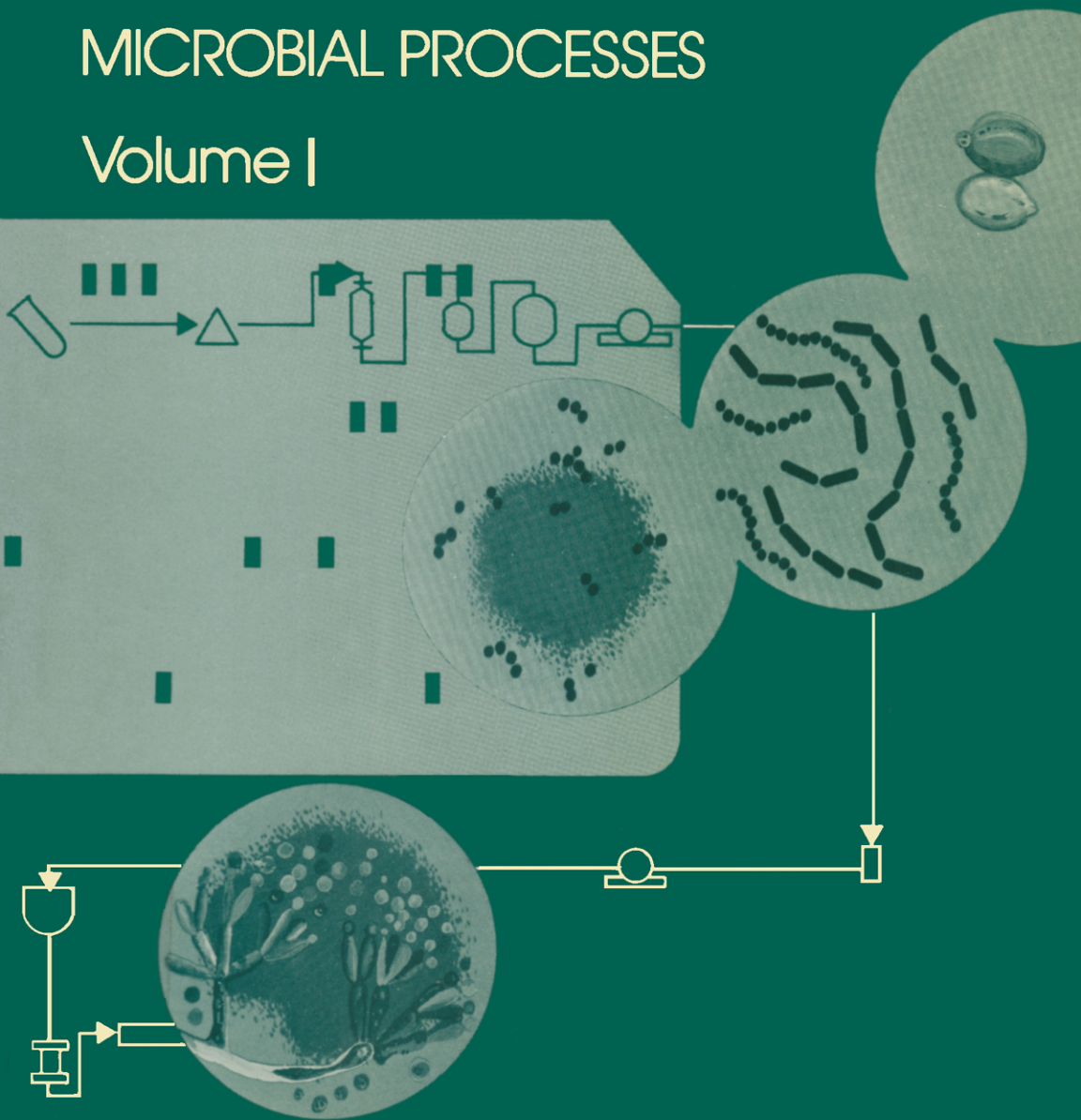
SECOND EDITION

MICROBIAL TECHNOLOGY

MICROBIAL PROCESSES

Volume I



H. J. PEPPLER and D. PERLMAN, Editors

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Microbial Technology

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Second Edition/Volume I

Microbial Technology

Microbial Processes

Edited by

H. J. PEPPLER
Universal Foods Corporation
Milwaukee, Wisconsin

D. PERLMAN
School of Pharmacy
University of Wisconsin
Madison, Wisconsin

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List of Contributors

Numbers in parentheses indicate the pages on which the authors' contributions begin.

