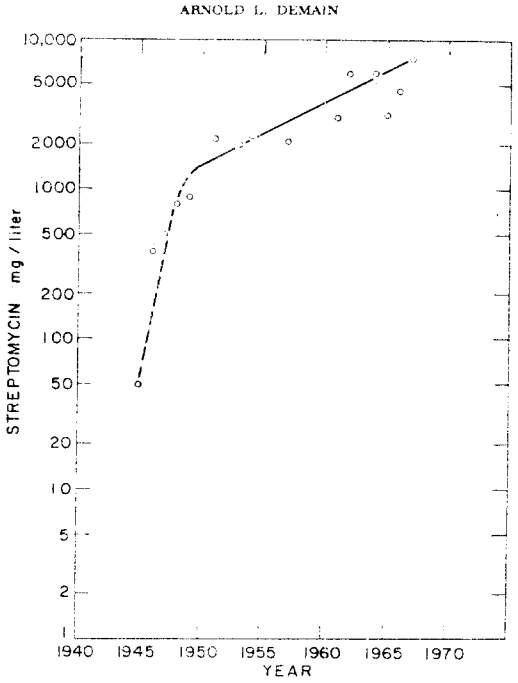


Fig. 2—Maximum literature values for ability to produce streptomycin from 1945 to 1967 (Demain[1])

Catabolite repression of phenoxazinone synthetase, an obligatory enzyme of actinomycin synthesis, has been demonstrated[10]. Inhibition of antibiotic synthesis by glucose has been observed with violacein[11], mitomycin[12], and bacitracin[13]. In the streptomycin fermentation, both streptomycin and mannosido streptomycin are produced concurrently. Towards the end of the fermentation when glucose is depleted, there is a sudden appearance of mannosido-streptomycinase (α-D-mannosidase) which converts the mannosido-streptomycin to streptomycin. Thus, if too much glucose is added, one ends up with an undersirable mixture of the two antibiotics.

