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REGIONAL NETWORK ON SAFE PESTICIDES PRODUCTION AND INFORMATION FOR ASIA AND THE PACIFIC

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Subprogramme of Farmer Centered Agricultural Resource Management Programme (FARM)

Technical report: Workshop on production and quality control of bio-pesticides (*Bacillus thuringiensis*) Wuham, China, 31 October - 9 November 1995*

Prepared for the Governments of the Member States of the Regional Network
(Afghanistan, Bangladesh, People's Republic of China, India, Indonesia, Islamic Republic of Iran, Myanmar, Malaysia, Nepal, Pakistan, Philippines, Republic of Korea, Sri Lanka, Thailand and Viet Nam)
by the United Nations Industrial Development Organization,
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Based on the work of Professor Xie Tianjian, China

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CONTENT

I. INTRODUCTION

While the development of pesticides and its large scale application in the field has contributed greatly towards increased food production and improvement of health conditions on a global basis, the continued use of these chemical agents has resulted in environmental pollution, human safety and public health. The resurgence of new pests, resistance development, contamination of soil, water and air, destruction of predators and other non-target organisms including wildlife brought about the realization that these chemicals are not exclusively doing the job they are intended to do but also cause damage to the environment.

The countries in the Asia and the Pacific region, being primarily dependent on agriculture, have taken to the increased use of pesticides to meet the basic food requirements. The growing concern on environmental protection is now manifested in efforts to restrict or eliminate the use of pesticides that persist in the environment over a longer period of time involving risk to various forms of life.

While the use of safe and environmentally friendly chemical pesticides continue, there is a pressing need for complementary use of bio-pesticides and botanical pesticides in support of the Integrated Pest Management (IPM) programme. RENPAP has all along been advocating "Clean Technology" with emphasis on the production of safe, low volume user and environmentally friendly pesticides and their formulations. The development and promotion of bio-pesticides in the Asia and the Pacific region has been identified by RENPAP as yet another effective means to reduce environmental pollution since these natural products are relatively more specific, bio-degradable and relatively non-toxic to non-target organisms.

The Bt Research and Development Centre of Hubei Academy of Agricultural Sciences P.R. China had been engaged in the development and production of Bt insecticides since the early eighties and possess appropriate equipment and research facilities required for the purposes. The Bt Research and Development Centre offered to host this workshop on Production and Quality Control of *Bacillus thuringiensis* from October 31 - November 10, 1995 for the delegates of the member countries of the RENPAP.

II. OBJECTIVES

The main objective of workshop was to assist the member countries for developing the capabilities of bio-pesticides. The workshop was intended to cover :

1. Production development of Biopesticides at laboratory, pilot plant and at the commercial level.

2. Quality control methods & standardisation procedures for bio-pesticides.

3. Tailor made formulation development for biocidal material (Bt) and its application methods / technology.

4. Occupational health, safety and hygiene relating to development, production, use and distribution of Bt bio-pesticides.

The list of participants is attached at Annexure I of the report.

III. OPENING OF THE MEETING

Prof. Xie Tianjian while welcoming the delegates from the member countries of the network briefly introduced the Bt Research and Development Centre of the Hubei Academy of Agricultural Sciences. He said that this institution is the oldest one focusing on the research, development and production of Bt insecticides in China. He said that there are 50 technical staff belonging to the various fields in the Bt Research and Development Centre and has at its disposal necessary installation including research, production and trade. The main aim of the Bt Research and Development Centre is to develop biopesticides, needed for augmenting agricultural production and at the same time protecting the environment for the overall benefit of the man-kind. As a result of its sustained efforts for the development of Bt insecticides, the institution has secured the second award for progress of science and technology of the Ministry of Agriculture three times. He said that in 1995 for its achievements the Institute has been given the second award of National Progress of Science and Technology and that the Institution has also been listed in the State Extension programme of Key Scientific and Technological Achievements by the State Science and Technology Committee of China. He mentioned that there are several research activities at the Bt Research and Development Centre which includes screening of Bt strains from dead insects, soil and granary dust and fundamental research for increasing industrial scale production of Bt pesticides.

Prof. Ye Zheng Chu in his address stated that since 1980 China has made considerable progress in the research and development of biological control with the support of the government and this has helped in ensuring environmental protection, thereby, benefiting the whole society. He said that at present the application area of biological control has reached nearly 20 million hectares annually and has played a significant role in

increasing agricultural production. He said that in order to control cotton bollworm large quantities of Bt insecticides have been used covering an area of 1.5 million hectares in 1994. He said that research and development of Bt insecticides in China has made significant achievements over the past 30 years and presently there are about 60 different factories of different capacities producing Bt pesticides with annual production of over 10,000 MT. He emphasised that the Chinese govt. has attached considerable importance to Bt Research & Development and has listed Bt research on the state key projects.

Mr. He Gengfa on behalf of the Science and Technology Committee of Hubei province extended welcome to the delegates from the member countries of the network and expressed his sincere thanks to the UNDP and the UNIDO for sponsoring the workshop. He said that the Hubei Academy of Agricultural Sciences has made considerable progress in Bt Research and Production and that with the spreading of the technology for producing Bt amongst the member countries of the network will not only enhance cooperation in science and technology within the developing countries but also accelerate the process of industrialisation and internationalisation of Chinese Bt pesticides.

Prof. Marion, Dean and Professor, Auburn University, USA said that the world of bio-technology is extending rapidly and that Bt is a leader in the extension of this bio-technological movement and the Bt insecticides at the Hubei academy is at the heart of this movement. While the world of bio-technology is claimed by many disciplines of science, the accomplishments in the microbiological world of Bt and the rapid expansion of Bt in the industrial world are solid evidence that Bt Science and Technology is at the fore front of bio-technology today. He complemented the Hubei Academy for being the world leader in Bt. He mentioned that during this year Bt from the academy was supplied to the fields of cotton in the state of Alabama USA. He highlighted that Bt is a commercial product today because agricultural chemicals do not remain effective over long periods of time, thus, biological methods of insect and disease control are a necessity for plant and animal protection.

Mr. Liu Dingfu, President of Hubei Academy of Agricultural Sciences, while welcoming the delegates mentioned that this training course is sponsored by the State Science and Technology Committee of China, UNDP, UNIDO and hosted by the Hubei Academy of Agricultural Sciences. He, on behalf of 3500 technical staff and employees extended welcome to the participants from the member countries of the network, the officials from the UN, and the delegation from Auburn University. He highlighted that his academy is the first institution accomplishing the industrial production of Bt in China and that the Bt R&D Centre is the oldest Institution focusing on the research, development and

production of Bt insecticides in China. He said that Bt has become the most important pesticide for controlling the chemical resistant diamond back moth, cotton bollworm, pine moth and other various insects in China. He said that Bt products are being exported to South-East Asia, USA, Switzerland etc. He was happy that with this international training course his institution would be actively promoting the technology on production and application of Bt insecticides in the 15 member countries of the RENPAP.

The Regional Coordinator RENPAP in his address traced the history of development of pesticides and highlighted that the old and time tested methods of biological suppression of pests have come to the forefront and the advances in biotechnology research have shown the possibility of better use of biologicals. Biopesticides being natural derivatives, are bio-degradable and do not necessarily contaminate the environment like the chemical pesticides. Therefore, these products have fairly high levels of safety for humans, animals, fish and other non-target organisms. While recalling the previous workshop organised by the RENPAP on the development and production of bio-botanical pesticides he said that lot more work has since been done but commercialization of these technologies are yet to set deeper roots in the member countries of the network. The need of the hour is for installation of industrial scale production and formulation units of bio-pesticides and in this context the workshop organised by the Bt Research and Development Centre is very timely and would be of immense help to the member countries of the member network who are seriously perusing the possibilities of promoting the use of bio-pesticides. The present programme would greatly help the participants to not only understand the production of Bt for controlling pests but also would enable them to learn the highly specialised quality control methods and the marketing of the products for large scale utilisation.

The UNIDO representative while welcoming the participants to the workshop highlighted the importance of this workshop on developing and producing bio-pesticides which would play a dominating role in the IPM programme of the developing countries. On behalf of the UNIDO he deeply appreciated the assistance of Govt. of P.R. China and particularly Hubei Academy of Agricultural Sciences for extending the excellent research and development facilities and for conducting the workshop for the benefit of the member countries of the network. He mentioned that while on the one hand this training would help the experts to understand the production and quality control technology, on the other hand it would help greatly towards promoting environment protection through increased use of Bt with concomitant reduction in the use of chemical pesticides.

Mr. Hong, National Programme Director of Sustainable Pest Control and Soil Fertility programme and National Coordinator, RENPAP offered the existing facilities of his pesticide formulation project for improving the quality of formulations of Bt produced commercially by the Bt Research and Development Centre. He highlighted that a good formulation of the Bt is the need of the hour and would greatly improve the performance of this bio-pesticide in the field.

The Vice Governor Han of the Hubei Province complemented the UNIDO and UNDP for selecting the Bt Research & Development Centre of the Hubei Academy of Science for conducting the workshop and exhorted the participants of the workshop to make full use of the proceedings for the benefit of their respective countries. He wished the participants a pleasant stay and a successful meeting and a declared the workshop open.

IV. COUNTRY REPORTS

The Country papers presented by the delegates are summarised below :

India

Consumption of pesticides in India has been increasing at a great pace and from a consumption level of just 2000 MT in 1955 it has gone up to 85000 MT in 1995. The domestic market is one of the fastest growing one in the world. Out of 147 pesticide products registered in India, about 60 technical grade pesticides are manufactured by different units. Newer and safer bio-botanical pesticides like *Bacillus thuringiensis*, *B. sphaericus* and Neem based pesticides has been granted provisional registration in the country. Studies show that there is great potential of Bt based biopesticides in the country for use against various pests of economically important crops. There are good prospects of using biopesticides in India. Because biopesticides being natural products, are biodegradable and do not leave toxic residues or by - products to contaminate the environment.

The development of the biotechnology industry is gaining momentum in India. In order to promote the use of biopesticides, the data requirements for grant of registration under Insecticides Act 1968 for pesticides based on Bt have been liberalised by the Registration Committee and at present one Bt based biocide is commercially available in the country.

It was pointed out that there is a greater need for quality assurance and ensuring safety in the production of bio-pesticides. Biopesticides did not gain much popularity in India because of their possible adverse effects on silkworm, higher cost of production and low

field stability due to exposure of ultraviolet rays.

Indonesia

Presently about 500 pesticides products are registered in Indonesia with the total annual capacity of producing approximately 180,000 tonnes of formulated pesticides. Indonesia produced around 70,000 tonnes of formulated pesticides during 1995 thereby utilising only 35% of the installed capacity. There are 18 pesticide formulation and 5 technical grade manufacturing plants in the country.

Bacillus thuringiensis was introduced in the country some time in 1980 and there are about 15 Bt based formulations namely Centari G, Florbac FC, Turea WP, Beempe 12 AS, Bactis WP, Bactis S, Bactimos WP, Tecknar 1500 AS, Vectobac 4G, Vectobac 100AS, Dipel WP. Delfin WDG, Bactospeine WP, Bactospeire ULV and Thuricide HP, are registered in the country for use. Amongst the various varieties of Bt strain, Aizawai is widely used because of better efficacy. Formulations available for Bt based products are granules, flowables, wettable powder, suspension, aqueous suspension, water dispersible granules and ultra low volume. The delegate highlighted some of the deficiencies of Bt formulations particularly the effect of ultra-violet rays of the sun and heat towards reduction in its efficacy. The delegate also highlighted the availability of a number of botanical pesticides particularly Neem available in Indonesia for pest control purposes.

Iran

Problems associated with the use of synthetic pesticides like resistance development, destruction of beneficial and useful species and non-target organisms, and environment pollution are well known. With the government's new policy, biological control agents specially bio-pesticides are being given high priority in integrated pest control programmes. Farmers are being encouraged and educated to use judiciously the pesticides with a view to keep pollution free environment and a balance in the eco-system through adoption of IPM with biopesticides. The delegate felt that the optimum microbial / biopesticide should be easily applicable, economical, maintainable and fit well with other control measures in IPM.

Korea

Pesticide is one of indispensable means for a steady production of agricultural food by controlling harmful diseases, insect pests and weeds. The consumption of pesticides on the active ingredient (a.i.) basis had increased from 17000 MT to 25000 MT per annum during the past 10 years. However, in value term it has increased about 26% during the

same period.

To reduce the adverse impacts on non-target organisms and the environment of the pesticides the government has, of late put much emphasis on the development of sound alternative pest control agents based on low input usage and sustainable agriculture. Biopesticide, the delegate said, is one of the promising pesticides for this purpose.

Out of the 568 pesticide products registered in the country, biopesticide account for 3% (15 products). Production of microbial pesticide has increased over 200 fold in the last 10 year period. Quality control of pesticides is regulated under Pesticide Management Law through the National Agricultural Science & Technology Institute (NASTI). The concept of Bt based pesticides quality control has recently switched over to diamond Back moth unit (DBMU) from BIUL (Biological International Unit). Significant research work is being carried out to screen the potential natural sources as bio-pesticides since 1980. However, the delegate felt that the integrated guidelines including biological testing methods are required for quality assurance of the pesticides.

Myanmar

Myanmar economy is predominantly based on agriculture. To increase the agricultural production, dependence on pesticides is growing very fast. Over dependence on chemical posed a lot of undesirable problem and the principle of Integrated Pest Management (IPM) was introduced in 1980s through the assistance of UNDP/FAO. IPM has been successfully launched on crops such as rice, groundnut, seasamum, cotton, potato, chickpea & cabbage. In various programmes emphasis have been laid on biological control agents including biopesticides.

Myanmar has got minimal mass rearing facilities for Bt production. The delegate felt that technical cooperation / assistance is required to upgrade the laboratory facilities to produce large quantities of good quality Bt based pesticides.

Nepal

In Nepal, pesticide consumption is estimated to be 1000 MT per annum. Nearly 50% of it is consumed in agriculture followed by public health, industry, forest etc. During the 8th five year plan the Government plant protection policy has been changed from chemical dependant technology to IPM technology where pesticides are only used as and when really needed. IPM programme designed for fruits, vegetables & cotton crops are being launched in various parts of the country. Bt has been found to be quite effective as a bio-pesticides in the IPM approach for the control of lepidopteran pests namely

Pieris brassicae, Plutella xylostella, Heliothis armigera.

Nepal does do not have any technology or facility to produce bio-pesticides. Because of its superiority over the chemical pesticides in terms of susceptibility towards development of pesticides resistance, toxicology and environmental pollution, it can fit very well in the IPM programmes in the country.

Pakistan

The use of microbial insecticide has been adopted in Pakistan as part of an Integrated Pest Management approach to provide an environmentally - suitable alternative to the generally hazardous, broad-spectrum insecticides used against *Helicoverpa (Heliothis) armigera* (Hubner). Laboratory bioassays using spore-crystal preparations of *Bacillus thuringiensis* var. *kurstaki* (Berliner) indicated high mortalities of the lst instar larvae of *H. armigera*. Potted chickpea *(Cicer arietinum L)* plant tests revealed that Dipel 2X and Dipel ES (Bt var. kurstaki) at the rates of 1.6 kg/ha and 2.0 liters/ha caused 81.48 and 84.0% larval mortality, respectively. Field tests of *B. thuringiensis* on chickpea crops (three consecutive seasons) indicated that Dipel 2X and Dipel ES at the rates of 1.6 kg/ha and 1.5 liters / ha (with and without molasses), respectively, caused significant increase in grain yield as compared to control plots. At least one Dipel treatment was not significantly different from the best synthetic, broad-spectrum insecticide treatment insecticide treatment in terms of yield in all field evaluations.

Philippines

With the increasing realisation of the need for the protection of the environment as well as the health concerns due to pesticide application, the use of bio-pesticides is increasingly becoming important in the Philippines. *Bacillus thuringiensis* (Bt) is one of the popular bio-pesticides commercially sold in the country. The use of Bt (var. kurstaki/ aizawai) is primarily in cabbage against diamond back moth and other worms. There are about 5 Bt based bio-pesticides available in the country. The total market share of Bt in the insecticide market has increased form 4% in 1992 to over 9% in 1995.

In conjunction with the Philippines government programme to encourage the use of biological control agents, various government institutions and agricultural companies are continuously evaluating / developing new serotypes & strains of Bt. Xentari, Delfin, Florbac, Condor, Cultas are some of the Bt. based products now under development by various companies.

Lack of facilities and financial support for Bt research and development and lack of

technology on bioassay and commercial production of Bt are the major constraints in the development of Bt and their formulations.

Thailand

IPM programme is promoted in the country by the government to restrict the use of chemical pesticides. Biological control methods have become essential & important component in the IPM programme due to the host specificity and environmental safety. Microbial biopesticides such as Bt are used as an alternative or in combination with chemical insecticides. The entomopathogenic bacteria, *Bacillus thuringiensis* (Bt) was introduced in 1972 in order to control the diamond back moth, *Plutella xylostella* and cabbage looper, *Trichoplusia ni* Hubner. Presently there are more than 30 species of insect pests which are controlled by Bt in Thailand.

Department of Agriculture of the Govt. of Thailand looks after the production, implementation and evaluation of bio-pesticides, which is then promoted for large scale production by the Department of Agricultural Extension. Considerable research work is being carried out to search effective local strains of Bt and extend large scale production of those effective local strains. Over 100 to 200 isolates of Bt have been found by the Deptt. of Agriculture and the Kasetsant University. A pilot plant with 500 lt capacity has been commissioned in Chaingmai Province for production of Bt and has started producing Bt. This facility would provide Bt at low cost and provide quality products to upland vegetable growing farmers.

Vietnam

Research work on the utilisation of bio-pesticides have been initiated in 1970 in order to develop domestic production of Bt. In Vietnam, IPM has become a National strategy in crop protection in which biological control agent is the main tool to protect the ecological system and to keep the pest population below economic threshold level. Bt application is quite effective and popular in controlling vegetable pests like *Plutella xylostella* (Diamond back moth) & other lepidopteran pests.

In collaboration with Food Industries Institute, a batch of 300 ltrs of Bt var. kurstaki was produced through fermentation with a shelf life of 7 months under low temperature. The product has been commercialised in the country. Though the locally produced Bt is cheaper than imported Bt, product standardisation and contaminations are some of the problems yet to be solved.

V. TECHNICAL SESSION

The Technical Session was conducted by Prof. Xie Tianjian and the session covered production and application of Bt insecticides including Commercial production and application of Bt insecticides in China, biological properties of B.*thuringiensis*, fermentation technology, industrial production of Bt insecticide, bioassay B. *thuringiensis* insecticide, mechanisms of the action of *B. thuringiensis*, Bt for control of pest in China, Bt for control of Malaria vector- mosquitoes, resistance of DBM to *B. thuringiensis*, safety of Bt and its preparations and *B.thuringiensis* past, present and future.

1. BACKGROUND

Bt insecticides are the most produced and the most widely used microbial insecticide in China. In 1964 the first pilot plant was established in Wuhan. Since then Wuhan has been the main base for producing Bt insecticides. During the past several years, Bt output has grown steadily. For example, in Microbial Pilot Plant of the Hubei Academy of Agricultural Sciences, the output has gone upto from 800,000 kg in 1990 to 1000,000 kg in 1992 and to 1,285,000 kg last year respectively. Bt insecticides have been used for control of agriculture, forestry and public health pests among twenty-eight provinces and big cities. Bt insecticides have shown good results and have been exported to South East Asian countries.

COMMERCIAL PRODUCTION

1. Strain

Bt galleriae was the main strain used for production in the 1960's. Bt galleriae, Bt wuhannensis, Bt dendrolimus, HD-1 and 7216 were used in 1970's. In the 1980s HD-1 was the main strain and in the 1990's, Bt aizawa was the main strain produced in the pilot plants.

2. Fermentation

Agricultural by-products are used as main raw material, such as defatted soybean cake, peanut cake, cotton seed cake etc. Some other waste materials are also used successfully for production of Bt mosquitocides. At the initial stage, the concentration of nutrient ingredients are kept between 3-4%, beer spore count being about 20 x 10^8/ml. The concentration is 7-9% and spor count is 50-70 x 10^8/ml now. Most fermenters used for Bt commercial production are 5,000-7,000 liters. With the market growing, 20,000 liters fermenter has been used.

3. Phage

Phage once threatened Bt commercial production seriously. The failure rate caused by phage was even as high as 30% in a typical factory which had to be closed finally. Now almost no damage is caused by phage. For example. The failure rate was reduced to 1.5% in 1986 and zero in 1987. The main measures taken include strengthening the air filter system, heating spore suspension before inoculation, changing two-stage fermentation to one stage fermentatin and so on.

4. Formulations and recovery procedures

Different formulation must be processed using different recovery techniques. In the 1980's, flowable formulation was produced mainly in China. The flow chart followed in Microbial Pilot Plant, Hubei Academy of Agricultural Sciences has been as follows:

Soil culture ---- formulation ----- fermentation ----- screening ----- centrifuging ------- formulation------ quality checking ---- packing

Comparing with powder formulation, flowable formulation consumes less energy to produce and its recovery rate is also higher, therefore, the production cost is also lower. But the stability is not as good as the powder. Usually, the storage period is one year. Flowable formulation is very popular in China. About 70-80% of total quantity of Bt insecticide belongs to flowable formulation which is produced by Microbial Pilot Plant, Hubei Academy of Agricultural Sciences.

Wettable powder of 16,000 IU/mg has been produced using the spray drying technique. Wettable powder is easy to transport and also very stable. Membrane, granule and briquet formulations have been studied for control of mosquitos. Membrane formulation have been specially used to treat larvae habitat of water bodies in South China.

5. Quality Control

Several years ago, spore count was the only means of ensuring quality standards for Bt insecticide in China. It has been replaced by bioassay recently. There are two procedures followed according to Bt strains and testing insects. For *Bt kurstaki* product, cotton bollworm, which could be reared on artificial diet, is used as to testing insect. For *Bt kurstaki* product, diamond back moth is used as the testing insect. The Bt producer can get standard sample (CS_3ab, CS_5ab) in China for conducting bioassays. It has been demonstrated that bioassay is very important for developing Bt production. Based on bioassay, the potency of liquid formulation is maintained at 2500IU/ul, wettable powder

at 16000 IU/mg to 32000 IU/mg and tech-powder at over 50000 IU/mg.

APPLICATON

Low cost stimulates farmers to use Bt insecticide for the control of pests. During the past several years, the area treated with Bt insecticides has been about 900,000 hectares per annum.

1. Grain crop pests

In north China, granule of Bt insecticides have been applied to control corn borer for more than twenty years. Flowable formulation has also been sprayed by aircrafts recently. It has been found to be very effective to control rice leaf tier, rice leaf folder and sorghun spotted borer. Progress has also been made using a mixture of Bt and chemical for control of rice steamborer.

3. Cash Crop pests

Good results have been obtained for the control of tobacco budworm, tea caterpillar, soybean caterpillar and cotton geometrid. High dosage of Bt can work well for the control of cotton boll worm. Recently, it has been replaced by a mixture of Bt and chemical pesticides which resulted in the lowering of the control costs and this method has been expanding in the Hebei province.