Ralph F. Anderson* (1), Velsicol Chemical Corporation, Chicago, Illinois 60611

Otto Andresen (281), NOVO Industri A/S, Bagsvaerd, Denmark

Knud Aunstrup (281), NOVO Industri A/S, Bagsvaerd, Denmark

Joe C. Burton (29), Research Department, The Nitragin Company, Milwaukee, Wisconsin 53209

F. Constabel (389), Prairie Regional Laboratory, National Research Council of Canada, Saskatoon, Saskatchewan S7N OW9, Canada

I. W. Cottrell (417), Research and Development Department, Kelco Division, Merck & Company, Inc., San Diego, California 92123

Edvard A. Falch (281), NOVO Industri A/S, Bagsvaerd, Denmark

J. Florent (497), Centre Nicolas Grillet, Rhône-Poulenc Industries, Vitry-sur-Seine, France

Yoshio Hirose (211), Biochemical Department, Central Research Laboratories of Ajinomoto Company, Ltd., Kawasaki-ku, Kawasaki 210, Japan

Carlo M. Ignoffo (1), U.S. Department of Agriculture, Science and Education Administration, Federal Research, Columbia, Missouri 65205

K. S. Kang (417), Biochemical Development, Kelco Division, Merck & Company, Inc., San Diego, California 92123

W. G. W. Kurz (389), Prairie Regional Laboratory, National Research Council of Canada, Saskatoon, Saskatchewan S7N OW9, Canada

John H. Litchfield (93), Battelle, Columbus Laboratories, Columbus, Ohio 43201

Lewis B. Lockwood (355), Biology Department, Western Kentucky University, Bowling Green, Kentucky 42101

* Deceased

J. L. Martin* (187), International Minerals & Chemical Corporation, Terre Haute, Indiana 47808

Yoshio Nakao (311), Microbiological Research Laboratories, Central Research Division, Takeda Chemical Industries, Ltd., Jusohonmachi, Yodogawa-ku, Osaka, Japan

Tage Kjaer Nielsen (281), NOVO Industri A/S, Bagsvaerd, Denmark

L. Ninet (497, 529), Centre Nicolas Grillet, Rhône-Poulenc Industries, Vitry-sur-Seine, France

Hiroshi Okada (211), Biochemical Department, Central Research Laboratories of Ajinomoto Company, Ltd., Kawasaki-ku, Kawasaki 210, Japan

H. J. Peppler† (157), Universal Foods Corporation, Milwaukee, Wisconsin 53201

D. Perlman (241, 483, 521), School of Pharmacy, University of Wisconsin, Madison, Wisconsin 53706

Randolph S. Porubcan (59), Chr. Hansen's Laboratory, Inc., Milwaukee, Wisconsin 53214

J. Renaut (529), Centre Nicolas Grillet, Rhône-Poulenc Industries, Vitry-sur-Seine, France

O. K. Sebek (483), Infectious Diseases Research, The Upjohn Company, Kalamazoo, Michigan 49001

Robert L. Sellars (59), Chr. Hansen's Laboratory, Inc., Milwaukee, Wisconsin 53214

M. T. Walton** (187), International Minerals & Chemical Corporation, Terre Haute, Indiana 47808

* Present address: 1334 S. Center Street, Terre Haute, Indiana 47802.

† Present address: 5157 N. Shoreland Avenue, Whitefish Bay, Wisconsin 53217.

** Present address: RFD No. 3, Shoals, Indiana 47581.

Preface

In the decade since the first edition of "Microbial Technology" appeared, applied microbiology has changed, expanded, and diversified. As new products were introduced in this period, and greater demand for some of the old ones developed, the total fermentation capacity increased at about the same rate noted in the previous 25 years. The number of fermentation products, it is estimated, has quadrupled, while the volume of products manufactured has increased tenfold. This growth has prompted publication of a second edition, completely revised and enlarged.

To accomplish a worldwide survey of industrial microbiology and to describe its contributions to agriculture, industry, medicine, and environmental control, the editors are indebted to 57 willing and expert contributors. Their comprehensive reviews of traditional fermentations and propagations, as well as newly developed microbe-dependent processes and products, are presented in a two-volume set.

Volume I, subtitled "Microbial Processes," describes the production and uses of economic bacteria, yeast, molds, and viruses, and reviews the technologies associated with products of microbial metabolism.