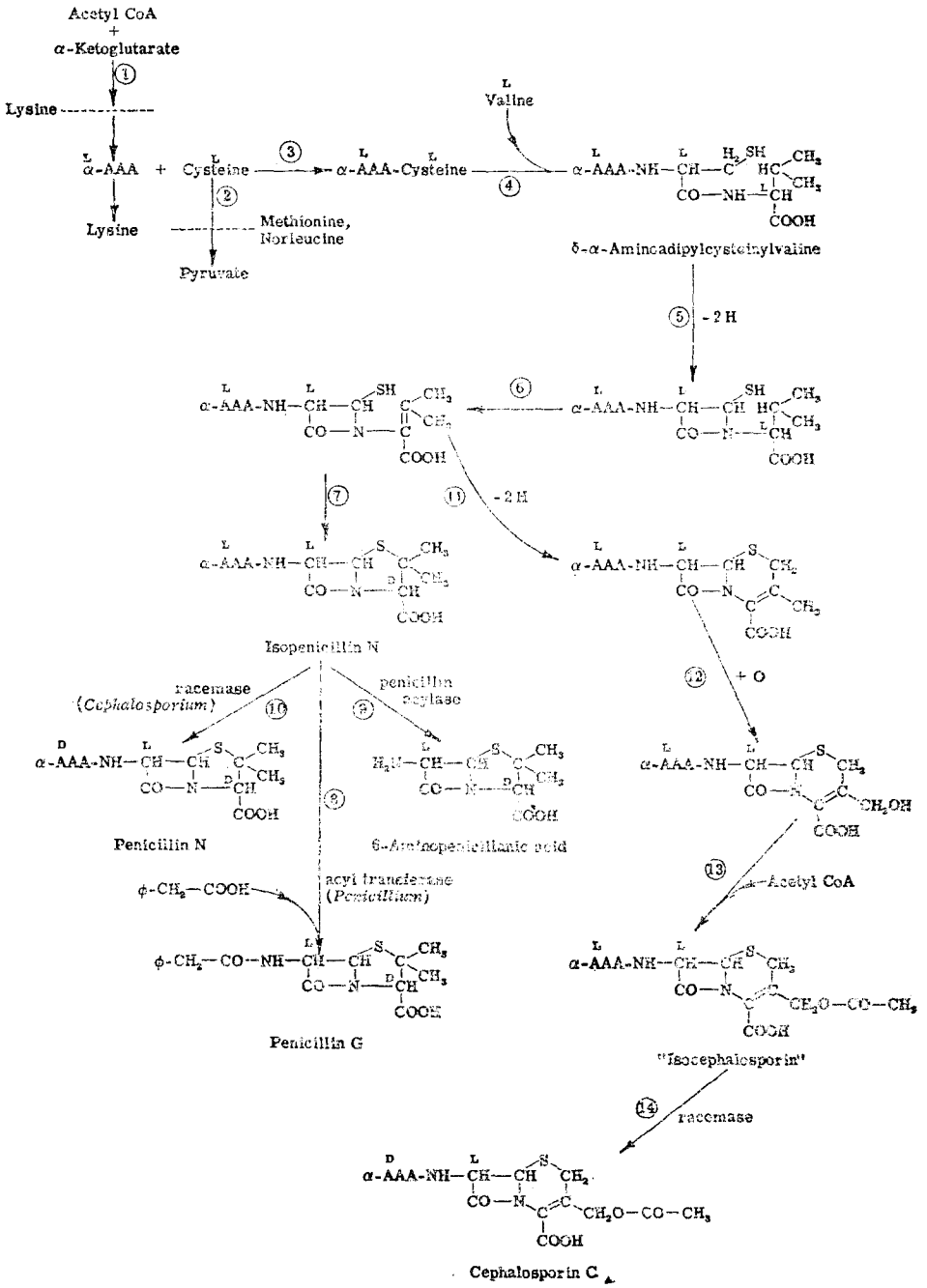


Fig. 3—Hypothetical pathway for the biosynthesis of penicillin G, penicillin N, and cephalosporin C. Dotted lines indicate inhibition. Abbreviations: α-aminoadipic acid, α-AAA; phenyl, φ; coenzyme A, CoA.(Demain[16])

Induction is also important here since the enzyme is inducible as well as repressed by catabolites. The inducer is mannan which is usually supplied by addition of distiller's dried solubles to the medium.

Feedback regulation—Feedback regulation also appears to play a role in secondary metabolism. It was shown many years ago that chloramphenicol inhibits its own production at concentrations nontoxic for growth of *Streptomyces venezuelae*[14]. The inhibition of penicillin production by lysine[15] seems to be due to feed-back regulation by the amino acid of a branched pathway (Fig. 3)[1] leading to both lysine and penicillin[17]. This hypothesis is supported by the several experimental results. In addition, this type of feed-back control does not appear to be so rigid in *Cephalosporium*, where lysine is only a weak inhibitor. In step 2 on Fig. 3 the degradation of cysteine is postulated at the site of methionine or norleucine action in cephalosporin C formation. Several reports on the role of methionine in the cephalosporin C fermentation were argued[18].

By-passing the control of secondary metabolism—Since the production of secondary metabolites is affected by genetically determined mechanisms, derepression, induction, catabolite regulation, and feed-back regulation, it is clear that mutation should have a major effect on the production of secondary metabolites. Although most industrial strain improvement programs are based on mutation and random selection, several novel techniques designed to bypass regulation are worth considering. The technique of using analog in the development of an antibiotic fermentation was demonstrated by Elander *et al*[19].

Tryptophan (a precursor of the antibiotic, pyrrolnitrin) had to be added to the medium for optimal yields. By selecting for *Pseudomonas aureofaciens* mutants resistant to fluorotryptophan. Elander and his coworkers obtained a strain which produced three times as much antibiotic and no longer required supplementation with tryptophan. In those cases where an antibiotic is toxic to young cultures of the producing organism, the antibiotic itself can be used to select resistant cultures, some of which are higher producers. This has been done with streptomycin[20,21] and ristomycin[22]. The technique of mutation and reversio nhas been successfully used to obtain superior producersspau of chlorotetracyclines[23]. Mutation to methionine auxotrophy and reversion increased yields three-fold while mutation to nonproduction of the antibiotic followed by reversion resulted in a ninefold increase in their studies.

3. Control of Cellular Environment

Control of simple process variables such as temperature and air flow and pressure regulation has been accomplished since the early days of the submerged penicillin production. However, only in a few years has it been possible to consider using automatic pH control in the plant, mainly due to the limitations of the asepsis required. Electrodes that could withstand repeated steam sterilization were not available. At present steam-sterilizable electrodes are supplied by several instrument manufacturers and pH can be controlled automatically in industrial processes by adding acid/base or nutrient feeding (*e.g.* NH_3, glucose, etc.).