3. Vegetable pests

This is the most successful area in China. Usually more than 90% of mortality can be obtained in spring, summer and fall for control of the cabbage caterpillar and diamond back moth. Nearly 50% of the pests are controlled now in big cities such as Shanghai, Beijing and Lanzhou. The cost of applying Bt insecticide is now less than those of the chemical pesticides. It is also very effective against pickle worm etc.

4. Forest pests

Nearly 90% of mortality can be obtained for the control of poplar looper, poplar caterpillar, oriental moth, orange dog etc. About 80% control of pine caterpillar can be obtained with the use of Bt insecticides. Bt also works well for the control of bag moth.

5. Mosquito

Bt has been used for the control of mosquito for five years in Hubei province. It has been found to be highly toxic to Anopheles sinensis and Culex fatigans.

PROSPECT

The prices of Bt insecticides are so low that the control cost of using Bt is cheaper than using chemicals for several pests, such as cabbage looper, cankworm, corn borer etc. The natural enemies of the pests in the area using Bt obviously will be more than that on the area using chemicals therefore, less of insecticides are needed. The economic reasons will encourage farmers to use more and more Bt insecticides. During the 1990's, Bt output would continue to grow steadily.

2. BIOLOGICAL PROPERTIES OF *B.THURINGIENSIS*

Since the early 1900's the *Bacillus thuringiensis* group of bacteria has received the most attention and for a number of years commercial formulations of *B.thuringiensis* have been produced for use in both agricultural and forest insect pest control. As the name indicates, and in common with other members of the genus Bacillus, it is a spore-forming, rod-shaped bacterium, motile by peritichous flagella. At the time of sporulation, *B.thuringiensis* forms a protein parasporal body termed the delta-endotoxin, commonly referred to as the crystal. When sporulation is complete, the sporangium lyses and both the spore and crystal are released into the surrounding growth medium.

In 1901, Ishiwata first isolated this type of microorganism from dying silkworm larvae and named it *Bacillus sotto*. Approximately 10 years later, it was found that only sporulated cultures of the bacteria could induce disease in the silkworm. In 1911, a German entomologist named Berliner isolated a similar spore-forming bacterium from diseased Mediterranean flour moths (*Anagasta (Ephestia)kuhniella*) in Thuringia, hence the name *Bacillus thuringiensis*. This original culture was lost, but in 1927 Mattes reisolated this microorganism and it soon became the source of extensive research and early commercial development of *B.thuringiensis*. In the mid 1950's it was determined that *Bacillus sotto* of Ishiwata and *Bacillus thuringiensis* of Berliner were varieties of the same species. Now *Bacillus thuringiensis* Berliner is regarded as the type species of these bacilli collectively called the "crystalliferous bacteria".

The use of these bacteria for practical pest control has both advantages and problems. Their common advantage is the production of stable spores and crystalline endotoxin which are readily formulated for use with conventional pest control application equipment and which are remarkably safe for humans, other mammals and non-target fauna.

On the other hand, *B.thuringiensis* rarely induces epizootics expect in cases where insects are in confined or crowded areas such as insect-rearing facilities, or stored grain bins. Further preparations of *B.thuringiensis* spores and parasporal crystals applied to foliage can be washed off by rain and may be inactivated by sunlight. Thus single application provide only short-term protection from pest population. This requires that *B.thuringiensis* must be applied repeatedly for long-term protection of agricultural crops. While isolates of *B.thuringiensis* with broad-spectrum toxicity for lepidopteran pests are available (i.e. the HD-1 isolated of *B.thuringiensis* subsp. *kurstaki*), no single isolate is active against all pest species.

Toxins of *B.thuringiensis*

In *B. thuringiensis* strains, seven different toxins(Table 1) have been described: phospholipase C (a-exotoxin:Toumanoff, 1953), a thermostabile exotoxin (B-exotoxin; McConell and Richards, 1959), an unidentified enzyme that may not be toxic (y-exotoxin; Heimpel, 1965), the protein parasporal crystal (S-endotoxin; Hannay, 1953), a "labile toxin" (Smirnoff and Berlinguet, 1966) a, water-soluble toxin isolated from a commercial formulation (Fast, 1971), and a mouse factor exotoxin (Krieg (1961) has also described a bacillogenic antibiotic and a proteinase produced by *B.thuringiensis.* The best known *Bt* toxins are a-exotoxin, B-exotoxin and the S-endotoxin.

d-exotoxin: this toxin was identified in the supernatants of fermentations by Toumanoff in 1953 as the enzyme lecithinase C. It is water soluble and heat-labile and is also produced by bacteria other than *B.thuringiensis.* A toxin considered identical to this but not lecithinase C was reported by Krieg in 1971 as being toxic, per os, to mice and diamondback moth (*Plutella maculipennis*). He named it the mouse factor of the thermo-sensitive toxin.

B-exotoxin:other than the spore and crystal, this was one of the first toxins found. It is produced and released in the fermentation medium during vegetative growth of some strains of *B. thuringiensis.* (Table 1). It is water-soluble and heat-stable. The exotoxin shows high activity against a broad range of lepidopteran and coleopteran insects and is highly toxic to flies. It has been identified as an adenine nucleotide-like compound. Synomys for this toxin are the McConnell-Richards factor, the fly knock-down factor and recently it has been renamed thuringiensin.

The *B.thuringiensis* B-exotoxin is characterized by its ability to prevent pupae of treated larvae of houseflies, Musca domestica, from developing into normal, complete adults, and it appears to act whenever cell mitosis occurs at molting or during metamorphosis; thus it's effect is often delayed. It can cause death during molting, completely prevent pupation, or cause chronic poisoning in adults. For example; when housefly larvae receive sub-lethal doses, vestigial wings and narrow, pointed abdomens develop in the adult.

This toxin is not absorbed or affected by passage trough the gut of cattle and at one time it was produced as a cattle feed additive for the control of flies in the feces. However, it can be toxic to vertebrates when introduced by parenteral injection and can have teratogenic effects in insects. The observations have led regulatory agencies to restrict preparations that contain this toxin from being used in the United States as well as

European countries expect Finland.

Table 1 Production of insecticidal Substances by subspecies of *B.thuringiensis*

Serotype H[b]	subspecies	α-Exoloxin	β-Exoloxin	χ-Exoloxin	δ-Endotoxin	Labile exotoxin	Water-soluble toxin	Mouse factor exotoxin
1	*thuringiensis*	+	+		+	+		+
2	*finitimus*	+	-		+			
3a	*alesti*	+	-		+		+	+
3a, 3b	*kurstaki*	+	-		+			
4a, 4b	*sotto*	+	-		+			
4a, 4b	*dendrolimus*	+	-		+			
4a, 4c	*kenyae*	+	-		+			
5a, 5b	*galleriae*	-	+		+			+
5a, 5c	*canadensis*	-	+		+			
6	*subtoxicus*	-	-	+	+			
6	*entomocidus*	-	-	+	+			
7	*aizawai*	+	+		+			
8a, 8b	*morrisoni*	-	+		+			
8a, 8c	*ostriniae*	+	-		+			
9	*tolworthi*	+	+		+			
10	*darmstadiensis*	-	+		+			
11a, 11b	*toumanoffi*	+	+		+			
11a, 11c	*kyushuensis*	+	-		+			
12	*thompsoni*	+	-		+			
13	*pakistani*	+	-		+			
14	*israelensis*	+	-		+			
15	*dakota*	+			+			
16	*indiana*	+			+			
-	*wuhanesis*[c]	-	-		+			
17	*tohokuensis*	-			+			
18	*kumamotoensis*	+	±		+			
19	*tochigiensis*	+	-		+			

[a] + = positive; - = negative; blank = not determined.
[b] Based on classification according to H. de Barjac, Institut Pasteur, Paris, France
[c] No flagellar antigen.

Source: Faust, 1975

Table 2 Susceptibility [a] of certain organisms to the β-exotoxin produced by a subspecies of *B. thuringiensis* [b]

Phylum	Organisms	Injection	Feeding
Annelida	*Tubifex* sp.		0
Arthropoda	*Aedes aegypti* (L.)	0	+
	Apis mellifera L.	++	+
	Blatta orientalis L.	++	
	Bombyx mori (L.)	++	+
	Diprion pini (L.)	++	+
	Drosophila melanogaster Meigen		+
	Estigmene acrea (Drury)	0	+
	Euxoa segetum (Denis & Schiffermuller)	0 to +	+
	Galleria mellonella (L.)	++	+
	Lepinotarsa decemlineata (Say)	++	+
	Locusta migratoria (L.)	++	+
	Lymantria dispar (L.)	+	+
	Malacosoma neustria (L.)	++	+
	Peridroma saucia (Hübner)	0 to +	+
	Phaenicia sericata (Meigen)	++	+
	Pieris brassicae (L.)	+	+
	P. oleracea Harris	++	+
	Musca autumnalis De Geer	++	+
	M. domestica L.	++	+
	Ostrinia nubilalis (Hübner)	++	+
	Periplaneta americana (L.)	0	+
	Plodia interpunctella (Hübner)	0	+
	Plutella maculipennis (Curtis)		+
	Prisitiphora pallipes Lepeletier	++	+
	Reticulitermes flavipes (Kollar)		+
	Reticulitermes hesperus Banks		+
	R. virginicus (Banks)		+
	Scarophaga bullata Parker	++	
	Tetranychus urticae Koch		+
	Zootermopsis angusticollis (Hagen)		+
Chordata	*Mus musculus* (L.)	+	+
		Ova	
Nemathelminthes	*Bunostomum trigonocephalum* (Rudolphi)	+	
	Chabertia ovina (Fabricius)	+	
	Cooperia (Ransom)	+	
	Haemonchus venulosum (Rudolphi)	+	
	Ostertagia (Ransom)	+	

Burgerjon et al. (1969) found that the effect of *B.thuringiensis* B-exotoxin in the Colorado potato beetle, *Leptinotarsa decemlineata*, involved the buccal parts, eyes and antennae and that these organs atrophied or morphogenetic anomalies such as clubs or claws occurred in the antennae. Table 2 lists the susceptibility of some organisms to the B-exotoxin produced by strains of B.thurigiensis.

Spore:originally *B.thuringiensis* was considered only as an infective agent but with the realization that the S-endotoxin was one of the principal factors in the insecticidal activity, interest in the spore diminished. Recently proteins have been found on the spore coat that are homologous to the S-endotoxin. The spore has also been found to be toxic to some lepidoptera larvae, and so interest in the spore has been revived. The spore is formed at the termination of growth at the same time the crystal is produced. In some insects, death can come very quickly after ingestion of the spores and crystals. This is due to the action of the crystal alone. In other insect species, both the spore and crystal are necessary for optimal potency.

S-endotoxin: the crystal, as it is also called, is a broad spectrum toxin. Until a few uyears ago S-endotoxins active against lepidopterian, dipteran and coleopteran insects were known. However, a broader activity spectrum is suggested by recent reports of *B.thuringiensis* strains active on mites, nematodes, aphids, etc. All safety data collected within the last thirty years have shown that the crystal has no adverse effect on non-target invertebrates or on vertebrates. After dissolution of the insoluble protei matrix, a smaller polypeptide is often produced by proteolysis. Therefore, susceptibility of an insect may in part, or perhaps entirely, depend on the insect's ability to digest the crystal into its toxic sub-units. The observed potency may actually reflect the rate at which the insect's digestive system brings about this dissolution. In addition the S-endotoxin from different strains of *B.thuringiensis* can differe quantitatively and qualitatively in their insecticidal activities.

The S-endotoxin is the major component of the characteristic parasporal crystals produced by the species. Heimpel and Angus have classified lepidopteran larvae into three types based on their susceptibilty to crystalline endotoxin, bacterial spores, or mixtures of the two. Type 1 insects are killed by preparations of crystalline S-endotoxin alone and spores of the bacterium do not increase toxicity; type II insects are susceptible to endotoxin but the effect is enhanced by the presence of spores; and type III insects are only killed by spore-endotoxin mixtures. The high midgut pH of most susceptible larvae

may prevent spore germination but is suitable for dissolution and activation of protoxin. The action of endotoxin may cause a decrease in pH so that germination can occur, nevertheless most susceptible insects fall into the type 1 class. In some insects, midgut pH may be closer to neutrality, allowing germination.

Classification of *B.thuringiensis* strains

Taxonomically, *B.thuringiensis* is closely related to *B.cereus*, except that the species *B.thuringiensis* is characterized as producing parasporal crystal bodies (*B.thuringiensis* S-endotoxin) whereas the "B.cereus group" is not. Division into subspecies of *B.thuringiensis* is primarily based on H serotypes. Esterase types, and production of toxins other than the parasporal crystal and on a few biochemical characteristics have also been used to characterize strains.

Differentation of *B.thuringiensis* strains by flagellar agglutination has been widely used. Since its introduction in 1963 (de Barjac & Bonnefoi), serotyping has brought some order to the *B.thuringiensis* group, by basing its classification on a specific, stable and reliable character, the H antigen. Before this, the same strain was frequently given different names. Similarly different isolates were sometimes given the same name. These misidentifications were parly responsible for the failure of some of the first *B.thuringiensis* field applications. Serotyping has become a powerful tool for the classification of *B.thuringiensis* strains and has limited the proliferation of the supposed varieties.

In recent years, strains have been discovered that are active against mosquito larvae (*B.thuringiensis serovar israelensis*. Goldberg & Margalit, 1977; de Barjac, 1978) or Coleoptera larvae (*B.thuringiensis* var. *tenebrionis*, Krieg et al., 1983 - *B.thuringiensis* var. *san diego*, Herrnst dt et al., 1986). This destroyed the long-established belief of Lepidoptera specificity of *B.thuringiensis* and in addition, it has created some confusion between new supposed varieties and existing serovars.

Properties of *B.thuringiensis* subspecies

There are literally thousands of isolates of B.thuringensis. Some of the more common studies subspecies are listed in Table 3.

Table 3 Plasmid content and location of protoxin genes in *B. thuringiensis* subspecies

Subspecies			Plasmid: no.(size range in MDa)	Location of protoxin gene (size of plasmid in MDa or chromosomal) by:	
Flagella serotype[a]	Crystal serotype[b]	Epithet		Curing/transfer	Hybridization
1	thu	*thuringiensis* (mattes)	10 (5-150)	75	Chromosome, 42,55
		(berliner)	17 (3.9-180); 7 (5.4 -58)		
2		*finimitus*	77,98	98, chromosome	98
3a	ale	*alesti*	12 (2-105)	105	
			6 (4-120)		~120
			10 (2.6-44.6)		
3a,b	k-1	*kurstaki*	12 (1.9-120)	44,110	44, ~120
			8 (1.5-54)		
3a,b	k-73	*kurstaki*	6 (4.9-50)	50	50
4a,b		*sotto*	3 (5.2-43)		33-38
4a,b		*dendrolimus*	4 (33-73)		Chromosome
4a,c		*kenyae*			
5a,b	gal	*galleriae*	4 (5-130)	130	
			5 (6.3-74)		~160
			3 (5-160)		
6		*entomocidus*			Chromosome
6		*subtoxicus*	2 (52,56)		Chromosome and plasmid
7		*aizawa*	12 (4-80)		
			8 (3.9-60)		45
8a,b		*morrisoni*	5 (10-160)		160
9		*tolworthi*	5 (5-150)		45, 50, 150
			6 (5.2-45)		
10		*darmstadiensis* 73-G-10-2[d]	4 (43-90)		47
11a,b		*toumanoffi*	5 (10-160)		150
		kyushuensis[d]			Chromosome
		wuhenensis	4 (50-150)		
12		*thompsoni*	4 (4-100)	100	
14		*israelensis*[d]	6 (4-72)		
			9 (3.3-135)	75	75

De Barjac and colleagues have provided some order on the basis of flagella serotypes Krywienczyk et al. have extended this typing to include parasporal antigens. In general, they found a correlation between flagella(H) serotype and crystal serotype. For example, 38 of 56 *B.t subsp. thuringiensis* (serotype H-1) isolates were of the thu crystal type; 8 of 9 *B.t.* subsp. alesti isolated were of the ale type and 6 of *B.t.* subsp.*galleriae* isolates were the gal crystal type. The exception to this predominance of one crystal type in each serological group was found among *B.t.* subsp *kurstaki* isolates where there was a major division between K-1 and K-73 crystal types. These types had very different activity spectra for larvae of *Trichoplusia* ni and *Heliothis virescens.*

Serologically identicial crystals sometimes appeared in different H serotypes; e.g. K-1 crystals were present in both *kurstaki* and *thuringiensis* subspecies. Furthermore some isolates contained a mixed crystal type, indicating that either one isolate may contain two protoxin genes of a given protoxin may share both major antigenic determinants.

The K-1 crystal type is particularly interesting since it was originally found to contain two distinct S-endotoxins, the major lepidopteran protoxin (P1 now Cryl) and a minor toxin (P2 now Cryll) active on both Lepidoptera and Diptera. K-1 crystals are also present in other subspecies and its likely that both Cryl and Cryll types of endotoxins will be found in these non-*kurstaki* isolates. Indeed, mosquitocidal isolates from subspecies thuringiensis and kenyae (as well as one from tolworthi) contained both Cryl and Cryll endotoxins. Presumably these isolates were of the K-1 or mixed thu-plus-K-1 crystal type and thus fit with the correlation among K-1 type, mosquitocidal activity, and presence of Cryl and Cryll endotoxins.

Differences between Cryl and Cryll endotoxins are summarized in Table 5. Both appear to be protoxins, although only a small fragment of about 3 KDa is removed from Cryll. These two toxins are almost certainly encoded by distinct genes probably residing in different plasmids. The major *kurstaki* HD-1 Cryl gene (CrylAb) is in a 44-MDa plasmid, perhaps with another gene in a larger plasmid. The location of the gene for Cryll has not been definitely established. Loss of a 110-MDa plasmid from *kurstaki* derivative, HD1-7, after growth at 42°C was accompanied by loss of Cryll production.

While mosquitocidal activity is often correlated with the K-1 crystal, it may not be restricted to this type. Two of three aizawai-type preparations were found to be mosquitocidal, although it was not determined whether Cryl and Cryll were present. In addition, there are at least three subspecies (israelensis, kyushuensis and 73-G-10-2) all in different serotypes that are toxic only for mosquitoes. The morphology of the

inclusions and array of parasporal proteins among the three are very similar and clearly different from K-1 types.

Crystal types have been further sub-divided on the basis of activity spectra. Dulmage examined the activity spectra of 38 isolates containing thu-type crystals and 36 K-1 types. They used activity ratios relative to a standard (HD-1-S-1971) for the larvae of two insect species to compare the relative potency of the toxins. The larvae compared were the following: (i) *Trichoplusia ni/Heliothis virescens*; (ii) *Hyphantria cunea / Bombyx mori*; (iii) H.*cunea / T.ni*, (iv) *B.Mori / H.virescens* and (v) *Ephestia cautella / Plodia interpunctella*. The 38 thu preparations could be divided into 13 activity groups, the largest consisting of 10 isolates. These sub-groupings emphasize once again the complexity and variability of the *B.thuringiensis* crystals in that two isolates of the same crystal serotype may differ significantly in activity spectra.

A paracrystalline inclusion comprised of 130- to 140- kilodalton (KDa) polypeptides (S-endotoxin or Cryl) is the predominant parasporal component of most *B.thuringiensis* sub-species. There are variations, however, in the number, shape and composition of the inclusions (Table 4).

Table 4 Morphology of crystalline inclusions of selected bacilli

Strains	Flagella serotype (H-type)	Crystals			
		Shape	Approximate length (μm)	Site of deposition	Time of formation
B. thuringiensis susp. *kurstaki*, P1 (δ-endotoxin)	3a,3b	Bipyramidal	1-1.5	Outside exosporium	Stage II-III of sporulation
kurstaki, P2 (mosquitocidal factor)	3a,3b	Cuboid	0.1-0.4	Outside exosporium; often embedded in bipyramidal crystal	Just before P1
israelensis	14	Cuboid, bipyramidal, ovoid, or amorphous	0.1-0.5	Outside exosporium	Stage II-III of sporulation
	2	Bipyramidal	1-1.5	Within exosporium	Stage II-III of sporulation
Other bacilli					
B. sphaericus		Polyhedron or no crystal		Outside exosporium	
B. medusa				Outside exosporium	At the end of vegetative growth

In most cases the crystal is found outside of the exosporium, major exceptions being sub-species finitimus. This sub-species is not toxic for the larvae of Lepidoptera commonly tested, i.e. *Manduca sexta* or *T.ni*. The only significant toxicity reported has been for larvae of the cotton bollworm *Pectinophora gossypiella*. Recently, sub-species finitimus was found to produce both attached (within the exosporium) and unattached

(outside the exosporium) inclusions each containing predominant 135-KDa polypeptides with unique immunological properties. Plasmid-curing experiments indicated that the gene(s) necessary for formulation of the larger, attached inclusion was encoded on a 98-megadalton (MDa) plasmid, while the gene(s) encoding the unattached body was chromosomally located.

A distinct class of inclusions is found in *B.thuringiensis* susp. *israelensis*. There are usually two to four inclusions per cell which vary in shape from cuboidal to bipyramidal, ovoid, or amorphous. They are relatively small (0.1 to 0.5 um) and the toxic component is active against dipteran but not lepidopteran larvae. There is a broad spectrum of polypeptides extractable from these inclusions including a major 26- to 28- KDa protein that appears to be the toxin, although a 65-KDa species has recently been implicated. The complexity of both the inclusions and polypeptides is reduced in partially plasmid-cured variants.

Protein solubilized from inclusions of *B.t.* subsp. *israelensis* did not react immunologically with antibody to the *B.t.* subsp. *kurstaki* HD-1 S-endotoxin and the overall amino-acid compositions differ. In addition, there was no hybridization of a probe derived from the cloned *kurstaki* HD-1 or HD-73 protoxin genes with either total or plasmid DNA from subsp. *israelensis*. The S-endotoxin appears to be a dimer of 230 KDa as shown by X-ray diffraction and gel electrophoresis. In SDS gels the predominant component is 130 to 140 KDa. In the case of *B.t.* subsp. *israelensis*, the major polypeptide in polyacrylamide gels (whether under native or denaturing conditions) is about 28 KDa.

Table 5 Differences between CryI and CryII of subspecies *kurstaki*

Parameter	CryI	CryII
Toxicity	Lepidoptera	Lepidoptera and Diptera
mol wt (SDS-Page)[a] mol wt of protoxin	135,000 55,000-70,000	65,000 62,000
Shape of inclusion body	Bipyramidal	Cuboidal
Solubility	pH 10-12 + reducing agents	pH 10-12
Times of synthesis		Begins before CryI
Percentage of total crystalline inclusion protein	70-90	10-30
Isoelectric point	4.4	10.7
Chromatography (sephacry S-300)	Eluted before CryII	
Serology (rocket immunoelectrophoresis)	mobility toward (+)	mobility toward (-)
Tryptic peptide mapping	Complex pattern with resistant core	Relatively resistant

[a] SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The mosquitocidal toxins of *B.t.* subsp. *kurstaki* and *israelensis* differ by a number of criteria. The molecular weight of the *kurstaki* CryII is 65'000, while for *israelensis* values of 26'000 to 28'000 or perhaps 65'000 have been reported. The *israelensis* toxin is further differentiated from the *kurstaki* CryII and the B.sphaericus toxin by immunological reaction and DNA hybridization. In addition,their toxicity spectra are not identical, since *B.t.* subsp. *israelensis* is more effective against Aedes spp. and is also effective against blackfly larvae.