Volume II, subtitled "Fermentation Technology," deals principally with fermentations and modifications of plant and animal products for foods, beverages, and feeds, while reviewing salient aspects of microbial technology: general principles, culture selection, laboratory methods, instrumentation, computer control, product isolation, immobilized cell usage, economics, and microbial patents.

H. J. Peppler
D. Perlman

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Microbial Technology

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Chapter 1

Bioinsecticides

CARLO M. IGNOFFO
RALPH F. ANDERSON*

I. INTRODUCTION

Control of insect pests costs more than one-half billion dollars annually in the United States. Yet less than 5% of this is spent for microbial insecticides, despite the fact that entomopathogens (pathogens of insects) have been suggested as controlling agents of insect pests for over a century. Synthetic organic chemical insecticides in vogue today were not even available until about 50 years after control of an insect pest was demonstrated by using an entomopathogen. Obviously, the development of microbial insecticides has been pitifully slow. However, within

* In Memorium: Ralph F. Anderson (1924–1978).

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the last decade there has been a significant revival. This was largely brought about by recent successes in developing entomopathogenic viruses and bacteria and was further fortified by the public's increased awareness of the impact of toxic, broad-spectrum chemical insecticides.

Of the nearly 1 million species of known insects only about 15,000 species are considered pests and only about 300 are sufficiently destructive to require some control. Fortunately, most insect pests have pathogenic microorganisms associated with them. About 1500 entomopathogens belonging to the fungi, viruses, protozoa, or bacteria are known. Of these, bacteria and viruses, because of their known effectiveness and relative lack of toxicity or pathogenicity to nontarget animals and plants, have been developed into commercial products in the United States.

A candidate entomopathogen, whether it is a bacterium, virus, fungus, or protozoan, must meet certain technical prerequisites before it can be developed into a microbial insecticide. Three of the most important technical prerequisites are: (1) availability or feasibility of a systematic, continuous production technology; (2) minimal or no toxicity or pathogenicity to man, nontarget animals, and plants; and (3) proved effectiveness against the intended target pest. However, from a commercial, nontechnical viewpoint the ultimate and most important question is whether this development will result in a reasonable profit to the industrial developer. Obviously, then, the decision whether or not to develop a candidate entomopathogen into a microbial insecticide is based upon favorable answers to both technical and nontechnical questions.

II. HISTORICAL BACKGROUND

Aristotle's description of diseases of the honey bee in *Historia animalium* was probably the first documentation of an insect disease. Observations of insect maladies, particularly those of bees and silkworms, were reported in early Greek and Roman literature as well as in the works of various poets and naturalists of the sixteenth to nineteenth centuries. For example, Kirby and Spence (1826) included a chapter called "Diseases of Insects" in their classical text "*Introduction to Entomology*."

Insect pathology per se probably had its beginning in the nineteenth century under the stimulus of Bassi (1835) and Pasteur (1870). Bassi was the first to demonstrate that a microorganism, the fungus *Beauveria bassiana,* could cause an infectious disease in an animal (*the silkworm*).

Bassi not only contributed to man's understanding of infectious diseases but also implied that infectious diseases could be used to control insects. Pasteur's investigations were concerned not with insect control but with control of diseases in populations of the beneficial silkworm and honey bee. These studies drew attention to the impact of diseases on insect populations and their feasibility for use as microbial insecticides.

A significant contribution to microbial control of insects was made by Metchnikoff (1879) and Krassilstschik (1888). They were the first to document that an entomopathogen, a muscardine fungus, *Metarrhizium anisopliae,* could be mass produced and applied as a microbial insecticide to control the grain weevil and the sugar beet curculio. These early efforts inspired Giard (1892), Forbes (1898), and Snow (1891) to widely use another muscardine fungus (*B. bassiana*) in an unsuccessful attempt to control chinch bugs.

Control of insect pests with bacteria was probably first attempted by d'Herelle (1914) approximately 35 years after Pasteur's description of silkworm diseases. Apparently, control was not consistent and therefore interest in bacterial pathogens was muted. However, after a lag of nearly 30 years, White and Dutky (1940) succeeded in demonstrating control of the Japanese beetle by distributing spores of the milky disease bacterium, *Bacillus popilliae.* Undoubtedly, this success stimulated other investigators to reinvestigate bacteria, and literature began appearing on the effectiveness of *Bacillus thuringiensis.* Issuance of eight patents between the years 1960 and 1963 for *B. thuringiensis* further attested to the revived interest in bacterial insecticides (Briggs, 1964). A more detailed history of the use of bacteria to control insects is presented by Steinhaus (1964), Heimpel (1965), and DeBach (1964).