The actual control of dissolved oxygen (DO) in a fermentation has been attempted in many ways but the major difficulty has been inadequate measurement. It is, of course, prerequisite that any system used for control have an adequte measuring device that is proportional to the changes in the variable that is controlled. Thus, sulfite oxidation method is useless in this case. Nowadays, satisfactory oxygen electrode is available and is used to monitor the concentration of dissolved oxygen (DO) in fermentation broth. Control of the dissolved oxygen could be accomplished by varying power input to the agitation and aeration system, oxygen enriched air, and back pressure based on the DO signal or respiratory signal with closed feed-back control loop.

The measurement of nutrient concentration in actual fermentations has again been limited by inadequate instrumentation. Refractometer was used to measure the concentration of sugar, but not accurately. More specific methods of measurements for some other nutrients might be developed utilizing continuous wet chemical analysis or continuous measurment of optical properties of certain component. Specific enzyme electrode would make it possible to follow up the concentration of nutrients during fermentation period.

It is possible that the cell concentration in a fermentation system may be controlled during the trophophase period by regulating the amount of nutrient feed (*e.g.* glucose, other carbon sources, and/or nitrogen sources, etc.) or other environmental factors.

In flask experiments, the optimal precursor concentration and temperature could be determined and applied to pilot and manufacturing scale processes.

In mold fermentations (*e.g.* penicillin, cephalosporin C, etc.), control of pellet size is very important to maintain the viable cells and to enhance the antibiotic productivity. Also the pellet formation is closely related to the broth viscosity. The structure, size and shapes of pellets can vary considerably from very loose and irregular to compact and spherical. If the pellet becomes compact and large, owing to the limited mass transfer of oxygan, the amount of oxygen as well as other nutrients present in the center of pellets is very low so that

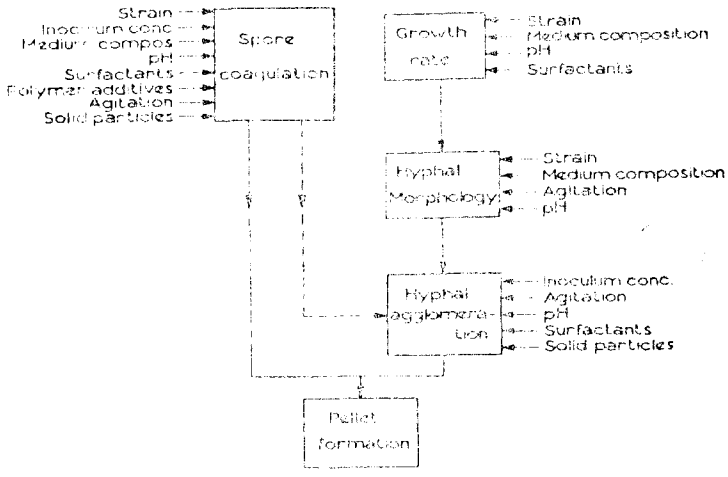


Fig. 4—Block diagram representing factors influencing pellet formation. (B. Metz, *et al.*[34])

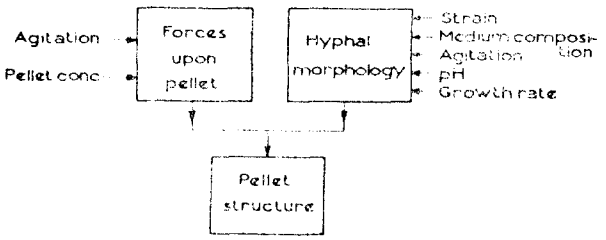


Fig. 5—Block diagram repersenting influences upon pellet structure. (B. Metz, *et al.*[24])

Table III—*Control and state variables of fermentation processes on different environmental levels*

Physical	Chemical	Molecular Biological	Biological
Temperature	pH of the broth	DNA level	Contamination
Vessel pressure	Redox potential	RNA level	Mutation
Power input	Dissolved oxygen	Total protein	
Agitation speed	Dissolved CO_2	Specfic protein	
Gas flow rates	Carbohydrate level (Enzyme activity)		
Liquid feed rates	Nitrogen source level		
Viscosity	Precursor level		
Liquid volume	Mineral ion level		

depletion of oxygen and nutrients can occur very easily and cells are autolysed. The pellt formation and its structure are very involved (Fig. 4, 5)[24]. In each case, several factors involved in the process should be considered simultaneously, for example, disolved oxygen concentration, medium composition, pellet characteristic and structure, etc.