3. FERMENTATION TECHNOLOGY

During the past few years, there have been major advances in the development and use of micro-organisms as microbial insecticides. Although several types of microorganisms can affect insects, only three types are being produced commercially at the present time; viruses, fungi and bacteria. Of these, only fungi and bacteria can be grown away from the insect in fermentations; viruses can be grown in large quantities only in host insects. A fermentation is a means of feeding suitable nutrients to a micro-organism in order to obtain useful or valuable end-products. It needs water, an adequate supply of carbon and nitrogen. If it is an aerobic micro-organism, as is *B.t.*, it needs a plentiful supply of air. It will usually be very particular about the temperature and pH of its environment. It must grow alone. The proper equipment and adequate support from various utilities are required. *B.t.* fermentation can be divided into four procedures, seed preparation, medium selection, fermentation control and recovery. In this section, the former three procedures will be discussed.

1. Culture Maintenance, Preservation and Preparation

1.1 Problems of Culture Variability

The most important single need for the production of microbial insecticides is a supply of reproducible, reliable, authentic cultures of the micro-organism to be grown. Maintaining such cultures can be a very troublesome problem, particularly with some of the fungi. Fortunately, the principle bacterium used in the control of insects, *B.thuringiensis*, is relatively easy to keep. Nevertheless care and skill are needed, and the person in charge of the culture collection should be a trained microbiologist. Many bacteria contain relatively small bodies of DNA in their cells, called "plasmids". Like chromosomes, these plasmids transmit genetic information to descendant cells. Unlike a chromosome, many of these plasmids can be altered or lost without harm to the continuity of the bacterium. Plasmids can, and do, direct some very important properties of the cells. Furthermore, plasmids can be, and now frequently are, moved to cells of other organisms in order to change the properties of the recipient micro-organism. Isolates of B.t contain plasmids. Some contain only one or two, while others contain more than 10 (Gonzalez, et al., 1981). Some of these plasmids appear to play a role in the production of &-endotoxin, so it is more than theoretically interesting that Gonzalez and Carlton (1982) have shown that merely growing two isolates of B.t together can bring about transfer of plasmids between the isolates by a process known as "conjugation". For the future, this discovery offers

great promise for the development of new, more potent, isolates of *B.t.* For the present, it imposes very strict standards on the maintenance of *B.t.* cultures to ensure that the various isolates are kept separate from each other without any cross-contamination. Otherwise,unwanted and unrecognized changes could occur that would affect future fermentations.

1.2 Differences in the Production of Toxin between Isolates.

The type of toxin produced and which insects it killed depended upon the isolate. Medium used in the fermentation could affect yields of toxin by the same isolate. Even natural isolates of the same serovar can differ in how much toxin they can produce. Any isolate, no matter how well protected, may weaken in storage in its ability to produce toxin. It is important to monitor the quality of key cultures. The scientists who are responsible for culture maintenance must have access to laboratory-scale fermentation equipment and must routinely test any cultures being used to produce larvicidal formulations to be sure that they have not lost any ability to produce insecticidal activity. However, unless some problem arises, such tests are not needed more than 3 or 4 times/year.

1.3 Methods of Preserving Microbial Cultures

The simplest way of maintaining cultures is to grow and store them on agar slants in screw-top test tubes. It is important to use an agar medium which will support good growth and sporulation of the isolates.

Nutrient Agar(NA), which is available as pre-mixed powder (Table 1), will suffice for most isolates of *B.t.* Tryptose-phosphate agar(TPA) is frequently used for growing *B.t.*, but TPA was found to be less satisfactory than NA for this organism. Formulae are given in Table 1.

Table 1. Agar Media Used for Preparing Stock Cultures of *B. thuringiensis*

	Ingredient	Grams/Litre
1. Nutrient Agar		
	Peptone, bacteriological	3.0
	Beef extract	5.0
	Agar	20.0
2. Tryptose-Phosphate	Agar(TPA)	
	Tryptose	20.0
	Dextrose(glucose)	2.0
	NaCl	5.0
	Na_2HPO_4	2.5
	Agar	20.0

Cultures of B.t are usually considered to be quite stable and to maintain their identity well under continuous transfer on agar slants. However, there is one serious risk; the production of delta-endotoxin by *B.t.* appears to be controlled by plasmid (Gonzales, et al., 1981) which can be lost on transfer. In spite of the supposed stability of *B.t.*, repeated transfers on agar slants may carry a significant risk of loss of activity. Lyophilization is safer, but more time-consuming. If a laboratory uses the same isolates on a regular basis, they may prepare a month's supply of slants from one tube from their lyophilized stock. To preserve the supply of lyo-stock tubes, it is possible to subculture enough slants from the first series of slants for a second month's use. This procedure is a practical compromise between the necessity of maintaining large number of lyophilized tubes of each culture and the risks of repeated transfers on slants.

B.t. grows and survives well in soil. Many alkaline to neutral soils have been used with success. Sterile soils offer a means of preserving *B.t.* A mixture of river silt loam and clay is prepared and distributed in 1 gm portions into test tubes. The tubes are sterilized 3 times by dry heat at 1600°C. Tubes are incubated for about 24 hours between sterilizations. To preserve a culture, a suspension of the isolate is prepared by adding 10 ml water to an agar slant of the bacillus in a 250 ml bottle. Then 0.2 ml of the suspension is added to each test tube of soil. The tubes are incubated for about 1 week and then dried under vacuum. However, note should be made that loss of plasmids has been observed in isolates of *B.t.* kept in soil.

Based on various reports the soil procedure seems better than continuous transfer of agar slants, but is not as good as lyophilization.

1.4 Seed Preparation

In Cotton Insect Research Laboratory (CIRL), USA, the standard procedure to inoculate all shake flask studies is as follows: a loopful of culture is taken from a nutrient agar slant and used to inoculate a 500ml Erlenmeyer flask containing 100 ml of tryptose-phosphate broth. The flask is placed on a rotary shaker and incubated at $30 \pm 1^{\circ}C$ at 340 rpm for 12 hours. At this time, 2 ml (2% by volume) of the first passage seed is used to inoculate a similar flask, which is incubated under the same conditions as the first-passage seed for 12 hours. Finally, 2% (by volume) of this second-passage seed is used to inoculate shake flasks in the fermentation series. This procedure works very well for all *B.thuringiensis* fermentations.

To inoculate pilot-plant scale fermenters, a loopful of culture is taken from a nutrient

agar slant and used to inoculate a 500 ml. Erlenmeyer flask containing 100 ml of tryptose-phosphate broth. The flask is placed on a rotary shaker and incubated at 30±1°C. However, in the case of seeds used for fermenters the primary seed flask is incubated for only 6-8 hours. At this time, about 13 ml of the first-passage seed is used to incoulate 670 ml of tryptose-phosphate broth contained in a 2000 ml Erlenmeyer flask (2% by volume incoculum size). This second-passage seed is incubated under the same conditions as the primary seed for 6 hours and then used to incoculate the fermenters. Fermenters are always incoulated with approximately 0.56% by volume of the second passage seed. Fourteen litre fermenters contain 10 litre of medium. To inoculate these, one should use a sterile 50 ml syringe to remove 50 ml aliquot from the seed flask and inject the seed into the fermenter through a rubber diaphragm that seals the port. A 200 litre fermenter contains 125-150 litres of medium, and an entire seed flask is used to inoculate this fermenter, approximating the 0.5% inoculum size. In BtRDC, 1 ml. spore suspension is used to incoculate a 500ml flask containing 25 ml medium. The tlask is placed on rotary shaker and incubated at 30 ±1°C at 240 rpm for screening isolates of and optimizing fermentation media. To inoculate pilot plant scale fermenters, spore suspension taken from several nutrient agar slant (250 ml bottle) which incubated at 30 +1oC for 72 hours is heated to 80oC for 20 minutes. This heat-treated spore suspension is used as inoculum which directly inoculates even 2000 liter fermenter.This method also maintains good growth.

2. Medium Selection

It is not possible to recommend specific nutrients for every laboratory to use. The choice of nutrients depends on three factors: availability, cost, and how well the microorganism can utilise them. The factors must be balanced against each other. Commonly available potential nutrients are shown in Table 2. Not all of these have yet been tried in the *B.t.* fermentations.

Local waste materials could be valuable and could markedly lower the cost of production of those microbial insecticides. Any change in fermentation conditions or nutrients must be evaluated in light of the following analyses:

1) Media costs vs. yields. As a result, media costs represent only a small portion of the costs. The energy input involved in running the fermenters, water chillers, steam generators, and centrifuges represents major costs. The equipment used is expensive, and overhead is high. There is cost of screening or grinding the final product or of processing it into a flowable formulation. Finally, there is the cost of packaging and

distributing the material. Since so much of the cost of a microbial insecticide is fixed by these other factors, increases in potency or yield must be relatively high before they make a major impact on the unit cost of the product.

Table 2 Examples of Potential Inexpensive Media Available in Developing Countries

Liquids
Conconut Milk *(waste product)
Crude sugar *(eg. jaggery)
Whey *(waste product)
Molasses *
Corn steep liquid *
Materials of plant origin
Legumes and other seeds: chick peas *, peanuts, lima beans*, cowpeas*, soya beans*, bambara beans*, kidney beans*, cotton seed meal*, peanut cake*
Cereals: corn*, guinea corn, millets etc., wet mash from breweries
Tubers: cassava, yams, sweet potatoes * etc.
Yeast powder*, wet yeast ex brewery (waste)
Materials of animal origin
Fish meal *
Blood

**Tried and found useful: the others either have not been tried or have given poor results but may be useful in combination with other ingredients.*

2) Interference in the recovery process. Whether the final product is a water-dispersible powder or a flowable formulation, it will be applied in the field as a spray, either from the ground or from the air. In either case, the product must be finely enough divided to ensure that it will pass freely through the spray apparatus. This will require that the material be finely divided enough to pass through a 200 mesh screen. This factor plays

a major role in dictating the nutrients used in the fermentation medium: a significant portion of the solids presenting in a harvested beer represent residual, unused, media ingredients. If these are coarse, grinding will be necessary. Grinding not only adds an extra and costly step to the recovery process, but also results in a significant loss of active ingredient because of the heat and dust generated in the grinding equipment. This loss can be avoided by using nutrients that are already been ground (cotton-seed flour instead of cotton-seed meal). This requirement for using finely divided nutrients is unique to the production of microbial insecticides and must be taken into consideration in any programme for developing suitable fermentation media.

3) Effect on the final formulation. There can be direct or indirect effects of fermentation ingredients on the final formulation. For example, water-dispersible powder formulation of *B.t.* are stable only when the moisture content of the power is less than 4%. Therefore, a water dispersible powder should not be strongly hygroscopic. All formulations can absorb moisture under certain conditions and therefore should be kept in sealed containers.

2.1 Carbon sources

Glucose, starch, and molasses have all been used as carbon sources in *B.t.* fermentations. The level of carbohydrate must be carefully chosen for two reasons: first, all the strains of *B.t.* that were examined so far produce acid from glucose. If the concentration of sugar is too high, the pH of the medium will drop below pH 5.6-5.8, and the acidity will inhibit or stop growth. It is difficult to define "too high". This depends on the concentration of the nitrogenous nutrients in the medium. *B.t.* tends to produce alkaline materials from these nitrogenous nutrients that can neutralize the acidic compounds produced from the sugar. Too low a level of sugar can terminate growth too soon. One should try to have a relative balance of sugar and nitrogenous materials so that the initial pH of the fermentation will drop to about 5.8-6.0, after which it will rise slowly and steadily to a level of pH 8.0. Proper concentrations are not difficult to determine by experimentation.

2.2 Nitrogen sources

Several nitrogen sources are used in *B.t.* fermentations. Three are proteins: soybean flour (soy flour), cottonseed flour, and fish meal. The soy flour and the cottonseed flour are both very good nutrients. Fish meal is also effective, but its use is restricted, parly because the meals contain significant levels of residual fish scales, which make it difficult to obtain a uniform product, and partly because of the residual odour carried over into the

final formulation. Corn steep is very useful as an additive to these media, and it may be satisfactory as the sole nitrogen source. (Corn-steep liquor is rich in many types of nutrients and its value in a fermentation is not restricted to serving as a nitrogen source). Corn-steep is acidic, being derived from the acid treatment of corn in the commercial production of starch, and it is important to neutralize any medium containing corn-steep.

2.3 Minerals

Minerals are essential in the nutrition of organisms. Five metallic ions are considered to be particularly important in the growth and sporulation of bacilli: Mg++, Mn++, Fe++, Zn++, and Ca++. These are all normally present in the carbon and nitrogen sources used in fermentations and there may be no need to include these ions in the fermentation media.

In many media 0.3 g/l MgSO4 7H20; 0.02 g/l MnSO4 H2o; FeSO4 7H2O;ZnSO4 7H2O and 1.0 g/l CaCO3 are added. Adding them to a medium will not damage a fermentation, even if there are already sufficient levels of these minerals present.

2.4 Formulae of selected media

Table 3 lists the formulae of several selected media used in *B.t.* fermentation. Some of these media are particularly significant in work with *B.t.* Medium B8 and B13 have been used in small scale pilot plant. Medium M, A and C have been used as production media in USA and European countries, while L1, L6 and L8 have been adopted by Chinese *B.t.* factories.

Medium can significantly affect fermentation potency. Medium optimization is needed for isolates used for production. Steepest ascent method and response surface method are proved to be effective in medium optimization.

3. Fermentation conditions

3.1 Shake-flasks

Shake-flasks are useful tools for screening large number of isolates of *B.t.* They are also useful in studies of fermentation media or other aspects of fermentation. It is recommended that 25 ml medium be used in 500 ml flask in order to get sufficient air supply in shake flasks.

2000 ml flasks are rarely used except the production of seed but, with this sized flask

one should attempt to get as high aeration as possible by not using more than 50-100 ml medium per flask. (table 4)

Table 3. Examples of Media Used in Fermentations of B.thuringiensis

Ingredient	Medium No: Level-grams/liter							
	A	C	B8	B13	M	L1	L6	L8
Soybean flour	25.0	22.5	-	40.0	-	18.0	25.0	40.0
cottonseed flour	-	-	30.0	-	14.0	-	-	-
corn flour	-	-	-	-	-	5.0	-	20.0
corn steep liquor (wet weight)	-	20.0	30.0	20.0	16.7	36.0	3.0	-
corn starch	10.0	15.0	-	-	-	7.5	-	-
Fish meal	-	-	-	-	-	3.0	5.0	10.0
Peptone	-	-	2.0	-	-	-	-	1.0
Dextrose	-	-	45.0	30.0	0	0	20.0	-
Molasses	-	-	-	-	18.6	-	-	-
Yeast powder	-	-	2.0	-	-	-	-	2.0
KH_2PO_4	1.0	-	-	-	-	2.16	1.0	1.0
K_2HPO_4	1.0	-	-	-	-	-	-	-
$FeSO_4\ 7H_2O$	0.02	-	0.02	-	-	-	-	-
$MgSO_4\ 7H_2O$	-	-	0.3	-	-	0.75	-	0.7
$MnSO_4\ 7H_2O$	-	-	0.02	-	-	-	-	-
$ZnSO_4\ 7H_2O$	-	-	0.02	-	-	-	-	-
$CaCO_3$	1.0	1.5	-	-	1.0	1.5	1.0	2.0
$(NH_4)_2SO_4$	-	-	-	-	-	-	2.0	-

Table 4 Conditions for Shake-Flask Fermentations of B. thuringiensis

Condition	B. thuringiensis
Size of flask	500 to 2000 ml
Volume medium/flask	25 to 100 ml
Incubation temperature	28 - 30 °C
Agitation	240 rpm
Starting pH	6.8-7.2
Harvest pH	6.5-8.5 hours
Age at Harvest	24-48 hours
Usual spore count/ml beer	10-70 X 10^8

Table 5 Conditions for Fermentations of B. thuringiensis with 14-litre and 200-litre Fermenter

Condition	14 litre	200 litre
Volume of medium	10 litres	125-150 litres
Aeration	1 v/v/m	1 v/v/m
Agitation	700 rpm	400 rpm
No. of impellers	2	3
Incubation temperature	28 -30°C	28-30°C
Starting pH	6.8-7.0	6.8-7.0
Final pH	7.0-8.5	7.0-8.5
End of log-phase of growth	16-18 hours	16-18 hours
End of sporulation phase	20-26 hours	20-26 hours
Completion of lysis	35-40 hours	35-40 hours
Age at harvest	30-40 hours	30-40 hours

3.2 *Fermenters*

The 14 litre fermenter is a useful tool for beginning fermentation research. The only limitation is the very large jump in size experienced when one attempts to extrapolate data from the 14 litre fermenters to production-sized equipment, which may be 40,000 times larger or more. The 200 litre fermenter. It is large enough to predict and solve many scale-up problems.

Table 5 gives typical conditions when 14 litre and 200 litre fermenters were used.

The most common pattern of pH in B.t fermentations reflects the production of acid from glucose. After sterilization, the pH of the fermenter varies between 6.8-7.2. Immediately after inoculation, the pH falls steadily, as the glucose is utilized, reaching a pH of about 5.8-6.0 after 10-12 hours. At this point, the pH starts to rise at the same rate as it fell, reaching pH 7.5 by 25 hours. The rise in pH slows gradually, reaching a pH of 8.0 at about 30 hours. The pH may continue rising and reaches pH 8.8 after 50-60 hours. With some cultures, the initial drop in pH may only reach pH 6.5-6.6. In such fermentations, there may be little or no rise in pH as the fermentation continues.

In B.t fermentations the pH can be controlled to some extent by maintaining a proper balance of sugar to nitrogenous compounds. The sugar causing a drop in pH, and the nitrogenous materials partially balancing the drop by producing basic compounds. If too high a sugar level is used without an adjustment in the nitrogen source, the pH of the fermentation can drop below pH 5.6-5.8. At this pH, the organism will stop growing.Too much sugar may also inhibit the sporulation of the *Bacillus*, even though the pH may not drop to a low level.

The "log-phase" of any bacterial fermentation is that period during which the organism is vigorously growing and rapidly dividing. This first phase lasts 16-18 hours. Sporulation is complete by 20-26 hours after inoculation, although the cells have not yet lysed. Lysis is complete by 35-40 hours.

The following figure shows fermentation patterns of B.t MP-342 in 7000-liter fermenter. Toxins are produced in proportion to sporulations. During the end of sporulation phase, mortality reaches its peak value.

The oxygen uptake rate of *B.t.* in different growth stages varied significantly. The highest oxygen uptake rate occurred at the end of log phase. Oxygen supply in log phase should be strengthened especially by the use of high concentration medium. Agitation speed

could effectively influence the dissolved oxygen concentration. During the log phase, the deficiency of oxygen supply inhibited the reproduction of cells. When the concentration of dissolved oxygen was above the critical value, *B.t.* got sufficient oxygen supply, which led to an increase of fermentation level. Extra supply of oxygen showed little effect on viable cell count. Reasonable oxygen supply could be achieved by changing agitation speed.

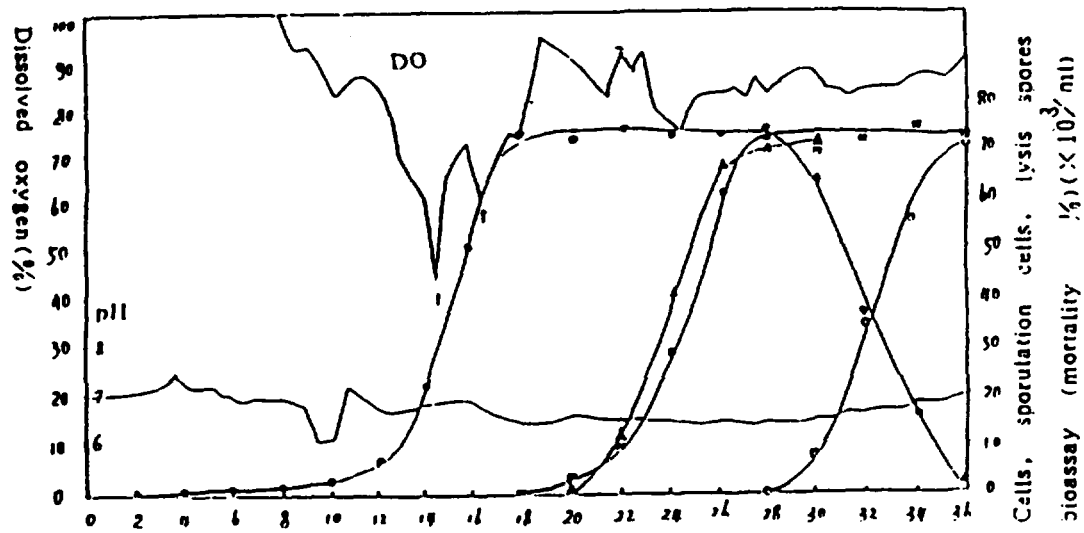


Figure. Fermentation patterns of Bt MP—342 in 7000—liter fermentor

During log phase, the agitation speed maintained at a high level assurees that the DO was over the critical value of oxygen concentration, while during other stage agitation speed could be adjusted to a relatively low speed. It could be achieved both in high fermentation level and with less energy consumed.

Many fermentations produce a considerable amount of foam. If foaming is left unchecked, the consequences can be disastrous. Excess foam lowers the oxygen transfer rate (OTR) with a direct effect on growth and yield. Foam will flow over into the air exhaust filters, clogging them and forming easy pathways for contaminants to enter the fermenter. Beer will be carried over as foam into the sewer lines, resulting in large losses in the volume of the beer. The same conditions that improve aeration the presence of baffles, the use of multiple impellers all stimulate foaming. Foaming can be controlled by mechanical devices and chemical antifoam agents which are added as needed during the fermentation process.

4. INDUSTRIAL PRODUCTION OF Bt INSECTICIDE

The basic principles of industrial production of *Bt* insecticide is illustrated as fig. 1.