Use of viruses to control insect pests was stimulated by the studies of Balch and Bird (1944) and Steinhaus and Thompson (1949) during the years immediately following World War II. This initial interest, which lagged after the first successful demonstration, is presently having a rebirth, as evidenced by the recent registration of the first viral pesticide (Ignoffo, 1973a, b,c) in the United States by the Environmental Protection Agency (EPA).

Documentation of the historical development and use of entomopathogens as microbial insecticides is an empty exercise without noting the contributions of an inspiring, enthusiastic giant, Edward A. Steinhaus, who died in 1969 (Linsley and Smith, 1970). Dr. Steinhaus not only developed the first university curriculum of insect pathology and laboratory of pathology in the United States but also wrote texts, reference sources, and numerous articles on invertebrate pathology. He was responsible for organizing the Society of Invertebrate Pathology and

starting the *Journal of Invertebrate Pathology*. His guiding principles and vision still inspire and influence invertebrate pathologists. Through his efforts there are laboratories throughout the world devoted to both basic and applied research in invertebrate pathology and a flourishing society and a journal.

III. CANDIDATE MICROBIAL INSECTICIDES

A. Entomopathogenic Bacteria

From a stockpile of nearly a hundred species associated with insects, only three bacteria (*B. popilliae, B. thuringiensis,* and *B. moritai*) were developed into commercial microbial insecticides (Table I). *In vitro* and *in vivo* production processes both are used (cf. Section V,A). The bacterium *B. popilliae*, produced only in larvae of Japanese beetles, is formulated into dust (Dutky, 1963). In contrast, *B. thuringiensis* and *B. moritai* are produced by conventional fermentation techniques. It is significant that the bacterial species that have been most useful for insect control have been sporeformers, perhaps because they can be

TABLE I. Trade Names of Commercial or Experimental Preparations of Microbial Insecticides Formulated from Bacteria

Bacterial species	Trade name	Producer
Bacillus moritai	Rabirusu	Sumitomo, Japan
Bacillus popilliae	Doom, Japidemic	Fairfax Biological Labs. (U.S.A.)
Bacillus thuringiensis		
β-Exotoxin	Biotoxksybacillin	All Union Inst. Agr. Microbiol. (U.S.S.R.)
	Eksotoksin	Glavmikrobioprom (U.S.S.R.)
	Toxobakterin	Glavmikrobioprom (U.S.S.R.)
δ-Endotoxin	Agritrol	Merck & Co. (U.S.A.)
	Bakthane	Rohm & Haas (U.S.A.)
	Bactospeine	Roger Bellon (France)
	Bathurin	Chemapol-Biokrma (Czechoslovakia)
	Biospor	Farbwerke Hoechst (Germany)
	Biotrol BTB	Nutrilite Prod (U.S.A.)
	Dendrobacillin	Glavmikrobioprom (U.S.S.R.)
	Dipel	Abbott Labs (U.S.A.)
	Entobacterin	Glavmikrobioprom (U.S.S.R.)
	Insektin	Glavmikrobioprom (U.S.S.R.)
	Parasporin	Grain Proc. Lab (U.S.A.)
	Sporeine	LIBEC Laboratoire (France)
	Thuricide	Sandoz-Inc. (U.S.A.)

easily mass produced and are stable enough to be handled in commerce. A relatively large number of entomopathogenic spore-forming bacteria were listed by Steinhaus (1959). However, none of the nonsporeformers, (which also can be mass cultured) have been commercialized, probably because they are not easily stabilized for field use. Safety to humans, other vertebrates, and plants was demonstrated for *B. popilliae, B. thuringiensis,* and *B. moritai* but has not been established for other candidate bacterial pathogens of insects (cf. Section V,B). Surprisingly, 19 proprietary names are reported for *B. thuringiensis* preparations (Ignoffo, 1967, 1975). Thirteen formulations are based upon the δ-endotoxin and three on the β-exotoxin. Two trade-name products are formulated from *B. popilliae* and one from *B. moritai.*

B. Entomopathogenic Viruses

Many insect pathologists believe viruses have the greatest potential for development into microbial insecticides. Entomopathogenic viruses are specific and are active against many economically important insects (Ignoffo, 1967, 1968, 1975). About 650 insect viruses are described (Martignoni and Iwai, 1977), and more are being discovered each year. In fact, there has been a phenomenal increase (over 250%) in the number of new viruses reported within the decade covered by this revision (Anderson and Ignoffo, 1967).