It is possible to control the foaming by the physical foam breaker attached to the agitator shaft in a fermentor or by non-toxic chemical antifoaming agents.

Some control and state variables are listed in Table III. Nowadays, with the aid of computer monitering system and variable control accessaries, fermentation variables and processes could be controled reasonably well, though not completly automatic.

4. Fermentor Design

Good design practice embodies several cardinal rules. The rules are directed mostly at aseptic practice, *i.e.*, keeping the fermentation process contamination-free. Microorganisms are a rich source of protein, some of which is foregin to the human body. Large inhaled doses of any microorganism will usually produce a reaction in most humans. Therefore, care must be exercised to avoid any design procedure which would permit the escape of aerosol from air outlet of the fermentor. This means that safe practice demands either incineration, filtration or dilution (by dispersing from a high effluent stack) of the exit gas of the fermentor. Some fermentor inherently produces large quantities of aerosols. It also means that

whenever centrifuges are used some consideration should be given to enclosing these units in a specially ventilated area. The cardinal design rules are following[25].

a. There should be no direct connections between sterile and nonsterile parts of the system. Bacteria have been known to grow through closed valves.

b. Minimize flange connections. Due to equiment vibration and thermal expansion, flanged connections do move and contamination can work its way through many flanged seals.

c. Whenever possible, use all welded construction. Be sure to polish all welds so that no reservoirs exist for the accumulation of solid medium that could resist sterilization.

d. Avoid dead spaces, crevices, pin holes, and the like. Solids can accumlate in these and provide an insulating environment for contaminants to escape sterilization.

e. Various parts of the system should be independently sterilizable.

f. Any connections to the vessel should be steam-sealed. For example, the valve on the sampling port should have live steam on the exit side whenever it is not in use.

g. Use valves which are easy to clean, maintain and sterilize. Examples are ball valves, diaphragm valves and globe valves with non-rising stems. (For items f & g above)

h. Maintain a positive pressure in the fermentor so that leakage is always out (with pathogens this is not applicable).

Fermentor geometry—In antibiotic fermentation a conventional fermentor is usually used. A conventional fermentor usually consists of an upright cylindrical tank fitted with four baffles, a jacket and/or coil for heating and cooling, an air sparger, a device for mechanical agitation and an air filter. Depending on the requirements, other accessories and systems may be included for control of foaming and addition of nutrient. In addition to the basic instrumentation and control of temperature, pH, air flow rate, equipments may be added for recording and/or controlling of back pressure, oxidation reduction potential, dissolved oxygen (DO), effluent gaseous oxygen and CO_2 concentrations, nutrients, and other variables of interest.

Illustrated in Fig. 6[26] are some of the more important dimensional ratios of a general-purpose fermentor. These ratios are not absolutely critical as indicated by the range of values given. The ratios may be deliberately changed for different fermentation processes (*i.e.* bacteria or mold.). The maximum value of Z may, in turn, be determined by the head space needed to control foaming. It would appear that the fermentation industry has not attempted to maintain geometric similarity of dimensional

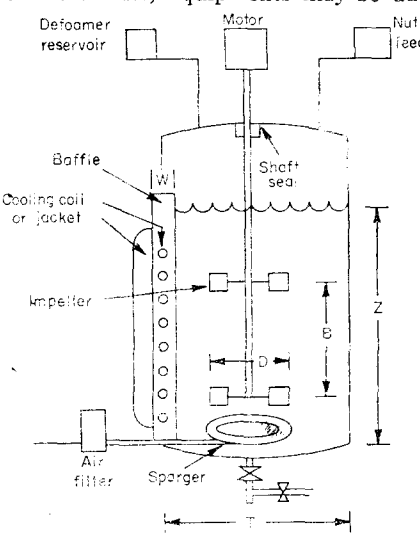


Fig. 6—Some dimensional ratios of a general purpose fermentor. (R. Steel, *et al.*[26])

ratios in scale-up of equipment from pilot plant to production scale. As far as the growth of unicellular organisms (*e.g.* yeast and bacteria) is concerned this does not pose a problem because the oxygen absorption rate correlates reasonably well with power input/unit volume for fermentors of different sizes. But in mold fermentation, it is very important that the number, size, and spacing of impellers are designed optimally because of the problems related to adequate oxygen transfer rate that depends on the characteristics of fermentation broth, aeration, and agitation.