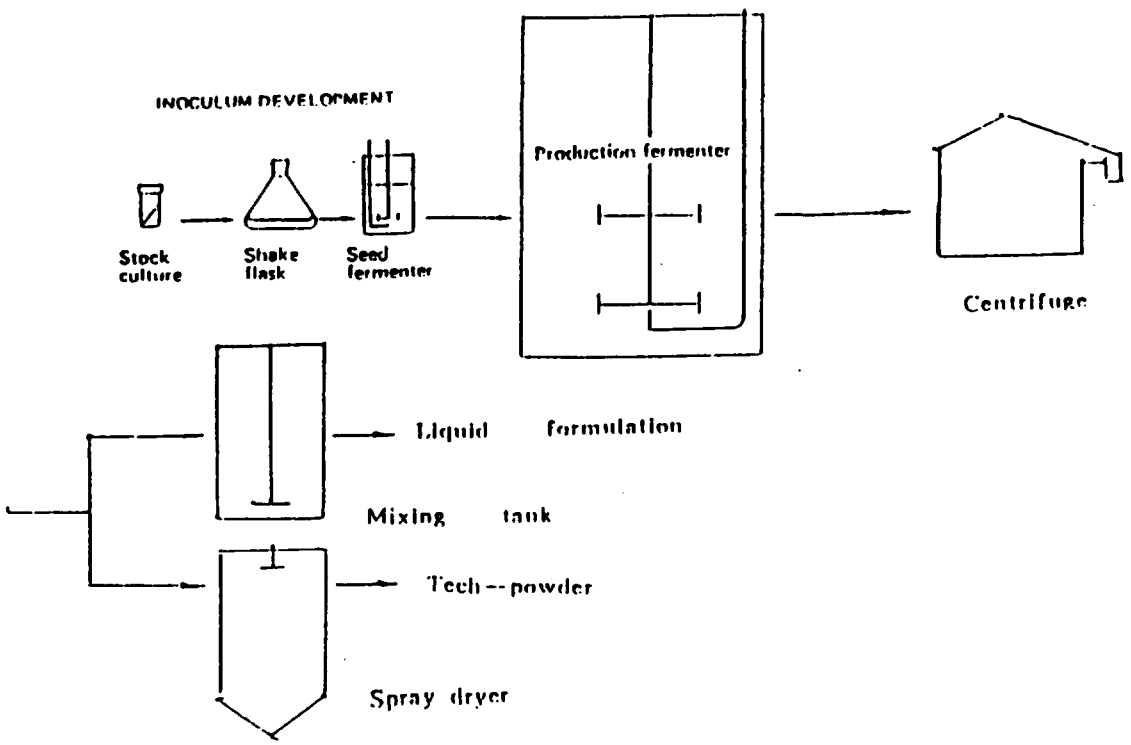


Fig. 1. Illustration of Bt fermentation procedures

The whole procedure is a means of feeding suitable nutrients to a micro-organism in order to obtain useful or valuable endproducts. It needs water, an adequate supply of carbon and nitrogen. If it is an aerobic micro-organism, as *B.t.*, it needs a plentiful supply of air. It will usually be very particular about the temperature and pH of its environment. It must grow alone. The proper equipment and adequate support from various utilities are required. In this section, some of equipment and utilities will be discussed. Also, some of recovery procedures will be reviewed.

I. Utilities

1. Steam boiler

Main function: Sterilization

During *Bt* fermentation, if the fermenter is invaded by foreign micro-organism, for example by phage, the contamination could result in lysis of *Bt*. To avoid contamination, sterilizing medium, fermenter, and all parts attached is a basic necessary way. Steam which supplied by steam boiler is used almost universally for sterilization. Choosing steam boiler size is decided not only by production scale, but also by sterilization model, (continuous sterilization or batch sterilization). For local production in developing countries, batch sterilization is preferably recommended. It has advantages of lower equipment cost, lower contamination risk and easier manual control. According to the guide of the New Brunswick Scientific Co., 90 kg. steam/hr is consumed for sterilization for 500 litre fermenter, 640 kg/hr for 5000 litre fermenter. With experience of HBMPP(Hubei Academy of Agricultural Sciences, Microbial Pilot Plant), 2000 kg/hr is suitable for 20,000 litre fermenter.

2. Compressor and air filter system.

Main function: Aseptic air supplier

Bt fermentation is aerobic process and, therefore, requires the provision of oxygen. If the stoichiometry of respiration is considered, then the oxidation of glucose may be represented as:

$$C_6H_{12}O_6+6O_2=6H_2O+6CO_2$$

Thus, 192 grams of oxygen are required for the complete oxidation of 180 grams of glucose. However, both components must be in solution before they are available to a micro-organism and oxygen is approximately 6000 times less soluble in water than in glucose (a saturated oxygen solution contains approximately 10 mg dm-3 of oxygen). Thus, it is not possible to provide a microbial culture with all the oxygen needed for the complete respiration of the glucose (or any other carbon source) in one addition. Therefore, a microbial culture must be supplied with oxygen during growth at a rate sufficient to satisfy the organisms' demand.

Oxygen is normally supplied to microbial cultures in the form of air, which being the cheapest available source of the gas. The method for provision of a culture with a supply

of air varies with the scale of the process:

a, Laboratory scale cultures may be aerated by means of the shake-flask technique where the culture (50 to 100 cubic cm) is grown in a conical flask (250 to 500 cubic cm) shaken on a platform contained in a controlled environment chamber.

b, Pilot and industrial scale fermentations are normally carried out by compressor. Air from compressor must be sterilized. Sterile air will be required in very large volume in many aerobic fermentation processes. Although there are a number of ways of sterilizing air, only three have found permanent application. These are heat, filtration through fibreous material and filtration through granular material. Heat is generally too costly for full scale operation.

Fig.2 An arrangement of packed air filter and fermenter.

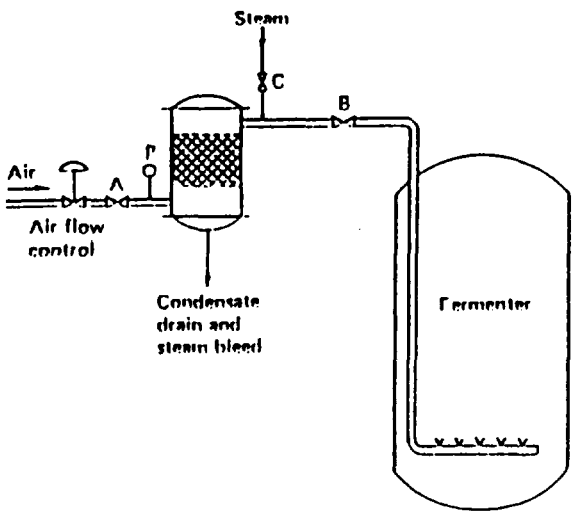


Figure 2 is a simple unit. During sterilization the main non-sterile air inlet valve A is shut and initially the sterile air valve B is closed. Steam is applied at valve C and air is purged downwards through the filter to a bleed valve at the base. When the steam is issuing freely through the bleed valve, the valve B is opened to allow steam to pass into the fermenter as well as the filter. It is essential to adjust the bleed valve to ensure that the correct sterilization pressure is maintained in the fermenter and filter for the remainder of the sterilization cycle.

3. Cooling water system

Large amount of water is needed to keep fermenters cool. *Bt* is usually grown at

To maintain close control over such temperatures in a fermenter requires that the temperature of the cooling water be about 18°C. Some areas of the world have ready access to water of this temperature, but in most cases especially in sub-tropical and tropical climates the only way to obtain such cold water is through the use of water chillers or cooling towers.

This problem should not be underestimated. Solving it can greatly reduce the cost of a fermentation. A chiller can adequately cool the water, but is expensive to operate. However, once the cooled water has passed through the fermenter cooling coils, it absorbs heat. The warming of water is particularly great during the cooling cycle after stilization and this poses a question: is it less costly to rechill the warmed water or to use fresh influent water reverse flow water and chill it. The cost of the water become important in answering this question.

Similar combinations may need to be developed even where the water supply is cold. Depending on the cost of water, it may prove more economical to recirculate some water and chill it. In deciding where to locate a fermentation plant, one must calculate the amount of influent water required and the cost of cooling.

4. Electric power

Fermentations are frequently spoiled due to the failure of electric power. This not only allows a sharp drop in air supply and pressure, it also stops the impeller or mixer used to stir the fermentation beer. If the local electrical supply is not highly reliable, it would be wise to install a back-up generator at the plant with an automatic start-up in case of power failure.

Fluctuations in voltage can lead to contaminated fermentations due to fluctuations in air supply and agitation, or possibly to burned-out motors of damaged electronic components. The solutions to fluctuating voltage are expensive: for example, portions of the equipment may need to be protected with constant voltage transformers. The frequency and seriousness of such fluctuations should be considered in valuating the local power supply.

II. Fermenter

Main function: Providing a controlled environment for the growth of *Bt.* In designing and constructing a fermenter, a number of points must be considered:

(a) The vessel should be capable of being operated aseptically for a number of days and should be reliable in long-term operation.

(b) Adequate aeration and agitation should be provided to meet the metabolic requirements.

(c) A system of temperature control should be provided.

(d) Sampling facilities should be provided.

(e) The vessel should be constructed to ensure smooth internal surfaces, using welds instead of flange joints whenever possible.

(f) The cheapest materials which enable satisfactory results should be used.

The first two points are probably the most critical. It is obvious from the above points that the design of a fermenter will involve cooperation between experts in microbiology, biochemistry, chemical engineering, mechanical engineering and costing. Although many different types of fermenter have been described in the literature, very few have proved to be satisfactory for industrial aerobic fermentations. The type of vessel can be produced in a range of sizes from one dm^3 to thousands of dm^3. More varied shapes are commonly used for alcohol, biomass production and effluent treatment.

1. **Body construction**

In fermentations with strict aspetic requirements it is important to select materials that can withstand repeated steam sterilization cycles. On a small scale (1 to 30 dm3), it is possible to use glass and/or stainless steel, Glass is useful because it gives smooth surfaces, is non-toxic, corrosion proof and it is usually easy to examine the interior of the vessel. Two basic types of fermenter are used:

(a) A glass vessel with a round or flat bottom and a top flanged carrying plate (Fig. 3). All vessels of this type have to be sterilised by autoclaving.

(b)A glass cylinder with stainless-steel top and bottom plates(Fig.4). These fermenters may be sterilized in situ, but 30 cm diameter is the upper size limit to safely withstand working pressures (Solomons, 1969).

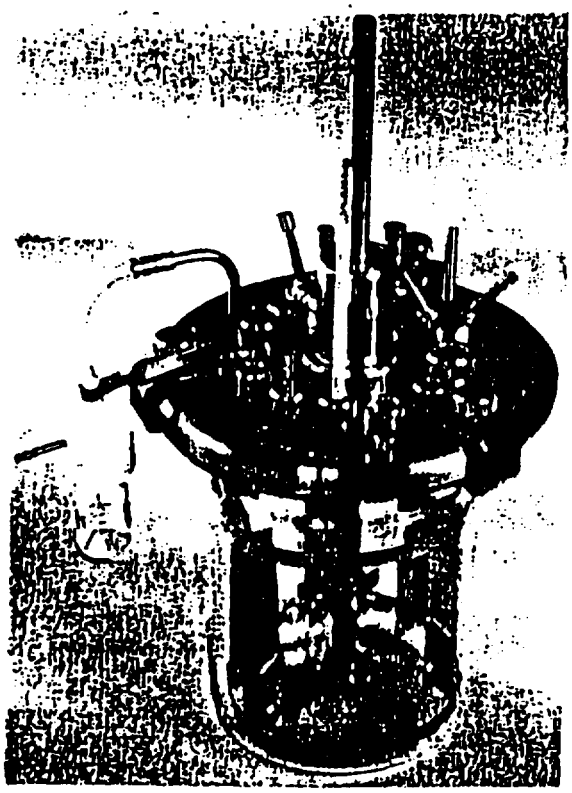


Fig. 3 Glass fermenter with a top-flanged carrying plate (L.H. Engineering, Stoke Poges, England).

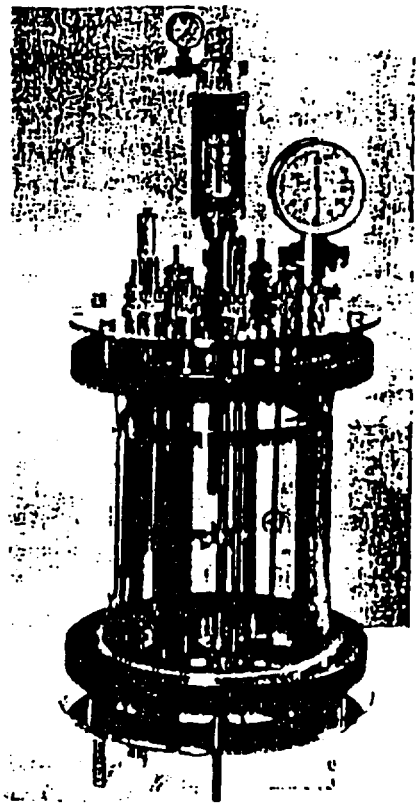


Fig. 4. Glass fermenter with top and bottom plates (L.H. Engineering, Stoke Poges, England)

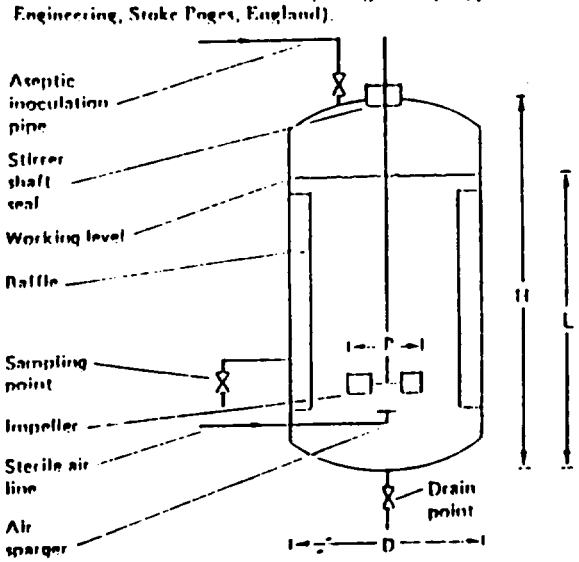


Fig. 5 Diagram of a fermenter with one multi-bladed impeller.

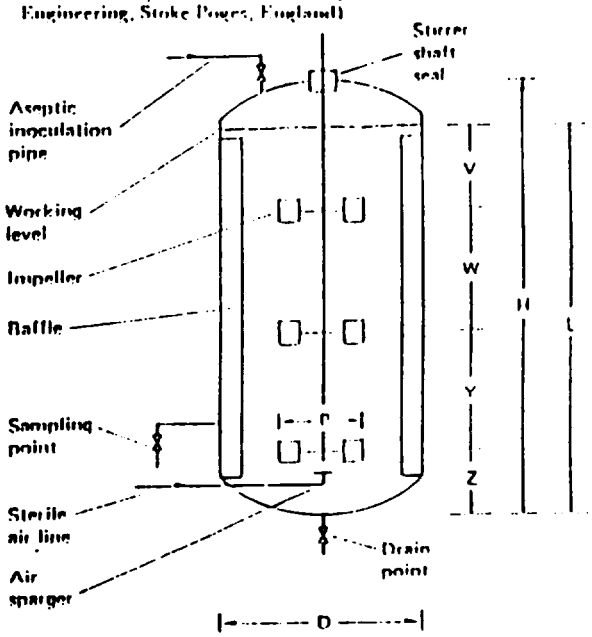


Fig. 6 Diagram of a fermenter with three multi-bladed impellers

On pilot and large scale, any materials used will have to be assessed on their ability to withstand pressure sterilization and corrosion and their potential toxicity and cost.

Figures 5 and 6 are diagrams of typical mechanically agitated and aerated fermenters with one and three multi-bladed impellers respectively. Although stainless steel is often quoted as the only satisfactory material, it has been reported that mild-steel vessels were very satisfactory after 15 years use for *Bt* fermentations.

Normally in the design and construction of a fermenter there must be adequate provision for temperature control which will affect the design of the vessel body. Heat will be produced by microbial activity and mechanical agitation and if the heat generated by these two sources is not ideal for the particular manufacturing process, then heat may have to be added to, or removed from the system. On a laboratory scale, little heat is normally generated and extra heat has to be provided by placing the fermenter in a thermostatically controlled bath, or by the use of internal heating coils. On a large scale, there is normally excessive heat production and the fermenter will be provided with a jacket or internal coils, through which cold water may be circulated to achieve the correct temperature. Fermenters of upto 5000 dm^3 are usually fitted with cooling jackets, while internal cooling coils or half tubes are used in large vessels(Muller and Kieslich, 1966).

It is impossible to specify accurately the necessary cooling surface of a fermenter since the temperature of the cooling water, the sterilization process, the cultivation temperature, the type of micro-organism and the energy supplied by stirring can vary considerably in different processes.

2. Impeller

The impeller has two main functions:

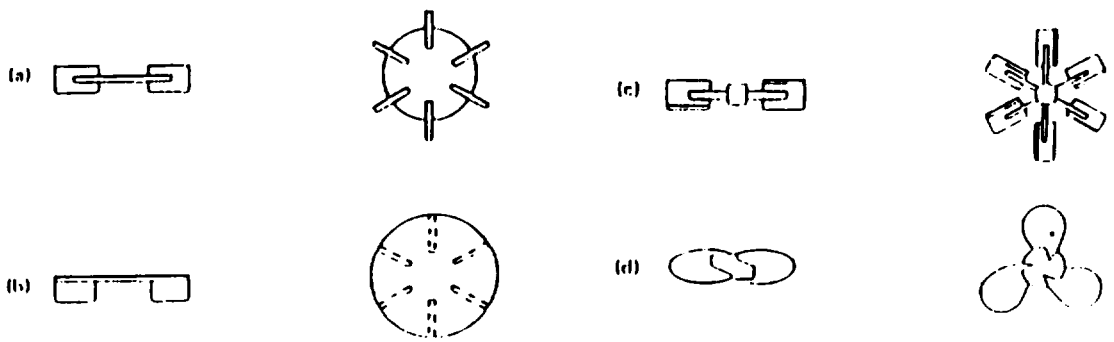
(a) To diminish the size of air bubbles to give a bigger interfacial area for oxygen transfer and to decrease the diffusion path.

(b) To maintain a uniform environment throughout the vessel contents.

Impellers may be classified as disc turbines, vaned discs, open turbines of variable pitch and propellers, and are illustrated in Fig.7. However, it has been established experimentally that the disc turbine is most suitable in a fermenter since it can break up fast air stream, without itself becoming flooded in air bubbles(Finn, 1954).

It is also necessary to consider the size of the impeller, and where to position it in the vessel. In tall vessels more than one impeller is needed if adequate aeration-agitation is to be obtained. Ideally the impeller should be one-third to one-half of the vessel diameter(D) above the base of the vessel. If the L/D ratio is increased above 1.0, additional impellers must be incorporated into the vessel. It has been common practice to space multiple impellers at 1.0 diameter(D) distance along the impeller shaft. Oldshue tested five variations of impeller position and found that similar mass-transfer coefficients were obtained, provided that power per unit volume was maintained at similar values. This implies that there is a degree of flexibility in impeller position on a shaft.

Fig.7. Types of impellers: (a) disc turbine; (b) vaned disc; (c) open turbine, variable pitch; (d)marine propeller (Solomons, 1969).



III. The recovery of Bt fermentation broth

The main objective of the first stage of recovery is to separate water from B.t spores, Crystals and medium residue, which can be completed by filtration or centifugation. Then according to market demand, slurry will be processed as liquid formulation directly or be further processed as technical powder with spray dryer.

1. Filter

Plate-frame filter was used as recovery equipment in early stage of *Bt* industrial production:

A plate-frame filter is a pressure filter in which the simplest form consists of plates and frames arranged alternatively. The plates are covered with filter cloths(Fig. 8) or filter pads. The plates and frames are assembled on a horizontal framework and held together by means of a hand screw or hydraulic ram so that there is no leakage between the plates and frames which form a series of liquidtight compartments. The broth is fed to the filter press through the continuous channel formed by the holes in the corners of the plates and frames. The filtrate runs down grooves in the filter plates and is then discharged

through outlet taps to a channel. The solids are retained within the frame and filtration is stopped when the frames are completely filled.

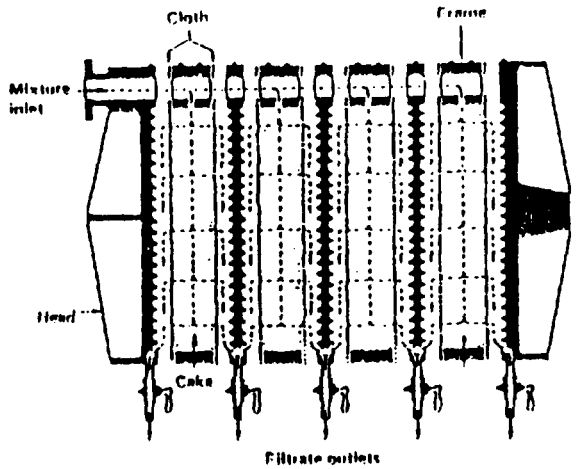


Fig. 8. Flush plate and frame filter assembly. The cloth is shown away from the plates to indicate flow of filtrate in the grooves between pyramids (Purchas, 1971).

On an industrial scale, the plate-frame filter is one of the cheapest filters per unit of filtering space and requires the least floor space, but because of high labour costs and the time involved in dismantling and reassembling, these filters should not be used when suitable size of centrifuge can be adopted.

2. Centrifuge

Continuous-flow centrifuges are used to separate *Bt* from broth in large scale production.

a. The tubular-bowl centrifuge

This is a centrifuge to consider using for particle size ranges of 0.1 to 200 um and upto 10% solids in the in-going slurry. The main component of the centrifuge is acylindrical bowl (or rotor)A, which may be of a variable design depending on application, suspended by a flexible shaft B driven by an overhead motor or air turbine C. Figure 9. Shows an arrangement used in a Sharples Super centrifuge. The inlet to the bowl is via a nozzle attached to the bottom bearing D.

The advantages of this design of centrifuge are the high centrifugal force, good dewatering and ease of cleaning. The disadvantages are limited solids capacity, recovery of solids. gradual loss in efficiency as the bowl fills, the solids being washed off the walls as the bowl is slowing down and foaming.

b. Disc-bowl centrifuge

This centrifuge relies for its efficiency on the presence of discs in the rotor(Fig.10). A central inlet pipe is surrounded by a stack of stainless-steel conical discs. Each disc has spacers so that a stack can be built up. The broth to be separated flows outwards from the central feed pipe, then upwards and inwards at an angle of 45° to the axis of rotation. The close packing of the cones assists rapid sedimentation and the solids then slide to the edge of the bowl, provided there are no gums or fats in the slurry, and eventually accumulates on the inner wall of the rotor. Ideally, the sediment should form a sludge which flows rather that a hard particulate or lumpy sediment. The main advantage of these centrifuges is the facility to remove solids automatically through a series of nozzles in the circumference of the rotor or by opening up the rotor in the intermittent discharge design. Unfortunately, the solid fraction is not completely dewatered and the arrangement of the discs makes this type of centrifuge laborious to clean. Feed rates range from 45 to 1800 $dm^3\ min^{-1}$.