Just as the more useful candidate bacteria are protected by spores, the most useful insect viruses are protected by a proteinaceous inclusion body. Those without this protective body are called noninclusion viruses. The inclusion body of insect viruses varies in shape from the irregular or regular polyhedrons (PIB) characteristic of the polyhedrosis and entomopoxviruses (EPV) to ovoid bodies representative of the granulosis viruses (GIV). The site of viral replication also is commonly used to describe insect viruses, e.g., nuclear polyhedrosis (NPV) or cytoplasmic polyhedrosis (CPV). Entomopathogenic viruses are currently placed in seven genera based upon the morphology and physiobiochemical characteristics of the virion (Ignoffo, 1973a; Vago *et al.*, 1974). Latinized binomials are used to name the viruses and the suffix-*virus* is used for all the generic names, e.g., *Baculovirus, Entomopoxvirus, Iridovirus.*

The NPV and GIV, because of their specificity, safety, virulence, and stability, are probably the most promising candidates for consideration as viral insecticides. The CPV and EPV are less virulent, but they have the other characteristics of a good microbial pesticide. Noninclusion viruses, although virulent, are not currently considered likely candidates because less is known about their safety, production feasibility, and

efficacy. Considerable information has been obtained on the safety and specificity of entomopathogenic viruses within the last decade (cf. Section V,B,3).

Because of their specificity, insect viruses are more difficult to produce on an industrial scale than bacteria or fungi. They are obligate parasites and consequently must be mass produced *in vivo,* i.e., in living hosts (cf. Section V,A,3). In spite of this inherent difficulty, insect viruses representing every major group have been produced and used in the field as viral insecticides (Ignoffo, 1967, 1975). In addition, more than a dozen commercial or experimental preparations of entomopathogenic viruses have been evaluated sufficiently enough to warrant a trade name designation (Table II). As of 1977, two insect viruses have been commercialized: the *Heliothis* NPV, trade-named Elcar (USA)*; and a CPV of *Dendrolimus* called Matsukemin in Japan. The U.S. Forest Service recently registered an NPV of the tussock moth, TM-BioControl-T (Martignoni and Iwai, 1978), and submitted a registration request to EPA for the gypsy moth NPV. Data are also being collated for submission of a registration of another NPV, i.e., *Autographa,* by the Agricultural Research Service. Insect viruses continue to be attractive candidates for microbial insecticides. Within the last decade production feasibility has been established, safety and specificity to nontarget organisms has been confirmed, and effectiveness against target pests has been demonstrated.

C. Entomopathogenic Fungi

No fungi are presently available as commercial microbial insecticides in the United States, but several were produced and used on a large scale in the Soviet Union (Table III). All classes are represented among the more than 500 entomopathogenic fungi (Ignoffo, 1967). Most of the promising fungi are in the class Phycomycetes and Deuteromycetes. Fungi associated with insects are considered effective pathogens if and when field conditions are optimal for spore germination, invasion, and growth. Because these conditions are not predictable, the usefulness of fungi for insect control is often questioned (Yendol and Roberts, 1970). Some mycologists (Bucher, 1964; Latch, 1965) contend that entomogenous fungi are ubiquitous and that field application for insect control is

* This paper reports the results of research only. Mention of a pesticide in this paper does not constitute a recommendation for use by the USDA, nor does it imply registration under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) as amended. Also, mention of a commercial product in this paper does not constitute an endorsement of this product by the USDA.

TABLE II. Trade Names of Commercial or Experimental Preparations of Microbial Insecticides Formulated from Viruses