Construction materials—For laboratory-scale equipment the fermentor body may be a Pyrex glass jar or a length of standard Pyrex pipe. The use of glass allows visual inspection of the fermentor interior whereas stainless steel construction offers zero breakage and better heat transfer. Manufacturing plant and pilot-plant fermentors are usually constructed with type 316 stainless steel, while production-scale vessels are often stainless clad, or 314 stainless steel. In most cases stainless steel satisfies the requirements of chemical inertness, ease of cleaning, absence of toxic effects on the fermentation, and long life.

Aeration-agitation systems—The usual aeration system is composed of an open-pipe sparger as a ring or cross sparger with holes to break the air stream into small bubbles. If the holes are situated on the bottom of the sparger ring it is a simple task to clean the sparger internally by passing steam through it. Aeration may also be carried out with metallic or ceramic discs or candles but these are difficult to clean properly. In addition to mixing generated by aeration, mechanical mixing is achieved by one or more impellers located on the agitation shaft. The size and shape of the impellers are very important considerations for aerobic mold fermentation to achieve uniform mixing and sufficient supply of nutrients and oxygen.

In Fig. 7, it is shown that the oxygen mass transfer coefficient (k_La) value decreases logarithmically with an increase in apparent viscosity within a certain range of apparent viscosity. Because of this reduction of k_La in actual fermentation broth, the rate of oxygen supply or the oxygen transfer rate by mixing and aeration, may become a very serious

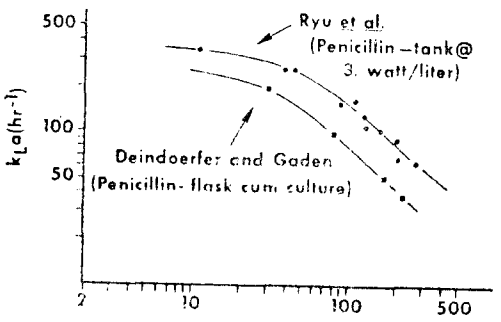


Fig. 7—Comparison of the effect of apparent viscosity at a reference shear rate for various cell concentrations of penicillin on the mass transfer coefficient. (Ryu, *et al.*[27])

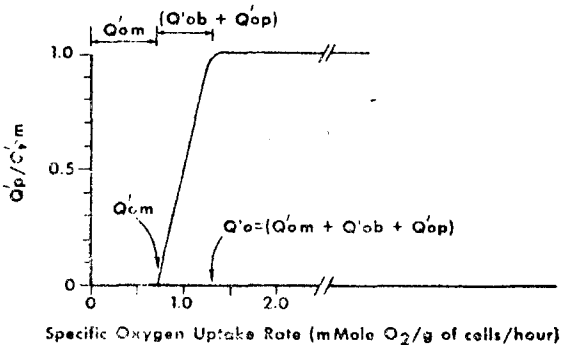


Fig. 8—Illustration of the effect of oxygen uptake rate on productivity of a typical fermentation. (Ryu, *et al.*[27])

problem in fermentation processes. The rate of oxygen supply, or the oxygen transfer rate, affects, in turn, the rate of production. In fact, it often becomes the rate-limiting factor in fermentation processes. For example, penicillin productivity depends on the oxygen uptake rate as shown in Fig. 8. Organisms require a certain amount of oxygen for maintenance of cells without any production of antibiotics. This quantity, Q'_{om} is called the specific rate of oxygen uptake for maintenance. Beyond this value of Q'_{om} the specific production rate of antibiotics increases proportionally to the oxygen uptake rate up to a certain level of oxygen uptake rate, namely Q'_o. The maximum level of specific production rate Q'_{pm} can be achieved when the oxygen uptake rate is at least equal to or greater than Q'_o. To satisfy the value of Q'_o, we have to ensure that the oxygen transfer rate (OTR) is at least equal to or greater than the oxygen uptake rate (OUR), if the maximum production rate is to be ensured and maintained[27~29].

Therefore, viscous antibiotic fermentation needs the efficient facilities for aeration and a good fermentor design.

PROBLEMS TO BE SOLVED FOR THE FUTURE

Although many successes have been reported and difficulties have been recognized, the application of genetic engineering to the selection of microorganism with greater capacity for carrying out the biosynthesis of desired antibiotics should be made seriously and continually. It is also desirable that we screen and develop a new antibiotic producing strains from manyspdifferent sources.