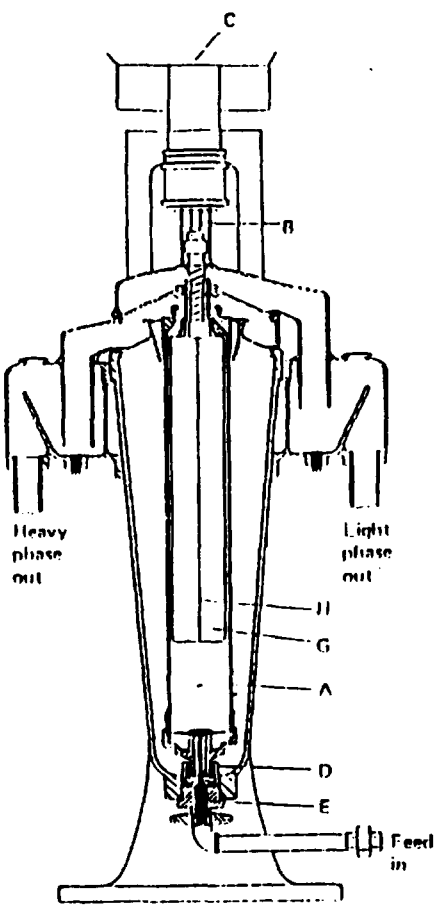


Fig 9. Tubular—bowl centrifuge

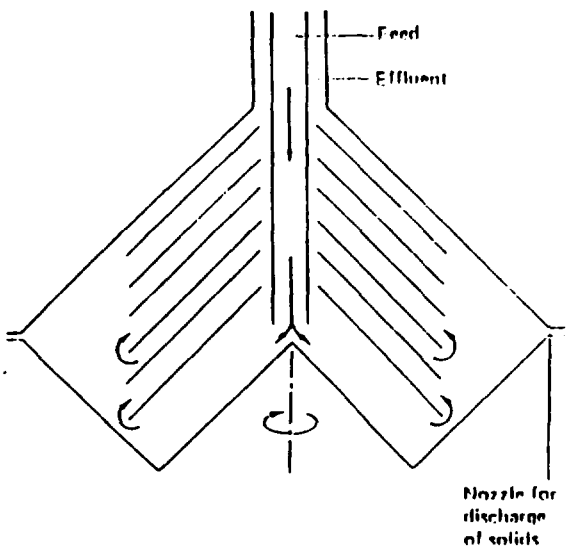


Fig 10. L.S. of disc-bowl centrifuge with nozzle discharge

3. Spray dry System

Spray drying is often the last stage of manufacturing process. It involves the final removal of water from a heat sensitive material ensuring that there is minimum loss of viability, activity or nutritional value. Drying is undertaken because;

a) The cost of transport can be reduced.

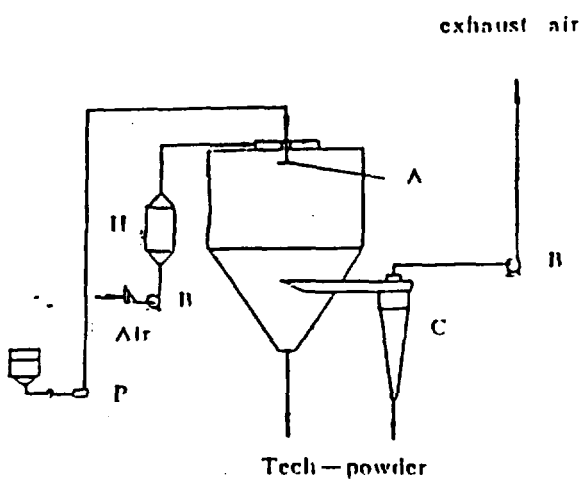
b) The material is easier to handle and package.

c) The material can be more conveniently stored in the dry state.

d) To ensure stability of the product.

It is important that as much water as possible is initially removed by centrifugation or in a filter press to minimize heating costs in the drying process.

A spray drier(Fig. 11) is most widely used for drying of biological materials when the starting material is in the form of a liquid or paste which can be initially atomized into small droplets through a nozzle or by contact with a rotating disc. The droplets may fall into a spiral stream of hot gas at 150°C to 250°C. The high surface area; volume ration of the droplets results in a rapid rate of evaporation and complete drying in a few seconds. The evaporative cooling effect prevents the material from becoming overheated and damaged. The gas-flow rate must be carefully regulated so that the gas has the capacity to contain the required moisture content at the cool-air exhaust temperature (75°C to 100°C).



A. Atomyzer B. Blower C. Cyclone

F. Feeding tank P. Pump

Fig 11 Spray dry system

5. BIOASSAY OF BACILLUS THURINGIENSIS INSECTICIDE

For microbilogical pesticides as well as chemical pesticides, we have to measure the potency and to translate it into valuable units which could be used and compared all over the world. The microscopic examination of B.t cultures appeared to show one crystal of the -endotoxin for every spore of the *Bacillus*, it was hoped that counting of viable spore presenting in a preparation could be used to determine its insecticidal activity. For some time, spore count became a generally accepted method of measuring the potencies of *B.t* preparations. But the toxicity of crystal is out of proportion to spore number and spore or crystal can vary in virulence, depending on variation in strain or in the fermentation, so the spore count is not a reliable index of potency. No chemical methods have yet been proved to measure activity of the spore-crystal complex except bioassay.

"Biological assay is the method for the estimation of the nature, constitution, or potency of a material by means of the reaction that follows its application to living matter". Bioassay of B.t is to measure its potency to insect and to be expressed in arbitrary units which must be related to a standard preparation. Statistically, the relation between the dose and response may be concisely expressed by an equation, Y=a+bx, when doses are transformed to log values and mortalities(responses) to probit values. The quantitative relation between the dose and the mortality is determined by performing parallel sets of operations with a known amounts of the standard preparation and by measuring mortalities, the ratio of the two equally effective doses is an estimate of the potency of the test preparation relative to that of the standard. In other word, the purpose of bioassay of B.t is to discover equally effective doses of the standard and test preparations.

Design of bioassay of B.t

In an bioassay of *B.t.*, the accuracy of the results are affected by the following factors: test insect, response forms, infection forms, doses of test preparation, and standard preparation. The design of bioassay of B.t must comply with following principles.

1. Test insect

Test insect include four respects: species, age, number and quality.

An insect species for test must meet following three requirements:

(a) It is a representative of target of control, so that the results can be used as reference for controlling it in field;

(b) It is a medium sensitivity to *B.t.* High or lower sensitivity will make bigger deviations;

(c) It can be reared on large scale with artificial diet in order to supply enough test insect in any season. According to the principle, in United States, cabbage looper *Trichoplusia ni* and in France, Mediterranean flour moth *Anagasta kuehniella*, and in China, both cotton bollworm *Heliothis armigera* and diamond back moth *Plutella xylostella* are selected as official test insects.

Young larvae tend to have greater sensitivity to B.t and have less differences in physiology between individuals than that of elder ones. Young larvae have short feeding time or needn't feed them, so young larvae are good for bioassay of *B.t.*

The number of insect used for bioassay will depend on the insect species and upon the accuracy needed. Generally speaking, the number of insect is determined by experiments. For example, 25 to 50 larvae of *Trichoplusia ni* are used per dilution to standardize *B.t.* H3a3b, 40 to 50 for *Heliothis armigera*, while 100 larvae of *Aedes aegypti* per dilution are used to standardize *B.t.* H-14.

In bioassay, the insect population used must be vigorous and disease-free. This requires clean, protected rearing areas with good control of temperature, humidity and lighting.

2. Methods of infection

There are many methods of infection in bioassay. Exact methods will depend on the nature of pesticides and on eating behaviours of the insects. B.t belongs to a stomach poison agent and midgut of the insect is the place of action. Therefore, the agent must be fed to the insect in any bioassay using the three possible methods: (a) force feed the test sample; (b) administer the test substance on top of the diet; (c) incorporate the test agent into the aritificial diet.

Force feed may injure the insect. Insect frequently regurgitate force-fed materials(unless such insects are eliminated from the assay), this can introduce an intolerable variable into the test. The second method-administering the test substance on top of the diet-may present some problems. If leaves are used, we must be sure that they are fresh and similar in thickness and condition. If the artificial diet are used, the principal danger is that the individual insect within the group may consume different quantities of the test agent. The third method, namely incorporating the test agent into the artificial diet, can attain more uniform diet-sample complex than those above mentioned, so ones usually use this

method.

The length of exposure time will depend on the insect being tested and its response to *B.t.* This will be determined by experiments. For example, *B.t.* H-14 assay are complete in 24 hours and *B.t.* H3a3b against *Heliothis armigera* require 3 days (at 30°C).

3. Forms of the responses

A variety of responses-death, retardation of development, deformation might be presented in the insects when infected with *B.t.* As the purpose of application of *B.t.* is to kill pests, so naturally mortality is chosen as the form of responses in bioassay of *B.t.* A bioassay of B.t has been designed to determine the amount required to cause death in a population of target insect. Statistically, the most accurate measurement of death response of an insect population is the concentration of toxin which kill 50% of the insect population.

4. Test doses

In bioassay, serial dilutions of the test samples and of a standard preparation are incorporated into an artificial diet respectively. It is important that the assay be standardized and that a minimum of five, and preferably seven dilutions be tested. The maximum killing rate of any dilution must be Z% and the minimum 10%. The dilutions should be selected, so that the calculated LC_{50} will fall in the middle range of the dilution series.

5. Standard preparation

In bioassay of *B.t.*, the LC_{50} of a single formulation can vary from day to day. Obviously no accurate measurement of potency can be obtained from the LC_{50} alone. The solution has been to compare the LC_{50} determined for a test sample with the LC_{50} of a standard formulation tested on the same day against the same population of insect. The presumption is made that under these conditions the ratio between the LC_{50} determined for these test sample and the LC_{50} of standard remain constant, regardless of any change in the condition of response of the insects. Therefore, the preparation of a standard formulation is required for bioassaying a new *B.t.* insecticide.

A standard preparation is that its potency has been determined by comparing other standard and be expressed with international units "or'IUS'". Several standard formulations have been used in the assay of *B.t.* The names of these standards and the potencies

assigned to them are given in table 1.

Table 1

Bacteria	Name of standard	Serotype	Potency-IU/mg
B.t.	HD-1-S-1971	H-3a,3b	18,000
B.t.	HD-1-S-1980[a]	H-3a,3b	16,000
B.t.	IPS-78	H-14	1,000
B.t	IPS-82[b]	H-14	15,000
B.t.	E-61	H-1	1,000

a: This is the current international standard for lepidopteran-active *B.thuringiensis* larvicides.

b: This is the current international standard for *B.thuringiensis* larvicides active against dipterons (mosquito and black fly).

Computing potency and evaluating accuracy of a bioassay

The LC_{50} of each sample and standard is computed from the data obtained on the percentage of kill in each of the series of dilutions of the sample and standard. The potency of the sample is then calculated by the following formula.

Potency sample(IU/mg)=

$$\frac{\text{LC50 standard X potency standard (IU/mg)}}{LC_{50}\text{ sample}}$$

The LC_{50} can be computed with a calculator which can compute the regression curves.

The procedures of computation are as follows:

The mortalities percentage are first transformed to probit values and doses to log values, then put these data into calculator and a,b (slope), LC_{50} can be respectively obtained by operating the calculator based on the program stipulated.

For evaluating the accuracy of a bioassay, Dulmage (1982) has listed some of the requirements used to determine if a bioassay should be considered valid. These are as follows:

i: There must be % dead in the control larvae-those that were grown on the same diet, but which were not exposed to toxin.

ii. Dilutions must be selected so that at least 5 concentrations of each sample and 7 concentrations of the standard are "valid" i.e. with no more than 90% or less than 10% kill.

iii: Slopes of the regression curves must be within the ranges expected by experience, and they must be parallel to each other within the error of the day's assays. Non-parallel curves are rejected.

iv: If computer analysis is available, the 95% Confidence Limits around the LC_{50} should be determined. They should be such that the Maximum Limit/Minimum Limit. If not, the assay is rejected.

v. Similarly, the 95% Confidence Limits around the IUS should be determined and the Maximum Limit/Minimum Limit should be . If not the assay is rejected.

vi: Assays should be replicated on 3 separate days, and the mean and standard deviation(SD) between the 3 assays should be determined. The Coefficient of Variation(CV) of each assay series should be determined(CV=SD/mean). The CV should be . If the CV is 0.2, the assays are accepted. If it remains 0.2, the assays are rejected. If this occurs with most of the samples being tested, it is evident that something has gone wrong with the bioassay procedure. Otherwise, one must suspect that the sample being tested is not homogeneous.

The confidence that we can place in a bioassay depends on the reproducibility and the reliability of the IU determined for each sample. Thus the CV is the key to the trust that we can place in our assays. The criterion that the CV should be was based on many years' experience with the *B.t.* bioassays against lepidoptera insects, which has demonstrated that if the assay procedures are being followed correctly and the test insects are of good quality, the CVs determined on the various sets of *B.t.* samples fall below . Experience with bioassays of *B.t.* H-14 against mosquitoes indicates that mosquito bioassays are more accurate than lepidopterous assays and that the requirement for the CVs in these assays might be reduced to 0.15.

Bioassays are used in many different situations with different kinds of products. It is generally considered that if the error in the assays can be held at 20%, the assays will permit good standardization of the product and will adequately support research to increase production. To achieve this goal with the B.t bioassay, assuming a CV of 0.2, we will need to replicate the assay between 3 and 4 times.

Bioassay Protocol for *Bacillus thuringiensis*

1. **Bioassay of B.t against cotton bollworm**

Preparation of diet

Ingredient of diet

Deionized water	1000 ml
Yeast flour	40 g
Soybean flour	80 g
Ascorbic acid	0.5g
Sodium benzoate1.4g	
Acetic acid	13 ml
Agar	15 g

Mix the yeast flour, soybean flour, sodium benzoate, acetic acid in a blender, and add 344 ml deionized water and blend. Dissolve agar in 666 ml deionized water in a beaker, move it into a microwave oven and heat to boiling and boil for 2 min. Add the agar solution into the blender and blend, then add the ascorbic acid after the blend has cooled to about 70°C, blend continuously for 1 min. Pour the diet into a big beaker and keep it in bath pot (at 60°C) until used.

Preparation of B.t inoculum

The stock solutions and all dilutions of the test samples and the standard are made in a buffered saline solution.

The buffered solution ingredients (grams per liter) are:

NaCl 8.5; K_2HPO_4 6.0; HK_2PO_4 3.0; and 1%Tween-80 solution 10ml.

Weigh a definite amount (based on activity of test sample) of B.t to be tested and standard, placed it into a separate container containing 20-30 bead (diameter 4mm), add appropriate amount of buffered saline solution, soak for 10 min, then shake with hand for 1 min. Serial dilutions are made from the stock solutions with buffered solution, an untreated control (with buffered solution only) must be included in each test. A minimum of 5 doses and a control must be used in each test.

Mixing the B.t inoculum into artificial diet

For each concentration, pipet 3 ml dilution into a 50 ml beaker and draw 27 ml of the diet into the same container, blend at high speed for 0.5 min, then distribute the sample-diet mixture into each well on the bioassay tray, 48 well per dose (24 well a tray).

Control is made with 3 ml buffered solution only. As a standard treatment, use CS_3ab-1991, potency 15,000 IU/mg, made in Hubei Academy of Agricultural Sciences using *B.t.k.* strain

Infection

Distributed sample-diet mixture is allowed to cool for at least 1 hour before introducing the larvae. Use only those larvae that climb to the top of the cup and hatchin less than 12 hours, one neonate per well, then cover with a lid which has a plastic sheet inside, overlap those tray and tie them with elastic rope, place the tray into incubator at 29-30°C.

Evaluation

After 72 hours, examine each tray and record number of living and dead larvae. Determine the LC_{50} by regression equation computed with log doses and mortality-probits. A test is valid only if control mortality is less than or equal to 10 percent. Potency of sample is calculated with following formula:

Potency of sample(IU/mg)=

$$\frac{LC50 \text{ standard} \times \text{potency of standard (IU/mg)}}{LC_{50} \text{ sample}}$$

2. **Bioassay of B.t H-14 against *Aedes aegypti***

Standard bacterial preparation

Use the material"IPS-82" as the standard, obtained from the Pasteur Institute. It should be stored and handled according to the suppliers' recommendations. These should include the necessity of storage at 2-5°C, equilibration at room temperature before opening the plastic box to avoid condensation and, after the taking of a sample, resealing the container and returning it to 2-5°C in darkness.

Assay species

Use early 4th instar larvae of the Bora-Bora strain of *Aedes aegypti*. Eggs to start breeding colonies can be obtained from the Pasteur Institute, or one of several other centres.

To obtain larvae at an equal developmental stage, induce eggs to hatch by a stimulant such as the addition of 100 mg ascorbic acid/litre of water. This deoxygeneates the water and all the eggs hatch very quickly. Purchase a large stock of suitable mosquito food to avoid variation due to the use of different batches of food in the course of time. Store this food in dry cool (5°C) conditions to prevent infestation by storage pests or infection by moulds. Feed larvae with standardized optimal quantities of food, using a standardized breeding routine at a constant temperature selected from a range between 20°C and 28°C. Do not vary this temperature for different experiments. Harvest the larvae on filter-paper, with a strainer or with a pipette, at a pre-selected age when all have just moulted into the fourth instar. If *Ae. aegypti* is unacceptable in a country for safety reasons, use *Culex quinquefasciatus* or *C.pipiens*.

Preparation of bacterial suspensions

The bacterial preparation to be assayed may be either a liquid suspension or a dry powder. If it is a liquid suspension, it is necessary to determine the dry weight per millilitre of the preparation. This is done by heating a small, known volume in a 100-110°C oven. The dry weight value is used later in calculation of the LC_{50}. Whether the preparation is a liquid or a dry powder, it must be thoroughly homogenized prior to being added to the assay cups. Homogenize a weighed quantity of powder or a measured volume of liquid in a known volume of chlorine-free water, preferably deionized or distilled (or tap water can be aerated in shallow trays for 24 hours). A sonifier, glass homogenizer, and ball mill or blender with rotating knives are suitable homogenizing equipment. Place a loop full of this initial suspension on a microscope slide, apply a cover slip and check under phase contrast illumination that all clumps have been broken into individual spores and crystals. Agitate the initial suspension thoroughly (eg. by vortex mixer) and prepare at least five serial dilutions at, for example, x 0.8 intervals in chlorine-free water. Reagitate every time a fresh dilution is made. For the standard IPS-82, a top concentration of 0.04 mg/litre is likely to be suitable.

A sufficient range of concentrations should be used for the test samples in order to obtain a good mortality rate distribution. Shake each dilution (eg. 20 circular shakes by hand), then apply early 4th instar larvae at a suitable density such as one larva per 8 ml of bacterial suspension (eg.25 larvae per 150 or 200 ml is suitable). The larvae should not be fed during the assay. Wax cups should not be used as they may trap the crystals. Keep at a constant temperature selected from the range of 23OC to 27OC in darkness or dim light: they must be kept away from sunlight. An exposure time limited to 24 hours is desirable. It should extend over an active feeding period, while avoiding the pre-pupal

period and pupation.

Numbers of insects and replicate assays

Sufficient insects and replicate assays should be used to achieve a coefficient of variation not exceeding 20%. For instance, four cups of 25 larvae per concentration, seven concentrations per test bacterial product are suitable, and with the assay repeated on three different days. The objective is to have at least two mortality rates on either side of the LC50 within the range of 10-90% mortality. It is essential that, when the assays are repeated on different days, it should be on a freshly-weighed batch of powder.

Reading the assay

After an exposure period of exactly 24 hours, count the numbers of live larvae (the results are based on counts of live larvae because of the cannibalistic tendency of larvae at lower concentrations). If the control mortality exceeds 10%, discard the assay. Calculate percentage mortalities and obtain LC_{50} values by plotting on log-probit graph paper, or by using a computer programme with a probit or logit transformation of mortality against log concentration.

Appendix I

Methods for large-scale Rearing of the Cotton Bollworm

Artificial Diet

The ingredients of the diet are as follows:

Ingredient	Amount
Soybean flour	80 g
Yeast flour	40 g
Barley flour	60 g
Ascorbic acid	5 g
Sodium benzoate	3g
Acetic acid	14ml
Benzoic acid	1g
water	1000ml
Agar	14g

Mix the yeast flour, soybean flour,barley flour, acetic acid, ascorbic acid, sodium benzoate and benzoic acid in a blender, and add 344 ml water in another container, put it into a microwave oven (or an electric oven) and heat to boiling and boil for 2 min. After the agar solution has cooled to about 70°C, mix it with all ingredients in blender and blend at high speed for 1 min. Pour it into diet-tray and cool for 2 hours.

Note: soybean flour must be treated by heating at 100°C at about 5 min to destroy toxin contained in it to insect.

Larval rearing

There are two kinds of trays used for larval rearing at our laboratory, small one hold 24 wells with diameter 1.6 cm and 1.7cm in height and big one holds 190 well with 2.7 cm x 2.7 cm x 3.0cm.

Cut the diet into small cakes with 1 cm x 1 cm x 1cm, distribute them into the well on the small tray, one per well, inoculate neonate into well, two or three a well. Move the tray into rearing room at 30°C and a photoperiod of 14 hours light for culture. After the larvae are reared 6 days, transfer them to a big rearing tray, one larva a well and culture continuously at same condition for 5-6 days until they mature.

Pupa treatment

Mature larvae are transferred to the sand which are contained in a plastic container to pupate. The container are placed in the larval rearing room and cover it by using black cloth to reduce the intensity of light. Pupa are held in that room for about 10-11 days, then move them in an adult cage, put the cage in the room at temperature of 25°C with a relative humidity of 85 percent and 24 hours darkness. About 1-2 day when adults emerge, a 10%-sucrose feeding solution, contained in a plastic cup filled with cotton, is placed inside the adult cage. This solution is changed daily. The gauze oviposition pads which is used as the cover of the cage, are changed daily.

Egg treatment

After the gauze oviposition pads have been collected, the egg are surface-sterilized in 4% of formalin for 20 min, then rinsed with tap water for 3 times, dried in air. The egg are maintained at a temperature of 30°C and hatch in 3 days.

Appendix II

Weighing coefficient

Y	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
1	0.001	0.001	0.001	0.002	0.002	0.003	0.005	0.006	0.008	0.011
2	0.015	0.019	0.025	0.031	0.040	0.050	0.062	0.076	0.092	0.110
3	0.131	0.154	0.180	0.208	0.238	0.269	0.302	0.336	0.370	0.405
4	0.439	0.471	0.503	0.532	0.558	0.581	0.601	0.616	0.627	0.634
5	0.637	0.634	0.627	0.616	0.601	0.581	0.558	0.532	0.503	0.471
6	0.439	0.405	0.370	0.336	0.302	0.269	0.238	0.208	0.180	0.154
7	0.131	0.110	0.092	0.076	0.062	0.050	0.040	0.031	0.025	0.019
8	0.015	0.011	0.008	0.006	0.005	0.003	0.002	0.002	0.001	0.001

Appendix III:

The probit transformation(Fisher & Yates(1948) & Finney(1952) have tabulated the probit at intervals of 0.001 in the response rate).