Viral type and host	Trade name	Producer
Cytoplasmic polyhedrosis		
Dendrolimus	Matsukemin	Chugai Pharmaceutical Co., Ltd. (Japan) Kumiai Chemical Industry Co., Ltd. (Japan)
Nuclear polyhedrosis		
Heliothis	Biotrol VHZ	Nutrilite Prod. (U.S.A.)
	Elcar	Sandoz, Inc. (U.S.A.)
	Virex	Hays-Sammons (U.S.A.)
	Viron/H	Int. Minerals & Chem. Corp. (U.S.A.)
Lymantria	Virin-ENSH	Glavmikrobioprom (U.S.S.R.)
Mamestra	Virin-EX	Glavmikrobioprom (U.S.S.R.)
Neodiprion	Polyvirocide	Indiana Farm Bureau (U.S.A.)
Orgyia	TM BioControl-1	Forest Service (U.S.A.)
Pieris	Virin GKB	Latvian Agr. Acad. (U.S.S.R.)
Prodenia	Biotrol VPO	Nutrilite Prod. (U.S.A.)
	Viron/P	Int. Minerals & Chem. Corp. (U.S.A.)
Spodoptera	Viron/S	Int. Minerals & Chem. Corp. (U.S.A.)
Trichoplusia	Biotrol VTN	Nutrilite Prod. (U.S.A.)
	Viron/T	Int. Minerals & Chem. Corp. (U.S.A.)

unnecessary. Others (Dunn and Mechalas, 1963; Müller-Kögler, 1965; Roberts and Yendol, 1971; McCoy *et al.*, 1976) are convinced that fungal insecticides are effective if properly used and that their usefulness can be increased when combined with chemical insecticides (Pristavko, 1963).

Fungal mycelium, spores, and toxins can be mass produced, either *in vitro* or *in vivo* (cf. Section V,A). Obviously, the *in vitro* method, either via submerged or surface fermentation, is preferred (Ignoffo, 1967; Dulmage and Rhodes, 1971; Roberts and Yendol, 1971). Some of the fungi that have been successful in controlling insects are *Aschersonia aleyrodis,*

TABLE III. Trade Names of Commercial or Experimental Preparations of Microbial Insecticides Formulated from Fungi

Fungal species	Trade name	Producer
Aschersonia aleyrodis	Aseronija	All Union Inst. Agr. Microbiol. (U.S.S.R.)
Beauveria bassiana	Biotrol FBB	Nutrilite Products (U.S.A.)
	Boverin	Glavmikrobioprom (U.S.S.R.)
Hirsutella thompsonii	ABG-6065	Abbott Labs (U.S.A.)
Metarrhizium anisopliae	Biotrol FMA	Nutrilite Products (U.S.A.)

B. bassiana, M. anisopliae, Entomophthora thaxteriana, and *Nomuraea rileyi* (Ignoffo, 1975).

One advantage of entomopathogenic fungi is that they are generally less specific than other groups of entomopathogens and consequently can be used against different types of insect pests. For example, species of fungi were successful in controlling scale insects, whitefly, plant hoppers, aphids, chinch bugs, phytophagous beetles, flies, caterpillars, and stored-products pests. More extensive development of fungal microbial insecticides, however, must await additional evidence of field efficacy (cf. Section V,C, 2) and safety (cf. Section V,B,3). Fortunately, there has been considerable effort in both these areas within the last decade, which increases the probability that entomopathogenic fungi can be commercially available within the next decade.

D. Entomopathogenic Protozoa

Protozoa of insects never have been industrially produced or sold as microbial insecticides, largely because they are low in virulence, unstable, and difficult to produce. Currently, production can be accomplished only *in vivo* (cf. Section V,A,3). In spite of this difficulty, at least 15 different protozoa were propagated for experimental use against insects (Ignoffo, 1967). Protozoa are promising candidates because many pest insects that are not attacked by other entomopathogens are susceptible to at least one of the 300 described species.

Only five species of protozoa are currently being seriously investigated for development into microbial insecticides. The protozoan *Nosema locustae* has been evaluated for production feasibility and safety and is currently being evaluated for efficacy against rangeland grasshoppers (Henry, 1976); *Mattesia trogodermae* is being evaluated for use against stored-products pests (Schwalbe *et al.*, 1973); *Nosema algerae* is a possible controlling agent of mosquitoes (Undeen and Maddox, 1973); *Vairimorpha necatrix* was suggested for control of caterpillar pests of soybeans (W. M. Brooks, North Carolina State University, personal communication) and *Nosema* (*Perezia*) pyrausta is being considered for control of corn borers (Frye and Olson, 1974; Lewis, 1975). Largely because of problems discussed earlier, none of these have been widely applied (Decker, 1960; Ignoffo, 1967; McLaughlin, 1973); however, successful uses were recorded against caterpillars (Weiser and Veber, 1956; Weiser, 1957; Wilson and Kaupp, 1975), grasshoppers (Taylor and King, 1937; Henry, 1976), and beetles (McLaughlin, 1966; Schwalbe *et al.*, 1973). More recently, favorable information on safety (cf. Section V,B,3), ease of production, environmental stability, and efficacy

(cf. Section V,C,2) have encouraged the reconsideration of protozoa as microbial insecticides.