The economics of the antibiotics derived from fermentation will change in the future as they have in the past. Some costs such as raw materials including carbohydrate and protein that have followed a cyclic pattern in the past will not do so in the future because they will be valued as food and will not be in oversupply as in the past. Therefore, it is important to develop novel carbon and nitrogen sources for a certain antibiotic production.

Using the knowledge of cellular physiology, idiophase period control by fermentation hardware such as pH controllers, aeration controllers, and nutrient control (*e.g.* fed-batch) must come into the picture to make fermentation processes more economical.

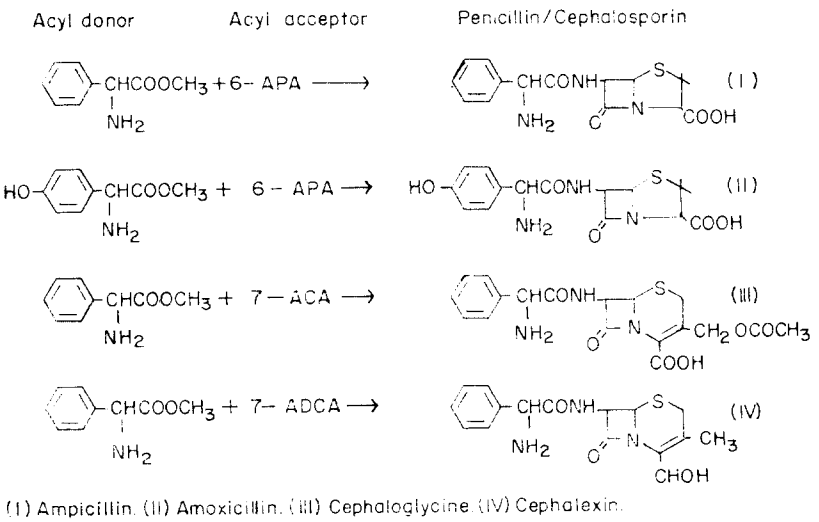
The increased costs of construction and fabrication of fermentation facilities has forced us to develop new fermentor designs that will have to be more efficient than the conventional stirred vats.

According to recent FDA regulation, penicillin, tetracyclines and erythromycin may be restricted as animal feed supplements. Therefore alternatives must be developed as soon as possible. Several drugs have already been approved for such a purpose in the U.S. virginiamycin, bambermycins; in Europe avoparcin, mocimycin in addition to bambermycins and virginiamycins already in use; and in Japan thiopeption, enduracidin, macarbomycin, and quebemycin in addition to the currently used mikamycins, and kitasatomycins. The large size of this maket for the animal feed antibiotics is attractive, and it is likely that other candidates will be evaluated and eventually introduced in the future. The candidate compounds for

animal feed antibiotics must be well tolerated when administered *per os*, effective when added at one gram per ton of feed, chemically and biologically stable in the feed mix, effective inhibitors of gram-positive bacteria, and inexpensive to produce in useable form.

Another field for antibiotic usage is to control plant pathogens and food spoilage organisms. Since more than 60% of fruit, vegetables, and grain undergo some microbial spoilage in growing areas or in transport to the consumer, there will be increased emphasis on the use of antibiotics to control these organisms. Among antibiotics already under extensive evaluation in Japen are ezomycin, milbemycins, the anisomycin analog NK-049, and the antiviral aabomycin and laurusin. Those used in Japan in 1974 are blasticidins (3 million lb), kasugamycin (16 million lb), validamycin (8 million lb), and polyoxins (1.7 million lb).

In connection with the fermentation engineering, enzyme engineering for semi-synthetic antibiotics is very promising and a very interesting field. Enzyme processes for 6-APA production and semi-synthetic penicillin (*e.g.* ampicillin, etc) and cephalosporin derivatives (*e.g.* cephalexin, etc) are already in use in the pharmaceutical industries. Intensive studies on enzyme production, immobilization process, and enzyme reactor system are carryied out worldwide. For example, penicillin amidase obtained from *B. megaterium* and *E. coli* and immobilized on the ion exchange resins and bentonite was thoroughly studied for production of 6-APA from benzylpenicillin[30]. Semisynthetic penicillins and cephalosporins can now be synthesized using immobilized enzymes at a very low process cost[31]. Some examples are shown in Fig. 9.



(I) Ampicillin (II) Amoxicillin (III) Cephaloglycine (IV) Cephalexin

Fig. 9—Typical examples of penicillin/cephalosporin synthesis by the Family Pseudomonadaceae bacteria (Takahashi, *et al.*,[31])

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