Response rate	0.00	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
0.00	-	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
0.10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
0.20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
0.30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
0.40	4.75	4.77	4.80	4.82	4,85	4,87	4.90	4.92	4.95	4.97
0.50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
0.60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
0.70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
0.80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
0.90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33

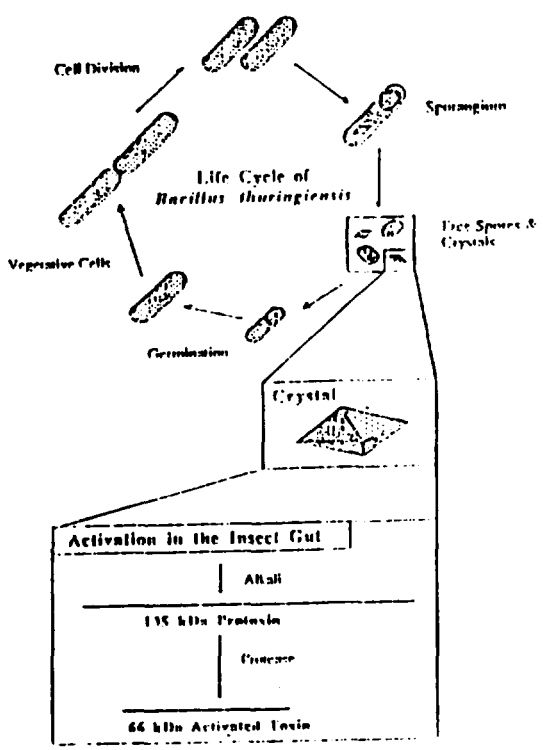
Response rate	0.000	0.001	0.002	0.003	0.004	0.005	0.006	0.007	0.008	0.009
0.97	6.88	6.90	6.91	6.93	6.94	6.96	6.98	7.00	7.01	7.03
0.98	7.05	7.07	7.10	7.12	7.14	7.17	7.20	7.23	7.26	7.29
0.99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

6. Mechanisms of the action of B.thuringiensis

At present, the most widely used bacterium in microbial control is *B.thuringiensis*. It is being applied in many countries throughout the world. Bacterial infections in insects can be broadly classified as bacteremia, septicemia and toxemia. Bacteremia occurs when the bacteria multiply in the insect's hemolymph without the production of toxins. This situation occurs in the case of bacterial symbionts and rarely occurs with bacterial pathogens. Septicemia occurs most frequently with pathogenic bacteria, which invade the hemocoel, multiply, produce toxins and kill the insect. Toxemia occurs when the bacteria produce toxins and the bacteria are usually confined to gut lumen, as in the case of brachytosis of the test caterpillar pathogenesis. *B.thuringiensis* does not exactly fit any of these classifications because it first creates a condition of Toxemia followed by a septicemia.

In a medium with a good supply of carbon, nitrogen and phosphorus, *B.thuringiensis* grows vegetatively and the sporulation is repressed. At the end of vegetative growth, the exhaustion of the medium induces the initiation of sporulation. During the process of vegetative growth and sporulation, many toxins will be produced.

Fig. 1 Life cycle of *B.thuringiensis*, showing the production and activation of endotoxin.



1. Mechanism of S-endotoxin

During the process of sporulation, all varieties of *B.thuringiensis* produce a proteinaceous parasporal inclusion body. In most varieties, this inclusion body is crystaline or quasicrystalline in structure and consists of one or more proteins. Shortly after ingestion by larvae of many species of the order Lepidoptera and Diptera, this inclusion dissociates in the environment of the larval midgut and the constitutent proteins then cleaved by larval proteases into several peptides, one or more of which is a cytolytic toxin for midgut epithelial cells, referred to as the S-endotoxin. The S-endotoxin causes the epithelial cells to lose the ability to regulate permeability and results in subsequent degeneration and desquamation of the cells from the midgut basal lamina, followed by death of the larvae.

a. Symptoms:

Insects killed by the S-endotoxin rapidly darken in colour and are often very soft. The internal tissues and organs are rapidly broken down to a viscous consistency, accompanied sometimes by a putrid odor. The integument remains intact. There is an abundance of bacteria in an insect shortly after death. The cadaver shrivels, dries and hardens.

b. Histopathological changes:

Histopathological changes of *Pieris rapea* treated with *B.t.* emulsion were observed in the laboratory studies. Two hours after the infection, the cytoplasm of midgut epithelial cells enlarged, forming many bubbles on the apex of the columnar cells. The epithelial cells began liquefaction 4-6 hours after the infection and all the epithelial cells of some larvae sloughed into the lumen by 8 hours. By 10 hours after the infection, the base membrance of the cells was broken followed by death of the larvae.

Observations on the histopathology caused by the S-endotoxin both *in vivo* and *in vitro* have demonstrated a similar sequence of pathological changes which occur in different cell types as well as in different species of susceptible insects. As a result of cytolysis and erosion of epithelial cells, the midgut loses its ability to regulate permeability and the alkaline contents of the lumen begin to flow into the hemocoel. The pH of the hemolymph rises from slightly below neutral to over 8 and the larvae subsequently dies.

c. Cytopathological changes

Although there is considerable controversy regarding the mode of the S-endotoxin, there

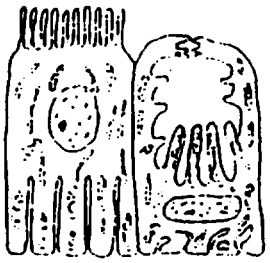
is, as a result of careful in vivo and in vitro studies by numerous investigations, general agreement on the pathological changes that result when cells are exposed to this toxin. For many years, it has been known that the toxin causes an almost immediate paralysis of the midgut epithelium and cessation of feeding. In highly susceptible insects such as *Bomby mori*, paralysis can result within a few minutes of ingestion of parasporal crystals. Histological and ultrastructural studies of treated Lepidopteran larvae reveal the following sequence of events. Shortly after ingestion of parasporal inclusions, columnar cells in the anterior region of the midgut begin to hyptertrophy. Concomitantly, the microvilli begin to expand forming bubble-shaped protrusions on the surface of the cell. Cellular organelles, particularly the endoplasmic reticulum, become increasingly vacuolated. Organelle membranes such as those of the nucleus and mittochondria, swell and separate from one another. As hypertrophy continues, basal infoldings and microvillar structure are completely lost. Subsequently, the cells lyse and are sloughed from the basement membrane. This process proceeds, eventually affecting the anterior two-thirds of the midgut. In contrast to the columnar cells, the goblet cells exhibit only minor changes consisting of enlargement of the goblet cavity and increased electron density in the cytoplasm. The sequence of events is summarized in Figure 2.

d. Mode of action of S-endotoxin

Workers have proposed a number of hypotheses on the mode of action of the S-endotoxins. These hypotheses are based largely on observations of the cytopathology of insect cells in vitro. As in the case of the larval midgut cells, the cultured cells swell, form vesicles and undergo lysis within a minute. The rapid cytopathology suggests that the toxin does not need to be internalized and that it causes a cytolygic mode of action. Accordingly, the toxin appears to affect the structure and permeability of the cell membrane and it does not directly affect the internal cellular structure.

The hypotheses that closely apply to the above cellular reactions to the toxin are (1) inhibition of the ion (K+) pump in the cell and (2) the formation of small holes or pores in the plasma membrane that results in colloid-osmotic lysis. In the first hypothesis, the K+ pump, located at the terminal ends of midgut epithelial cells, is irreversibly inhibited by the S-endotoxin. This inhibition prevents the movement of ions in the cell, leading to cytolysis.

normal

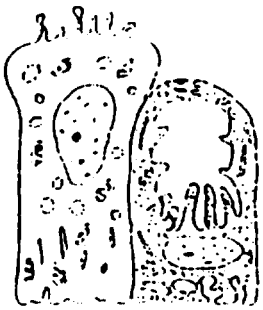


columnar cell-numerous microvilli at apical end deep infoldings of basal cell membrane.
goblet cell-numerous cytoplasmic projections into goblet cavity, infoldings of basal cell membrane. (mitochondrion in each projection).

stage 0
(~ 15 min)

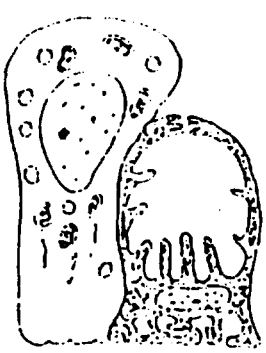
columnar cell-deformation of basal infoldings, transformation of some mitochondria at basal region into condensed form.
goblet cell increase electron density of cytoplasm.

stage 1
(~ 30 min)



columnar cell-enlargement of cell itself and nucleus, decrease of electron density of cytoplasm, decrease of number of microvilli, condensed from mitochondria, vacuole- like endoplasmic reticulum, destruction of basal infoldings.
goblet cell-high electron density of cyto-plasm, enlargement of intrapace of basal infoldings.

stage 2~3
(60~90 min)



columnar cell - disappearance of microvilli and basal infoldings; very coarse cytoplasm, swollen nucleus goblet cell - enlargement of goblet cavity.

Fig. 2 Pathological changes resulting in the midgut of *Bombyx mori* exposed to the δ-endotoxin of *B.t.*

The second hypothesis is based on (1) the attachment of the endotoxin to receptor sites in the midgut epithelial cells (2) the creation of pores in the cell membrane and (3) the free movement of ions and molecules trough the permeable membrane. This activity distrubs the colloid-osmotic equilibrium and results in cell lysis. Specific plasma membrane receptors for the S-endotoxin have been detected. The attached toxin generates small pores by inserting into the plasma membrane. The formation of pores causes the loss of permeability of the plasma membrane, resulting in a net inflow of ions, accompanied by the influx of water, cell swelling and cell lysis. Moreover, the recept sites differ with the various endotoxins and this may explain the variations in host specificity of the sub-species of *B.t.*

2. Beta-exotoxin

Beta-exotoxin was first detected in cultures of *Bacillus thuringiensis* as a heat stable, dialyzable toxin that killed insects when inoculated into the hemocoel. Subsequently, the toxin was detected in the *bacillus*. It was shown to be a soluble heat stable toxin that killed insects when it was fed to them. This heat stable toxin is the B-exotoxin.

Various names have been applied to the B-exotoxin, such as thuringiensin, heat stable toxin, thermostable toxin, fly factor, fly toxin, etc.

The general consensus is that the B-exotoxin(thuringiensin) is formed during the vegetative growth phase of the bacteria and is secreted into the medium. Very little or none is produced during sporulation. The exotoxin production is not connected with that of the parasporal body. Some strains that do not produce a parasporal body are known to produce the B-exotoxin. A 62-MDa(megadalton) plasmid may be carrying the B-exotoxin gene(exo) and the S-endotoxin gene (cry) in the sub-species darmstadiensis.

Not all sub-species of *B.thuringiensis* produce thuringiensin. Its production is a strain-specific property rather than a serotype specific property (Table 1). It is formed by isolates of serotypes 1 (thuringiensis), 4a,4c(kenyae),4a,4b(sotto),5a,5b(galleriae),7(aizawal),8a,8b(mirrisoni), 9(tolworthi), 10(darmstadiensis), 11a,11b(toumonaoffi) and 12(thompsoni). The possibility exists, however, that isolates of other serotypes may produce thuringiensin but at a low level.

Table 1 Serovar and its toxin of *B. thuringiensis*

H Serotype	Serovar	Toxin Type						
		1	2	3	4	5	6	7
1	*thuringiensis*	+	+	+		+		+
2	*finitimus*	+	+	-				
3a,3c	*alesti*	+	+	-			+	+
3a,3b,3c	*kurstaki*	+	+	-				
3a,3d	*sumiyoshiensis*	+						
3a,3d,3e	*fukokaensis*	+						
4a,4b	*sotto*	+	+	-				
4a,4c	*kenyae*	+	+	-				
5a,5b	*galleriae*	+	-	+				+
5a,5c	*canadensis*	+	-	+				
6	*entomocidus*	+	-	-	+			
7	*aizawai*	+	+	+				
8a,8b	*morrisoni*	+	-	+				
8a,8c	*ostriniae*	+	+	-				
8b,8d	*nigeriensis*	+						
9	*tolworthi*	+	+	+				
10	*darmstadiensis*	+	-	+				
11a,11b	*toumonoffi*	+	+	+				
11a,11c	*kyushuensis*	+	+	-				
12	*thompsoni*	+	+	-				
13	*pakistani*	+	+	-				
14	*israelensis*	+	+	-				
15	*dakota*	+	+					
16	*indiana*	+	+					
17	*tohokuensis*	+	-	-				
18	*kumamotoensis*	+	+	+				
19	*tochigiensis*	+	+	-				
20a,20b	*yunnanensis*	+						
20a,20c	*pondicheriensis*	+						
21	*colmeri*	+						
22	*shandongiensis*	+						
23	*japenensis*	+						
24	*neoleonensis*	+						
25	*coreaensis*	+						
26	*silo*	+						
27	*mexicanensis*	+						
28	*monterrey*	+						
29	*amagiensis*	+						
30	*medellin*	+						
31	*toguchini*	+						
32	*cameroun*	+						
33	*leesis*	+						
34	*konkukian*	+						

1. δ-endotoxin
2. α-exotoxin
3. β-exotoxin
4. χ-exotoxin
5. labile exotoxin
6. water-soluble toxin
7. mouse factor exotoxin

Thuringiensin affects a broad host spectrum both by per os and parenteral inoculations of insects as well as other invertebrates and vertebrates. The susceptible insect species are found in the orders Diptera, Lepidoptera, Hymenoptera, Coleoptera, Isoptera, Orthoptera, Hemiptera and Neuroptera. In Hemiptera, it was found that the nymphs, but not the adults, of the predaceous lygaeid bug, *Geocoris piunctipes*, are affected by the topical applications of thuringiensin. Bugs feeding of *Heliothis zea* larvae intoxicated with thuringiensin have been killed; surviving nymphs produced adults with reduced longevity. Larvae of the lace wing, *Chrysopa carnea*, also are killed after consuming the eggs of *Sitotroga cerealella* treated with the exotoxin.

The toxicity of thuringiensin is greater trough hemocoelic inoculation than by ingestion. The exotoxin is deactivated in the digestive tract very likely trough enzymatic activity. Most insects, however, are susceptible if given adequately high doses of thuringiensin.

Thuringiensin acts as a feeding deterrent with black cutworm, fall armyworm and the European corn borer. This feeding inhibition is evident with high concentrations of the toxin. Even tough only a small quantity is ingested, the larvae dies after a period of 7 to 14 days. In mosquitoes, B-exotoxin acts as larvicide and adulticide when ingested. Sublethal concentrations of exotoxin causes a delay in larval molting and induces teratological effects in larvae and pupae. After a sublethal application of exotoxin at the larval stage, the next generation of mosquitoes is much more susceptible to this toxin. When applied to piggeries, compost toilets, and hen houses, exotoxin has effectively controlled *Musca domestica* (house fly) and less effectively controlled *Fannia canicularis* and *Stomoxs calcitrans* (stable fly).

Besides teratological effects, when fed daily to Drosophila, thuringiensin reduces the longevity and fecundity of the flies and decreases egg size. The fecundity of noctuid adults developing from larvae fed the exotoin is also reduced. In the citrus red mite (*Panonychus citri*), the tetranychid mite(*Tetranychus pacificus*) and the phytoseld mite (*Metaseiulus occidentalis*), exotoxin kills larvae and adults and to a lesser extent the eggs. Exotoxin acts on the mites trough ingestion and by contact.

The insecticidal activity of thuringiensin is high (e.g. the LD_{50} by intrahemocoelic inoculations in larvae of *Galleria mellonella* is from 0.055 to 0.5 ug/larvae, about 1000-fold more toxic than DDT). It is less toxic than the S-endotoxin derived from the crystalline parasporal body (e.g., it is about one fifth as toxic to neonate larvae of *Heliothis virescens* as the S-endotoxin with a potency of 16,000 international units per milligram (IU/mg). When thuringiensin is combined with the sub-species *kurstaki*, a

potentation or enhancement develops against larvae of the beet armyworm, *Spodoptera exigua.*

Workers have speculated that more than one toxin is involved in the thermostable component. However, it has been concluded that all evidence points to a single heat stable exotoxin, thuringiensin. It is a toxic metabolite, a yellow solid, acidic in nature, and with a molecular weight (MW) 701. Its molecular structure is related to adenosine triphosphate and is composed of adenine, ribose and allaric acid. Thuringiensin is a specific inhibitor of DNA-dependent RNA polymerases. At high dosages, it may affect the biosynthesis of proteins and DNA.

Thuringiensin, through its interference with RNA transcription affects cell mitosis, particularly during molting and metamorphosis. If larvae are not killed by the toxin, they transofrm to deformed pupae and adults with teratologies, such as abnormal antennae, wings, legs or mouthparts. Affected adults are infertile or have reduced fecundity and longevity.

The presence of thuringiensin in *B.thuringiensis* is generally determined by a bioassay per os susceptible insects. Microbiological and biochemical methods have also been developed. The microbiological method is based on the growth inhibition of Sarcina larvae. The biochemical determination, based on the inhibition of bacterial RNA polymerase by the exotoxin, is much more precise than insect feeding tests.

As in the case with insects, thuringiensis is less toxic to vertebrates, through the mouth than inoculation into the body. It affects mammals by producing lesions mainly in the liver, kidney and adrenal gland. Chickens, when fed the toxin, exhibit a loss of vigor, reduced feeding and undersized eggs. Because of the vertebrate toxicity, most commercial preparation of B. thuringiensis are composed of sub-species that do not produce thuringiensin (B-exotoxin).

Insect resistance to *B.thuringiensis*

Recent studies show that insects have the capacity to develop resistance to B.t Laboratory studies have shown insects can develop resistance to the major toxins, B-exotoxin and the S-endotoxin of *B.thuringiensis.* House flies and *Drosophila melanogaster* have developed resistance to the B-exotoxin (thuringiensin). This resistance is not related to the resistance of the flies to chemical insecticides.

The first well-documented report of B.t-resistance was with the lepidopteran, *Plodia interpunctella,* where in laboratory selection of a field-exposed population with BTK

(Dipel) a 27-fold resistance was detected in two generations and a 97-fold resistance observed after 15 generations of selection. Further selection of this colony resulted in a 250-fold resistance after which the resistance level reached a plateau.

The tobacco budworm, *Heliothis virescens*, has developed resistance to the S-endotoxin produced in a recombinant *Pseudomonas flourescens*. Under continuous exposure of *P.flourescens*, the resistance increased 24-fold by generation seven and fluctuated by 13- and 20- fold thereafter.

Field populations with different levels of resistance of B.t have also now been identified. A field population of the diamondback moth, *Plutella xylostella*, in Hawaii, which had been treated 50 - 100 times during 1978 - 1982 with BTK, developed a resistance as high as 36 fold. In the Philippines, this same species developed resistance to Dipel after many years of use in the field and showed 200-fold resistance to CrylA(b), one of the proteins in Dipel.

The resistance was stable and inherited as a recessive trait. The resistance was not due to differential toxin processing in resistant as susceptible insects, but rather to a decrease in toxin binding affinity to receptors on the midgut membranes. However, the receptor number was unchanged in both populations. This resistant colony was, however, more susceptible to another *B.t.* toxin CrylC, where the enhanced suceptibility was apparently due to an increase in Cry1C receptor concentration. It was reported that the resistant insects to Cry1A(b) were susceptible to Cry1B and Cry1C toxins, which are not present in HD-1, the active ingredient in Dipel. Experiments demonstrated that the Cry1A(b) toxin did not bind to BBMVs from resistant insects, while the binding characteristics of Cry1B and Cry1C toxins and the LD50 values for these toxins, remained unchanged in resistant and susceptible strains. Thus, resistance to Cry1A(b) did not cause cross resistance to the other two related toxins. Heterologous competition experiments, in which one of the toxins is labeled and its binding competed with other toxins, showed that there were three distinct binding sites for these toxins, which probably accounts for the continued sensitivity to the Cry1B and Cry1C toxins.

Two mechanisms for resistance have been reported, in *Heliothis* virescens populations selected for *B.t.* resistance in the laboratory. A strain selected first with CrylA(b) for 13 generations, then with Dipel between 13 and 17 and finally for four generations with Cry1A(b) showed 71-fold resistance to Cry1A(b) and 16-fold to Cry1A(c). Binding parameters for Cry1A(b) and Cry1A(c) in the resistant strain showed a decreased affinity of 2- and 4-fold and an increase in binding site concentration of 6 and 4 times,

respectively. However, these changes are not sufficient to account for the 20- to 70-fold increase in resistance, indicating that altered binding is only a part of the resistance mechanism. In the susceptible strain, these two toxins appeared to compete for the same binding site, but in the resistant strain, the binding site was apparently differentially modified for Cry1A(b) because unlabeled Cry1A(b) was less effective in competing with labeled CryA(c) toxin than was unlabeled Cry1A(c). It has been suggested that the changes in receptor affinity and binding in the resistant strain appeared to be compensatory for the same toxin to which resistance was selected, in contrast to what was reported for *Plodia interpunctella*. In the latter case, sensitivity and receptor number increased for a different toxin. Cry1C, not present in Dipel, the insecticide use for selection.

A second type of resistance in *H.virescens* has also been reported. Broadspectrum resistance to other *B.thuringiensis* toxins was obtained after laboratory selection with Cry1A(c). After 13-15 generations, the LD50 of the selected strain for Cry1A(b) was 13 times higher than that for the control, and for CryIIA, it was 53 times higher. Contrary to what was reported for these cases of resistance, the binding parameter of both Cry1A(c) and Cry1A(b) to the resistant and susceptible strains were statistically similar. However, it was observed that the resistant strain had fewer high-affinity binding sites than the control strain. Cross-resistance to CryIIA was unexpected considering its sequence differs considerably from that of Cry1A(c). Results from genetic crosses indicate that resistance is not sex linked and is inherited as an additive trait, but the number of loci implicated is still unknown.

Recent comparisons of the heritability of the S=endotoxin resistance in *P.xylostella*, *H.virescens* and *Leptinotarsa decemlineata* suggest that the potential for resistance development can be detected in the first few generations of selection because heritability is higher in these generations.

7. Bt FOR CONTROL OF PESTS IN CHINA

China is one of the largest agriculture country in the world with 146.6 million ha. of cropping area Rice, wheat, maize, cotton, vegetables, fruits etc. are major crops (Table 1), and are usually infested by over 1350 species of pests including 700 insect pests, 550 diseases, 80 weeds and 20 rats, etc. With the rapid development of pesticides industry, large scale application of chemical pesticides was accompanied. Thus, the extensive use of pesticides exposed a series of adverse effects, such as environmental pollution, pest resistance to the pesticides, resurgence of the pests etc. Since the national policy of plant protection was confirmed to be "Integrated pest control with emphasis on prevention", biological agent, such as *B.t.*, for control of pests was greatly motivated as the core of integrated pest management, and lead to remarkable achievements.

Low cost stimulates farmers to use *Bt* insecticide for control of pests. During the past four years, the area treated with *Bt* insecticide was about 4,000,000 hectares.