IV. DEVELOPMENTAL PHASES OF A MICROBIAL INSECTICIDE

The initial impetus for development of a candidate microbial insecticide or any other pesticide is the demonstration of its activity against a target pest(s). Fortunately, most of the screening of candidate microbial insecticides has been done by nature, which has relieved developers of the inherent high cost of finding one potential candidate among thousands that would have to be screened. Approximately 10% of the cost of developing a chemical insecticide is that of synthesis and screening (Anonymous, 1969). Actual development toward commercialization of a microbial insecticide usually begins with a concept in the laboratory that progresses through a pilot-plant phase and attains technical realization as a registered product (Ignoffo, 1973a, c). Three technical parameters, i.e., production feasibility, safety (man, vertebrates, beneficial insects, and plants), and effectiveness against important pests (cf. Section I), are evaluated during each phase of development (Fig. 1). These technical parameters, broad generalizations during the laboratory phase, are more closely examined at each successive step as more data are accumulated. The questions asked at each phase are: Can technical success be attained? Can technical success be translated into commercial success? Technical success is attained when an entomopathogen can be continuously produced, is safe, and will effectively control a pest.

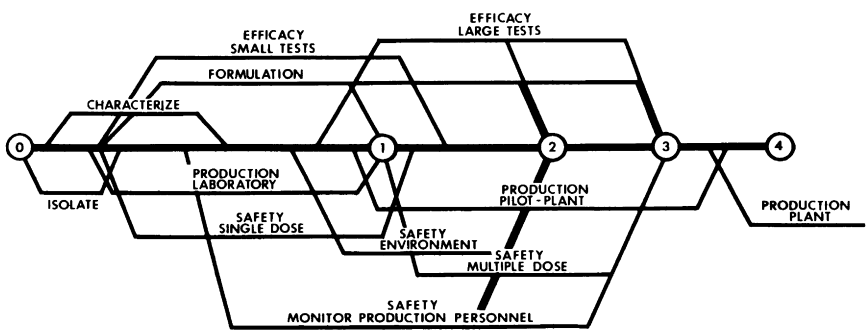

FIGURE 1. A critical pathway scheme for development of entomopathogens into microbial insecticides. (Reprinted from *Environmental Letters,* p. 27, by courtesy of Marcel Dekker, Inc.)

Commercial success is achieved when the product is produced, is effective, and can be used at costs that will return a reasonable profit to the producer. Generally, it takes 5–7 years to develop a known entomopathogen into a microbial insecticide at a cost two to five times lower than for a chemical insecticide. However, if the entomopathogen is similar to those previously investigated (*B. thuringiensis, B. heliothis*) and no problems of safety are encountered, costs may be less, and the entomopathogen may be available within 5 years. A previous success facilitates development of new agents because a framework has been established for constructive dialogue and exchange of information between persons responsible for development and registration.

The time required for development will undoubtedly vary for specific cases and for a variety of reasons, but the sequence (Fig. 1) is applicable to many candidate entomopathogens. Estimated dollar cost and research team years required to develop a candidate microbial insecticide during the first 3 years is about $1–$3 million (Ignoffo, 1975). Most technical questions concerning characteristics of the entomopathogen (biological identity, specificity, mode of action, stability, compatibility, etc.) can be resolved during the first year. Questions of production feasibility, safety, and effectiveness are resolved within the first 2 years. By the end of the third year, all major problems, both technical and nontechnical, should be defined or resolved. Therefore, shortly after the start of the fourth year, sufficient information should be available to decide whether to proceed toward commercial production, marketing, and sales. Optimistically, a product then could be available during the field season of the fourth year of development.

V. TECHNICAL PARAMETERS: MICROBIAL INSECTICIDES

A. Production Feasibility

1. General Comments

Entomopathogens are produced either by using fermentation (*in vitro* process) or in living insects (*in vivo* process). Fermentation technology (submerged or surface) has been used with facultative entomopathogens; *in vivo* technology with obligate entomopathogens; both processes have been employed to successfully produce commercial products. For example, submerged fermentation is generally the technology of choice for commercial production of the bacteria *B. thuringiensis* and *B. moritai;* surface fermentation is used for fungi, e.g., *B. bassiana* and