1. Grain crop pests

In North China, granual of *Bt* insecticide has been applied to controlcorn borer for more than twenty years. Flowable formulation is also sprayed by airplane recently. It is very effective to control rice leaf tier. rice leaf folder and sorghun spotted borer. Progress has been made with the mixture of *Bt* and chemical for control of rice stem borer.

2. Cash crop pests

Good results were got for control of tobacco budworm, tea caterpillar, soybean caterpillar and cotton geometrid. High dosage of *Bt* can work well for control of cotton boll worm. Recently, it has been replaced by mixture of *Bt* and chemical which results in lowering the control cost. This method has been expanding in Hebei province.

3. Vegetable pests

This is the most successful area in China. Usually more than 90% of mortality can get in spring, summer and fall for control of cabbage caterpillar and diamond buckmoth. Nearly 50% of those punts are controlled now in big cities such as Shanghai, Beijing and Lanzhou. The control cost of applying *Bt* insecticide is now less than chemicals. It is also very effective against pickle worm and so on.

The price of *Bt* insecticide is so low that the control cost of using *Bt* is cheaper than that

of using chemicals for several pests, such as cabbage looper, cankworm, corn borer and so on. The amount of nature enemies on using *Bt* area is obviously more than that on using chemical area so that less insecticide is use more and more *Bt* insecticide. There is no doubt, the *Bt* output will grow up steadily.

8. *BACILLUS THURINGIENSIS* FOR CONTROL OF MALARIA VECTOR-MOSQUITOES

Today, the malaria situation is serious and getting worse. Every year, malaria causes clinical illness, often very severe, in over 100 million people and over 1 million people die of it. 2200 million persons, about 40% of the world's population are threatened. Before the mid-1970s no bacteria demonstrated much promise for the control of mosquitoes. None of the more predators and parasites can be mass-produced and stored for long period. It is must rearer in viva. Since then, the most promising microbial agents for mosquito control are two bacilli. *Bacillus thuringiensis* Berliner Serotype H-1'4 de Barjac and *Bacillus sphearicus* Neids. In 1975-76, for *B.thuringiensis*, Drs Tahor and Margalit conducted a survey in Israel for biocontrol agents against mosquitoes larvae. A sample collected from the edge of the pool, containing dead and decomposing larvae. Water and silty mud was taken to the laboratory and refrigerated. Bacteria were isolated from this sample in the lab,in association with Mr. L H Goldberg, and purified to single colonies. Thus, from a single colony designated ONR 60A, were derived all the known culture of *Bacillus thuringiensis* var. *isrealensis* now in use. The larvicidal activity of this strain was tested in 1976 and found to be effective against five species of mosquitoes belong to the genera *Aedes*, *Culex*, *Anopheles* and *Uranotaensia*. Clones of this strain were delivered through WHO to Dr. de Barjac. It was identified as *Bacillus thuringiensis* var. *israelensis* Serotype H-14 (Bti).

TARGET ORGANISMS

Since BTI detection, it has been tested by scientists all over the world, and was found to be toxic against practically all filter-feeding mosquito and blackfly larvae tested. namely 72 species of mosquitoes: *Anopheles* (21 species), *Aedes* (21), *Culex* (17), Culisita (5), Limatus (2), *Uranotaensia* (1), and Coquilleidia (1). BTI also proved to be effective against 22 species of simulium blackflies; Simulium (14 species), Cnephia (2), Prosimulium (1), Austrosium (2), Eisimulium(1), Odogmia(1), and Strogoptera(1). Among the many other organism which have been tested for susceptibility to *Bti* only two filter-feeding chironomid species .and one species of Dixidae were found to be susceptible, but at a dose two orders of magnitude higher than that required to kill mosquitoes laver. All non-target organism tested, breeding in association with mosquito larvae, were not affected by *Bti*.

MODE OF ACTION

The principal agent of insecticidal activity of *Bti* is the parasporal body commonly known

as the crystal. In Diptera only the crystal is toxic. Sero var. *israelensis* parasporal bodies are irregular in shape and there are usually two or more such bodies associated with the spore. The basic elements are polypeptides with a molecular weight range between 128,000 and 136,000. After pathogen is ingested by a susceptible insect, gut secretion solubilitized the crystal and gut protases convet the protoxin into toxin which has a molecular weight of 65,000. This pattern of solobilization and protease cleavage is not seen in *Bti*. As mentioned above, the size of the suspected toxin is smaller (25,000) than that of the other varieties. It also does not appear to require solubilization or proteolytic activation.

The mode of actin of *Bt* is found in the large proteinaceous parasporal crystalline inclusion that is produced concomitantly with the spore. Upon ingestin by mosquito larvae the inclusion is solubilized in the high pH of the midgut. Lesions are caused by disruption of osmotic balance in the midgut epithelial. Crystal of *Bti* kill mosquitoes larvae within minutes. *Bt* causes toxin symptoms in lepidopterous larvae also within minutes.It is therefore assumed that the delta-endotoxin of both varieties have similar modes of action.

As mentioned earlier, the primary target organ of the delta-endotoxin of the *Bt* serotype active against lepidopterous larvae is the plasma membrane of get epithelial and of susceptible cells in vitro. For *Bti*, it was showed that the gut epithelium also appears to be primary target for its delta-endotoxin. Thomas and Ellar observed that a soluble preparation of *Bti* toxin caused rapid cytolysis of insect and mammalian cells in vitro, but had no effect on bacterial protoplasts. This toxin also showed haemolytic activity. A later work showed that identified certain membrane phospholipid as the primary target for the toxin. The interactin of the *Bti* toxin with specific plasma membrane lipids such as phosphatidyl choline, aphygomyeline and phosphatidyl ethanolamine, provided the lipids contain unsaturated acyl residues, causes a detagent-like rearrangement of the lipids, leading to disruption of membrane integrity and eventual cytolysis.

The biological and environmental factors that determine the efficacy of *B. thuringiensis* H-14. Some of th emost notable area : species, age, feeding behaviour of the target mosquito, water quality and depth. Vegetative cover and solar degradation of toxin.

PRODUCTION AND FORMULATION

In a general way, industrialised countries only three major firms commercially produced each year. Most manufacturers used *Bt* and *Bt*-187 (local strain) as the main production strain in China. The amount of toxin produced during the fermentation process depends

on the medium used, the temperature employed and the isolate used. The quantity and insecticidal activity of the toxin may also vary among different production lots and cannot be measured chemically at present. Therefore, potency is now determined by bioassay using insect larvae of an appropriate target species and calculated in international toxin units (ITU). Two main types of *Bti* formulation exist. In wettable powder the particles are large aggregates (10-30 u), whereas in suspension concentrates they are suspensions of isolated spores and crystals. At present, the main formulations available are water dispersible powders (wp) and suspension concentrates. The granules are now being developed to float on the water surface where the mosquitoes larvae feed. Concerning about the formulation mentioned earlier, there are several major differences between formulation of a chemical larvicide and formulation of *Bti*.

1. In contrast to chemical larvicides the primary in the formulation of *Bti* may show differences in their composition and physical properties. These variations strongly affect their biological efficacy.

2. The potency of *Bti* products is evaluated only by biological method.

3. *Bti* products is a natural microbial agent and cannot be patented.

4. As a microbial agent, *Bti* may be genetically engineered. Strains may be consists which can grow at low carbon source and oxygen concentrations found in mosquito breeding environments, offering long term vector control with low environments impact, Strains may also be constructed which grow faster, produce more toxin, or grow on cheapter carbon source, thus reducing the costs of production and formulations.

SAFETY

Due to bti specificity, it is remarkably safe to non-target organisms, including man. *Bti* has not showed a single case of human toxify after over several years of operational use.

FIELD TRIAL

(1)Protocol for field testing of *Bti*

This protocol designed to provide guidelines to scientists to mosquito control where these and other new tools can be evaluated in different situations and against different species of mosquito vectors.

A. Target vector species

Bti possess good activity against *Anopheles,Culex, Aedes* mosquitos. In large scale field trials, the bacterial agents should be evaluated against one or more species of mosquitos that are vector of human diseases. The control agent should be applied to a variety of habitats where one or more species of mosquito vectors breed.

B. Site selection

The selected area should be large enough for it to be possible to assess the efficacy of a bacterial agent applied to numerous breeding sites, using mass-application operational techniques. The area should preferably be isolated, in order to avoid the interference of vectors from adjacent non-treated areas. Ideally the area should have a relatively high level of endemicity and a population of epidemiological data. It is preferable to select an area where different types of breeding source are found. If possible, an area similar to the treated area should be selected and left untreated, to be used as a control area.

C. Pre-trial data gathering

C.1 Geographical survey : A geographical survey should be made by mapping all breeding places in both the treated and untreated areas. A full discription is needed of all types of breeding places, classified into (a) permanent, (b) semi permanent, and (c) temporary sources.

C.2 Entomological evaluation : Information on vector susceptibility, population levels and bionomics are needed. These baseline data should include species distribution in the selected area, their seasonal density, feeding habits, adult longevity and survival rates. It is preferable to select an area where most of this information is available.

D. Assessment methods

The efficacy of the larvicides used against mosquitos may be assessed by quantitative or observational of larvae density before and after application. Assessment of the pupal and adult populations before, during and after the experiment is a reliable method. Changes in pupal population sfollowing treatment are a good indicatin of efficacy of a larvicide. Measurements of other adult mosquito indices, such as parity rate and the level of adult emergence before and after application, as well as comparison of other parameters i both treated and untreated areas, well provide confirmatory evidence. Final

assessment of the success of the control agent can be achieved only through detection of a decline i the rate of disease transmission through active detection as well as parasitological and serological surveys in both the treated and untreated areas.

E. Methods of application

Selection of the method of application will depend on the type of breeding source to be treated and formulation of bacterial agent under test. Liquid concentrate or water dispersible powder (WDP) formulations should be diluted with water and applied with sprayers, and every effort should be made to achieve complete coverage of the breeding sites. In breeding sources covered with dance vegetation, granular or other solid formulations may be employed. Formulations giving slow release of the active materials may have to be employed in some special situations.

Diluted sprays or granular formulations may be applied by air blast equipment or air craft. This equipment is especially suitable for the treatmetn of large breeding sites or for those that are not readily accessible from the ground.

Regarding dosages, the instructions given for the particular commercial product should be followed. Period testing of the potency of the commercial preparation may be necessary for each batch, as is sometimes done with chemical insecticides.

F. Safety

Any observation indicating irritatino, allergic rations or other adverse effect in people exposed to the products must be recorded. Also, it is important to that or the target organisms, should be used for non-target fauna.

(2): Field evaluation of *Bti* formulations

In the present study, many small scale field trials all over the world have been carried out. Two lessons can be learned from these trials: Liquid concentrates and wettable powder were effective in controlling both anopheles and culicine larvae densities within 24 hours after. treatment. The percentage reduction of both anopheles and culicines ranged between 87-100 and from 80-100 in clean and polluted water, respectively. The impact on fourth instar larvae was relatively less than that observed on the first three instar, presumably due to the presence of a certain number of late fourth instar beyond feeding stage at the time of the *Bt* H-14 application.

In the case of all treatments of both polluted and non-polluted water, the larvae density

rebound to the pre-application level by the second day after treatment or shortly thereafter. Thus all formulatin had only a 24 hour residential larvicidal activity in natural mosquito breeding habitats. By the fifth to seventh day after application, both anopheline and culicine larvae densities were comparable to or greater than those in the untreated breeding places. Pupal started appearing by the sixth and eighth day so that effective control applications had to be repeated every five and seven days.

Most studies have showed that the effect to control anopheles larvae has a good instant effect with a little high dosage, but the persistence effect was too short. The mechanism seem to be a function of surface feeding habits and the particle of *Bti* sink rapidly after treatment, therefore, anopheles larvae are not able to have a long period of exposure to ingest sufficient amount to induce a high mortality. As a stomach toxin, the filtration rate of target larvae is a key factor for the ingestion rate of toxin particulate. Therefore, formulation of *Bti* as a floating formulation could increase activity of the toxin against anopheles larvae.

Large scale field trials for mosquitos control were carried out in Germany, USA, and China. Large scale field trials and routine treatments with different preparation for *Bti* have been showed that the effectiveness of *Bti* not only reduce the mosquitoes larvae densities, but also reduces the incidence of malaria in certain areas.

A: Wettable powder preparation of *Bti*: The wettable powder preparation of *Bti* (potency of the preparation was 1,000 ITU/mg was mixed with five portions of the fine sawdust and then dusted over an area of 8.4 Km2 (including 282.5 ha. of rice field and 24.6 m3 of standing polluted water. There were three main species of mosquitoes present in the trial area. *Culex* quinquefasciatus, *Culex* fuscanus and *Anopheles* sinensis). The treatment were made at 8 day intervals. The densities of larvae in polluted ditch water and in rice field were reduced by 90% and 76% respectively. Concomitantly, the densities of adult mosquitoes in the treated town areas and the treated rural area were reduced by 94% respectively. The cases of malarial cases between 1980 and 1979. The incidence was 40% and 33% more in both control town and rural areas; and 77% and 62% less in both treated town and rural areas.

B: Flowable preparation of *Bti* (emulsion concentration) : Flowable concentrate (FC) formulations have permitted a greater number of options for application and, consequently have resulted in more efficacious control. FC formulation have been used for ULV applications. Using a constant flow application appareatus, FC is added to irrigation water as it enters the field. The advantages of this methods are reduction of labour and

cost of application. Its major disadvantage is that large fields cannot be effectively treated due to settling of th eactive moiety prior to reaching a significant percentage of the larvae population.

In China, during the last 8 years, approximately 40,000 Kg of *Bt*-187 have produced (emulsion) each year using natural resources to treat 12,000 hectares of mosquito breeding sites. A good results have got for control *Culex* quinquefasciatus and *Anopheles* sinensis. For instance, in 1986, before application of the microbial control agents between April and October, a determined with a peak of 21.2 mosquitoes per person per night in June and a second peak of 16.5 mosquitoes per person per night in Sha Shi city, Hubei, China. After application, the densities were reduced. The seasonal averages (April to October) being 0.8, 0.76 and 0.52 mosquitoes per person per night, in 1991, 1992 and 1993 respectively. The incidence of malaria has reduced in the test area from 5.6 cases/10,000 people in 1986 before treatmetn to 0.27 cases / 10,000 people in 1993. A large scale field trial (there are 140 hectares with population of 1246) has been also carried out in Fang Ji township, Xiang Yang county, Hubei, China. The results showed that the emulsion of *Bt*-187 against *Anopheles* larvae, it was not only could reduced th epopulation of mosquitoes larvae, but also standing alone has reduced the mortality of malaria.

C: Granules preparation of *Bti* : At an application rate of 5 to 6.5 Kg/ha of the tailor made *Bt* - 187 granules yielded mortality of above 80% for more than 15 days in rice field.

ADVANTAGE AND PROBLEMS

Bti has many advantages such as lack of toxicity to not-target organisms, little potential for producing resistance among vectors and no cross-resistance to chemical larvicides. It can therefore be used with a high safety margin against multi-resistant malarial vector larvae.

A major advance of *Bti* is that it enable selective control of mosquito larvae with little or no effect on non-target fauna. An additional benefit of this bacterium is the possibility of long term effects on survival and vector competence of adults that survived exposure to it as larvae. Robert et al reported diminished vector competence in adult. An gambiae for p. faciparum after larvae exposure to a sublethal conentration of *Bti.*

The main disadvantages of *Bti* for large scale control of mosquitoes larvae are cost and short duration of larvicidal activity. Formulation of *Bti* are less effective against anopheles larvae due to the differential feeding behaviour among mosquito species.

Larvae of anopheles are adapted to collect particles from the air water interface. Feeding individual remain at the water surface in a horizontal position and filte only particulate presenting the uppermost layers of water; particulate which sink deeper than 1-2 mm are not ingested. Filtration rates of anopheles are 10-20 times smaller than filtration rates of *Culex* and *Aedes* larvae. Due to the particulate of *Bti* sink to the bottom of water quickly after treatment. A sustained release formulations of *Bti* is possible to prolongatin of larvicidal activity.

Biological method for the titration of Bti preparations with IPS standard

The bioassay principle is based on the comparison of mosquito larvae mortalities produced by a *Bti* preparation and by the iPS 82 bti standard titrating 15,000 ITU * *Aedes aegypti* (*Culex quinquefasciatus*) per mg.

1. 50 mg of the standard powder (15,000 ITU *) are weighed and placed in 20 ml penicillin flask, to which is added 10 ml deionized water and 15 glass beads (6 mm diameter).

2. The contents are thoroughly homogenised by shaking on a crushing vibrator machine (e.g. Dangoumau type) of 10 minutes at 700 strokes /minute.

3. From this homogenate, a stock solution is made in a tube (22 mm diameter) by adding 0,1 ml of the homogenate to 9.9 ml deionized water followed by maximum agitation on a Vortex agitator for a few seconds.

4. From this solution (50 mg L-1) subsequent dilutions are directly prepared in plastic cups, previously filled with 148 ml deionized water. To each cup, 25 early L4 larvae of *Aedes aegypti* (*Culex* quinquefasciatus) in 2 ml water are added by means of a Pasteur pipette.

5. With precision pipettes or micropipettes 120 ul. 90ul, 60ul, 30ul, and 15ul are added to the cups in other to obtain final concentrations of 0.04, 0.03, 0.02, 0.01, 0.008, 0.005 mg L-1 respectively of IPS 82.

6. Two or four cups are used for each concentration and for the control containing 150 ml deionized water only.

7. No food is added.

8. The choice of early L4 is more representative of the total susceptibility of the

target population and also allows for greater convenience in handling. It is very important to use an homogenous populatin of early fourth instar larvae which, in our standardised way rearing, are usually 5 days old and 4 to 5 mm length.

9. In bioassays with *Anopheles* sp. (e.g. *stepensi*), wich are less susceptible than *Aedes* or *Culex* sp., L3 larvae should be used. A small amount of food (e.g. ground mouse biscuit should be added, especially if observations are extended to 72 hours.

10. For bioassay of preparations of unknown activity, an initial homogenate is made and comparable dilutions prepared as for the IPS 82 standard. The range of dilutions should exceed that of the standard in other to obtain a reliable regression line.

11. Time can be saved by first making a range-finding bioassay, with widely soaked concentrations of the unknown. The data can be used to fix subsequent concentrations in an exact bioassay and as a partial replicate of this bioassay.

12. Each bioassay series preferably involves at least 400 larvae for the IPS 82 standard and 100 larvae for the contro; for preparations of unknown activity 500 to 1,000 larvae should be used. All tests should be conducted at 25 C + 2 C.

13. Mortality is determined at 24 and 48 hours and is based mainly on the counting of live larvae. Because of the very rapid killing action of *Bti*, usually there is no difference between the 24 and 48 hours mortality. But, 48 hours reading appears suitable intervention of factors other than *Bti* component. If pupation occurs, the pupal should be removed and their numbers excluded from the data base.

14. When control mortality exceeds 5%, the mortalities no treated groups should be corrected according to Abbot's formula. Tested with control mortality greater than 10% should be discarded. Mortality concentration regression lines should be drawn on gauss logarithmic paper. Based on the LC50s of the standard and unknown preparation, the litre of the latter is determined by the following formula:

Titre of unknown (ITU/mg)

$$= \frac{15{,}000 \text{ ITU} \times \text{LC50 of standard}}{\text{LC50 of unknown preparation}}$$

on *Ae. aegypti* (or other species)

For increased accuracy, bioassay should be repeated on at least three different day, and the standard deviation calculated.

* International Toxic Units. The titre of IPS 82 has been determined from the 1 st *Bti* standard IPS 78 which was assigned an arbitrary titre of 1,000 ITU per mg.

9. RESISTANCE OF DIAMONDBACK MOTH TO *BACILLUS THURINGIENSIS*

I. Introduction

Resistance is a genetically based decrease in susceptibility of a population to an insecticide. It is an evolutionary phenomenon. Pesticide resistance is an increasingly urgent worldwide problem, and resistance to one or more pesticide has been documented in more than 500 species of insects and mites (Georghiou and Lagunes-Tejeda 1991). Similar resistance to *Bacillus thuringiensis* (*Bt*) is a potential threat to the future of insect pest control.

Because *Bt* had been used commercially for more than 20 years without reports of substantial resistance development in open field populations, some scientists had presumed that the resistance to *Bt* was unlikely (Krieg & Langenbruch 1981). Recently, Tabashnik (1994) summarised that laboratory populations of at least 16 species of insect (ten sp. of moths, two sp. of beetles, and four sp. of flies) selected with *Bt* had developed resistance, 9 of whch developed more than 10-fold resistance. Dismondback moth (DBM), *Plutella xylostella* (L) was the only species of insect developed resistance to *Bt* under field conditions.

The diamondback moth is the most destructive insect of crucifers worldwide, and the annual cost for managing DBM is estimated to be US $1 billion. It is an especially serious pest in regions with warm climates where it has many generations per year and it has rapidly developed resistance to chemical insecticides (including organochlorines, organophosphates, carbamates, pyrethroids, and benzoylureas). DBM resistance historically occurs within 2-5 years where selection by one insecticide is intensive (Talekar & Shelton 1993). As an alternate, *Bt* insecticides became more and more important for DBM control, especially in tropical countries. The resistance of DBM to *Bt* has been reported in fields in the United States (Tabashnik et al. 1990, Shelton et al. 1993), Malaysia (Syed 1992) and China (Wang et al. 1993, Zhao 1993a & b), and in greenhouse in Japan (Hama et al. 1992). All of these cases were related with the intensive applications of *Bt* to control DBM.

To delay or reverse *Bt* resistance in pests, we must first understand the nature of resistance. The following is a review on the detection, monitoring, laboratory selection, cross-resistance, mechanism, genetics, and the management of *Bt* resistance in DBM.

II. Detection and Monitoring of *Bt* Resistance

1. China

Zhao et al (1993a) selected four areas in China for the detection of *Bt* resistance in DBM, i.e. Shenzhen (*Bt* used most intensively, as often as 6 to 10 times monthly in 1990-1992), Shanghai (intensively in limited periods), Wuhan (less intensively) and Beijing (not intensively). The DBM larvae and pupae were collected from *Brassica* fields in October and November 1992 in Shenzhen, Shanghai and Wuhan, and in May 1993 in Beijing. A susceptible (S) DBM strain was provided by Prof. Y.Q. Sun. DBM larvae were reared on fresh cabbage leaves at 25 $\pm$1°C and photoperiod 14:10 (L:D). Third instar of F_1 or F_2 offsprings and leaf disk dip method similar to Tabashnik et al. (1990) were used for bioassays. Wettable powder (WP) formulation with the potency of 1,5000 IU/mg of *Bacillus thuringiensis* subsp. kurstaki (*Btk*) from Hubei Academy of Agricultural Sciences was used for the study. DBM larvae were kept for 48 h at 25 $\pm$ 1°C before the mortality was determined. The discriminating concentration method for resistance monitoring was also used for comparison with concentration mortality tests.

Table 1 shows the resistance ratios (RRs) of DBM to *Bt* and their relationship with the % survival at a discriminating concentration. Compared with the LC_{50} of S strain, the RRs of DBM populations in Shenzhen and Shanghai were 42.1- and 6.1-fold, respectively, with Wuhan and Beijing populations not significantly different with S strain. These results were comparable to the fields *Bt* application history in each of the areas.

The surviva rates at the discriminating concentration of 25 IU/ul (equivalent to 50 mg [ai/litre) led to similar conclusions with the concentration tests on the relative susceptibility of five DBM populations to *Bt*. The concentration-mortality tests did not show significant difference in resistance between Wuhan and Shanghai populations, but the discriminating method did (Table 1). From this result we could get a conclusion that bioassays using short time intervals and a single concentration may greatly increase efficacy for routine evalution of resistance. We thought 25 IU/ul was a suitable discriminating concentration for on-farm resistance monitoring to *Bt* in DBM in China.

Wang et al. (1993) demonstrated that RRs of DBM to *Bt* increased from 21.1 to 35.0 fold in Shenzhen (from the same farm as Zhao et al. 1993a) and kept at 8.4-to 9.3-fold in Guangzhou in 1990-1992 (Table 2).

Table 1. Susceptibility of diamondback moth populations in China to Bt

Population	Slope(SE)	LC_{50} (95%FL) IU/μl	RR	% Survival at 25IU/μl
Susceptible	1.46(0.20)	0.50 (0.30-0.82)	1.0 c	0 c
Beijing	1.23(0.21)	0.54 (0.26-1.10)	1.1 c	0 c
Wuhan	1.39(0.23)	0.82 (0.41-1.66)	1.6 bc	0 c
Shanghai	1.92(0.36)	3.07 (1.62-5.83)	6.1 b	7.4 b
Shenzhen	1.86(0.27)	21.05 (12.68-34.95)	41.1 a	48.4 a

Table 2. Resistance of diamondback moth to Bt in China

Year/month	Population	Slope	LC_{50} (ppm)	RR
1990/12	Susceptible	1.38	0.8010	1.0
	Guangzhou	1.16	7.4635	9.3
	Shenzhen	1.49	16.9232	21.1
1991/12	Susceptible	1.39	0.8980	1.0
	Guangzhou	1.26	7.5102	8.4
	Shenzhen	1.55	24.1371	26.9
1992/3	Susceptible	1.38	0.7595	1.0
	Guangzhou	1.09	6.7876	8.9
	Shenzhen	1.59	26.5673	35.0

2. USA

Tabshnik et al. (1990) first report two DBM population from Hawaii that were treated repeatedly with commercial formulations of *Bt* developed resistance to *Btk* (Dipel, Abbott Laboratories). The LC50 of a field population (NO) collected from watercress in 1989 were 25.5 time greater than that of the susceptible strain (Lab-P) (Table 3). A rough estimate of various *Bt* formulations use at NO from 1982 to 1989 is 50-400 treatments (as often as 2 to 4 times monthly).

Table 3. Resistance of diamondback moth to Bt in Hawaii, USA[a]

Year	Population	LC_{50} (95% FL)	RR	Mortality at field rate
1986-1987	Lab-P	1.76(1.05-2.89)	1.0	95%
	SO	10.2 (5.7-16.9)	5.8	60%
	LH	11.9 (7.2-20.0)	6.8	60%
1989	Lab-P	2.51(1.89-3.22)	1.0	94%
	SO	24.1 (17.7-32.3)	9.6	35%
	LH	63.9 (46.1-89.0)	25.5	34%

[a] Modified after Tabashnik et al. (1990).

Table 4. Resistance of diamondback moth to various Bt products in Florida, USA[a]

Product	Strain	Slope	LC_{50} (95% FL)(ppm)	RR
		Bt subsp. *aizawai*		
Xen Tari	R	1.32	3.9 (1.0-10.8)	3.0
	S	1.66	1.3 (0.8-1.9)	
Agree	R	1.69	20.5 (14.1-28.3)	3.5
	S	1.91	7.2 (2.3-13.5)	
NB200 FC	R	1.57	4.5 (2.7-6.8)	4.1
	S	1.52	1.1 (0.7-1.6)	
		Bt subsp. *kurstaki*		
Biobit HP	R	1.25	161.7 (20.7-383.7)	461.6
	S	1.83	0.7 (0.5-1.0)	
Javelin WG	R	0.89	96.2 (54.8-163.5)	320.7
	S	2.25	0.3 (0.2-0.5)	

Shelton et al. (1993) reported field control failure and resistance of DBM in Florida to *Bt* subsp. *kurstaki* (*Btk*) formulations and low level resistanc to *Bt* subsp. *aizawai* (*Bta*). The RRs were 320.7-to 461.6 fold for *Btk* and 3-4.1 fold for *Bta* (Table 4).

III Laboratory Selection and Cross Resistance

Laboratory Selection : Tabashnik et al. (1991) reported that laboratory selection increased resistance to *Bt* from a moderately resistant field population of DBM in Hawaii. Five generations of selectin caused 5.5 - to 6.7-fold increases in LC_{50}, resulting in 150- to 190-fold resistance compared with a susceptible laboratory colony. Nine generations of selection caused 15.3 - to 29.7-fold increases in LC_{50} and produced 430- to 820-fold resistance (Table 5). In contrast, five foliar application sof *Bt* in the field did not increase the LC_{50}.

Table 5. Susceptibility of unselected and selected DBM larvae to Bt[a]

Strain	Generation	Slope	LC_{50} (95% FL)(ppm)	RR	RR/NO-U
LAB-P (S)	>60	1.5	2.3(1.0-3.9)	1	--
		Unselected subcolony			
NO-U	1	1.4	64 (46-89)	28	1.00
	4	0.9	29 (12-56)	13	0.45
	6	1.3	18 (7.8-32)	8	0.28
	9	1.6	38 (21-61)	17	0.59
	15	1.6	9.5(4.4-16)	4	0.15
		Selected subcolony			
NO-P	9	1.2	350 (180-24000)	150	5.5
NO-Q	9	1.9	420 (250-970)	180	6.6
NO-R	9	1.1	430 (260-1100)	190	6.7
NO-P	14	1.2	980 (450-27000)	430	15.3
NO-Q	14	1.0	1900(630-$7*10^9$)	820	29.7

[a] Modified after Tabashnik et al. (1991).

Cross-Resistance : Field collected population sof DBM from Florida with 300-fold resistance to *Btk* had less than 4.1-fold resistance to *Bta* (Shelton et al. 1993, see Table 4). DBM from Hawaii with 1000-fold resistance to Dipel and other formulations of *Btk*, as well as significant resistance to four separate toxins from *Btk* [CryIA(a), CryIA(b), CryIA(c), CryIIA], were not resistance to CryIC. However, extreme resistance to *Btk* conferred 3-fold cross-resistance to *Bta*, which contains CryIC and CryIA (Tabashnik et

al. 1993b). Ferre et al (1991) reported that 200-fold resistance of DBM to CryIA(b) did not affect the LC_{50}s of Dipel, CryIB, or CryIC.

IV. Mechanisms of Bt Resistance

Reduced binding of *Bt* toxin to the brush border membrane of the midgut epithelium has been identified as a primary mechanism of *Bt* resistance in DBM and the Indianmeal moth, *Plodia interpunctaella*. A dBM strain showed 200-fold resistance to CryIA(b) and little or o binding of CryIA(b) and CryIB fed DBM larvae revealed that CryIB in both susceptible and resistance larvae and CryIA(b) in the susceptible larvae, were found to the apical microvilli and peritrophic membrane. The CryIA(b) protein in the CryIA(b)-resistant strain did not bind to apical microvilli and binding to the peritrophic membrane was very low to absent (Bravo et al. 1992).

V. Genetics and Stability of Bt Resistance

Tabashnik et al. (1992a) reported that the resistance in a strain (NO-Q, RR=720 to Dipel) of DBM to *Bt* was recessive, autosomally inherited, and controlled by one or a few loci. Hama et al. (1992) concluded that *Bt* resistance of a DBM population from greenhouse (RR-704=-fold to Toarow CT, containing *Bt* crystal toxin only) was primarily controlled by an incompletely recessive, autosomal single allele.

Field-selected resistance of DBM to *Bt* declined slowly in the absence of *Bt* treatments (see Table 5). High level of *Bt* resistance selected in greenhouse could decreased significantly in 10 generation (Hama et al. 1992).

VI. Bt Resistance Management

1. General Considerations and Strategy

Management of resistance to *Bt* can be viewed as a special case of pesticide resistance management. Because insect resistance to *Bt* is a relatively recent phenomenon, the literature dealing with its management is mostly theoretical. The literature on resistance management strategies and tactics for synthetic organic insecticides is extensive. For conventionally sprayed *Bt* products, approaches used for managing resistance to chemical insecticides should be applicable. However, *Bt* differs from most other insecticides in terms of its effects on natural enemies and its persistence. The genes that code for *Bt* toxins can be expressed on natural enemies and its persistence. The genes that code for *Bt* toxins can be expressed in other bacteria and in plants. These feature are beneficial

for the *Bt* resistance management.

Resistance management within the context of integrated pest management (IPM) usually involves four strategies which may or may not be combined (1) diversification of mortality sources such that a pest is not selected by a single mortality mechanisms, (2) reduction of selection pressure for each major mortality mechanism, (3) maintenance of susceptible individuals through refuges and/ or immigration, and (4) development of resistance progress estimatin and / or prediction through development of diagnostic tool, monitoring, and models (McGaughey and Whalon 1992).

2. Tactics

Tabashnik (1994) summarised six tactics for managing resistance to *Bt*. (1) Mixtures of toxins: One conditions that is necessary for the success of mixtures is lack of cross-resistance between mixture components (2) Synergist : Serine protease inhibitor synergized *Bt* against four species of moths and the Colorado potato beetle (CPB), *L. decemlineata* (MacIntosh et al. 1990). (3) Mosaics : A mosaic consists of adjacent areas that are treated with different toxins. (4) Rotations : It is especially useful when large fitness costs are associated with resistance, and instability of resistance to *Bt* in DBM (Hama et al. 1992) indicated that rotations might slow resistance development (5) Ultrahigh doses: It is valuable in theory but not feasible in practice for high level of *Bt* resistnce in DBM (6) Refuges : To provide temporal and spatial refuges from exposure to *Bt* can slow resistance development.

VII. Conclusion and Discussion

The resistance to *Bt* in the field has been documented for only one species -- DBM. The level of *Bt* resistance in DBM under laboratory selection can be exceeded 1000-fold. Pests can develop resistance to a variety of *Bt* strains and toxins, even when many toxins are used simultaneously. Most of the knowledge of *Bt* resistance is based on studies with three species of moths: *P. interpunctaella,* DBM, and *H. virescens.* More empirical studies are especially important for *Bt* resistance management.

After 30 years of successful use, *Bt* is considered to be one of the safest pesticides available. When *Bt* products are used wisely in IPM programmes, and combined with a range of pest control measures, the risk of resistance will be minimal.

10. THE SAFETY OF BT AND ITS PREPARATIONS

Bacillus thuringiensis (Bt) and its preparations were first discovered in 1901, and have been studied deeply in many fields, e.g. ecological distributions, mechanisms of pesticide action, applications in industrial fermentation and production, and its molecular biology. *B.t.* is very effective against various pests in the agriculture, forestry and house-life without appreciable risks to human beings, animals, environments and ecological balances simultaneously. These advantages have attracted more and more scientists and governments of the countries in whole-world recently. The yields of *B.t.* preparations have been increasing year by year and the application areas have been spreading wider and wider in the last two decades. *Bt.* and its preparations have become one of the most important weapons for microbial control agent.

Why are *Bt.* and its preparations not toxic to human beings and mammals, and not harmful to the natural enemy insects? Scientists had perfectly explained. As early as in 1953, Hannay discovered that the active components of *B.t.* were the parasporal crystals, which are proved to be a kind of proteins in the chemistry nature later. After doing a lot of studies of the action mechanism of the parasporal crystals, Luthy P. and H.R. Ebersold (1981), Thomas W.E. and D.J. Ellar (1983) and Knowles B.H. and D.J. Ellar (1986), etc, hypothesised the patterns of the toxic effects of the *B.t.* parasporal crystals on the larvae of Lepidoptera. After entering the midgut of the larvae, the parasporal crystals were hydrolysed into toxic polypeptide fragments by the effects of the high alkali reaction and the specfic enzymes in the midgut. Then, the toxic polypeptide fragments combined with the specific receptors which were only on the membrane of the midgut epithelium of the sensitive larvae, and perforated the plasma membrane through some unknown mechanisms. Simultaneously, the ions and water flowed into the cell through the pores by the action of ionic equilibrium, and made the cell swelled and split finally. The pH, the enzyme systems of the chyle of the midgut and the specific toxic polypeptide receptors on the membranes of the midgut epithelium of the sensitive larvae are different from those of the human beings, animals, and unsensitive larvae. Therefore, the parasporal crystals are only toxic to the sensitive injurious insects and harmless to the human beings, animals, fishes, frogs and natural enemies of the pests. This is the outstanding advantage of *Bt* and its preparations.

B.t. is very safe in theory, but the theoretical reasoning are not enough to the evaluation of the safety of *B.t.* preparations. It is necessary to collect more experimental data or testing results to confirm it. As microbial insecticides, *B.t.* and its preparations are

produced by the fermentation procedures with some chemical auxiliaries; and they are possible to enter the ecological systems and the human bodies. Many countries catalogue the *B.t.* and its preparations into a special kind of pesticides, and anounce strict regulations about their safety testings. In 1973, the US Environmental Protection Administration (EPA) published the guideline to the safety testing of mcirobial insecticides, According to the regulations of EPA in 1973, in order to evaluate the safety of a microbial pesticide, the tests have to be done in five stages, e.g. the tests, long-term infection tests, etc. The major test animals may be rodents, such as rats, mice, guinea pigs or rabits. The safety of the *Bt* and its preparations to the beneficial insects, plants, aquatic and wild animals should be evaluated. In 1974, USA published the government registration of microbial insecticides. Both Food and Agriculture Organisation (FAO) and World Health Organisation (WHO) also agreed on these regulations. In the next years, USA suggested further in details about the safety testing for several times. In 1980's European Economic Community (EEC) considered and formulated the safety testing of microbial insecticides, and WHO also suggested the safety testing of the microbial insecticides used to prevent and cure the injurious insects. Generally, all of those regulations required to do the safety testing in several stages.

Most tests including in the safety testing of the chemical pesticides and some special tests of microbial insecticides have been performed. As the others, our country divides the whole toxicological safety testing into several stages.

Stage 1 Acute Toxicity Tests, Dermal and Ocular Tests

Stage 2 Cumulative Toxicity tests and Mutagenicity Tests

Stage 3 Subchronic and Metabolic Studies

Stage 4 Chronic Tests and Carcinogenicity Tests

1. The Oral Acute Toxicity, Dermal and Ocular Tests

(1) The results of the acute toxicity tests of the spore and parasporal crystals showed that the *B.t.* and its preparations were a kind of gastric toxins; the major infection was through mouth. Additionally, as the powder preparations have the important proportion in the *B.t.* preparations, and the spraying or dusting are usually used by the farmers, so that the inhalation infection is also important. Table 1 summarised the results of theacute oral toxicity tests of several important *B.t.* strains and their preparations; Table 2 showed the results of the inhalation and dermal tests.

Table 1 The Results of the Acute Oral Toxicity Tests of Several B.t. Strains and their Preparations

strains or preparations	flagellum(H) serum types	test animals	LD50 (mg/kg.b.w)	authors	notes
7216 germpowder	$H_{7a,7b}$	mice	>4830	HEPSHP	no death, body temperature, weight, blood were normal. gavaged for 7 times continually, no adverse effects were observed.
7216 germ powder	$H_{7a,7b}$	mice	>19260	HEPSHP	no death the appetite dropped in about 1 hour restored in 24 hours.
B.t.k raw powder	$H_{3a,3b}$	mice	>15000	TMU	No pathological changes were observed in necropsy
B.t.k raw powder	$H_{3a,3b}$	rats	>20000	TMU	as above
B.t.k wetable podwer	$H_{3a,3b}$	rats	>20000	TMU	as above
B.t.i germ powder	H_{14}	mice	>15000	HEPSHP	no death
B.t.i spore-crystals	H_{14}	mice	>180 billion live spores/ kg.b.w	Long Qixin,et al	no death

notes: Health and Epidemic Preventions Station of Hubei Province (HEPSH); Tongji Medical University(TMU).

Most of the LD_{50} listed in Table 1 were higher than 15000mg/kg.b.w. According to the level standards of the toxicity of pesticides of WHO and some countries, the oral acute toxicities of the *B.t.* strains and their preparations listed in Table 1 should be judged to belong to the slight toxic (or actual non-toxic) level.

The oral acute toxicity test of Thuricide performed by Smirnoff, etc, in 1961 was very interesting. After feeding the mice with large doses of Thuricide, the feces excreted by the mice and diluted with water was able to kill the Mediterranean flour moth (Angastra kiihniella), but no adverse effects were observed in mice during the test.

Table 2. The Results of the Inhalation, Injection and Dermal Acute Toxicity Tests of the Bt. Strains and their Preparations

strains or preparations	H serum types	test animals	exposure methods	LD_{50} (mg/kg.b.w)	notes
7216 germ powder	H_{3a3b}	mice	simple dynamic inhalation	>22700	no abnormality was observed
B.t.k germ powder	H_{3a3b}	mice	ultrasonic spraying dynamic inhalation	>28000	no abnormality was observed
B.t.i germ powder	H_{14}	mice	dynamic inhalation	>23500	no abnormality was observed
B.t.i germ powder	H_{14}	mice	i.p. injection	>25000	no death
B.t.i slope culture	H_{14}	mice	i.p. injection	>5000	toxic reaction was
B.t.k spore crystals	H_{3a3b}	rats	dermal	>5000	not observed no death
B.t. k germ powder	H_{3a3b}	guinea pigs	dermal	>5000	no death, local slight erythema

The test results presented in Table 2 also showed that the acute dermal and oral toxicities tests of *B.t.* strains and their preparations belonged to low or slight toxic level.

The results of both Table 1 and 2 suggested that the acute toxicity of *B.t.* insecticides is very low to human beings and mammals.

(2) **The results of the Local Irritation Tests**

The author collected some test results of the dermal and ocular irritation tests of *Bt.* preparations performed in China. The results showed that *Bt* itself induced no irritation to the skin and eyes of the test animals.

B.t.k. germ powder showed the low irritation to the abraded skin, but the germs themself did not. These suggested that the irritation of the germ powder might be caused by the chemical additives in the powders; and the ocular irritation of the raw fermentation liquid might be caused by the residues of solid materials in the fermentation liquid.

(3) **The results of the sensitization tests.**

Are the *B.t.* preparations sensibiligens and will they cause hypersensitivity reactions after contacting with the animals? The results of the sensitization tests in guinea pigs and mice showed that the *B.t.k.* germ powder had not apparent sensitization effects on the test animals; the sensitization rates were near zero, and no animals died. However, the sensitization rates of the positive control chemical, i.e. dinitrochlorobenzene, which was tested at the same time, was 100%; the test animals died in 72 hours, and the edema and lung bleeding were defined and apparent.

In tests, the male guinea pigs were injected intracutaneously with the 7216 (H_{3a3b}) germ liquid of 0.275 billion live germs/ml/2 days. The first injection was 0.05ml, then 0.1 ml every time. The last injection was on the 14th day. From the first day of injection to the 20th day, no edema, hard lump and hypersensitivity were observed.

2. **The Cumulative Toxicity and the Mutation Tests of the B.t Preparations**

(1) **Cumulative toxicity.**

In the oral acute toxicity tests, we determined that the LD50 of the preparation of the *B.t.k* spore-crystals was higher than 15g/kg.b.w. With the dose increasing, the prpeara-tion was administered continually for 21 days. Totally 153 g per kg. b.w. of dried powder of the *B.t.k.* spofe crystals was given to rats; and at the last day, a shock dose of one LD50 was given to the rats, no rats died.

As the dose was too high, the average body weight of the female rats decreased in the four days after gavage, but statistically non-significant. After the 27th test day, the test animals were sacrificed and the organ coefficients of the *B.t.* test groups and the control group were tested statistically non-significant, too. The tissues and organs of the rats in

the *B.t.* infection groups revealed no pathological gross changes. The cumulative coefficient(K) was calculated to be higher than 6.24, which suggested that the *B.t.k.* had no significant cumulative effect in the rats.

(2) **Mutagenicity tests**

a. **Ames test**

The *B.t.k* spore crystal preparations were used as the test materials and their mutagenicity were studied by the Ames test. The results revealed that the rates of the induced and the spontaneous mutation(MR), with or without S9, were all lower than 2, i.e. the results of the Ames test were negative.

b. **The effects on the micronucleus rates of the mice bone marrow cells**

The influence of the *B.t.k.* spores and crystals upon the micronucleus rates of the mice bone marrow cells was tested. The rates of the treatment groups were 3.33-5.00%, the negative control groups were 2.77-4.61%, the differnce between them was statistically non-significant.

c. **The influence upon the M1 spermatocidal chromosomes of mice**

Administering the mice with the *B.t.k.* spore-crystals, the aberration numbers of the test group were about equal to those of the negative control group until the dose increased to 1000 mg/kg.b.w. All were between 9.1 to 9.8% *B.t.k.* spore-crystals had no mutagenicity and could not cause the mutation of the mammalian cells and prokaryotic organisms.

3. **The Results of the Subchronic and Metabolic Tests of the B.t. and its Preparations.**

1) **The Subchronic Tests**

According to the registration regulations of the pesticides, the oral feeding tests of the dried powder of *B.t.k* spore-crystals were performed. The highest dose was 30g *B.t.* powder/kg per day. The observations at two stages, i.e., 90 days and 180 days after administration, were carried out. No apparent subchronic toxicity was observed. The indexes of the liver functions and the kidney functions were within the ranges of the normal values. The haematological indexes were normal, too. After administering large doses for a long time, it was discovered that the increments of the weights of the animals

in the treatment groups were lower than those in the control group; and the higher the dose was, the more feed the animals consumed; whereas the lower the increments of weights were, and the lower the feed utilization rates were.

It was observed that the average numbers of the leukocytes of the dose groups were significantly lower than that of the control group, after feeding the *B.t.* germ powder for 6 months. The organ coefficients of spleens of the test groups and the control group were different, but were statistically non-significant. All of the other organ coefficients were not different from those of the control group. The autopsy of the animals of the dose groups showed no apparent abnormality.

The results of the 21-day dermal test in rats were negative.

(2) **Reproduction tests**

The two-generation/one litter reproduction tests of the *B.t.k.* germ powder were performed in rats, The test dose of group A was 1000mg/kg per day, B 100mg/kg/d, C 10mg/kg/d. The activity, feed consumption and behaviour of the animals in every group of all generations were statistically not different from those of the control group. *B.t.k.* germ powder had no adverse effects on the weight increments of the offsprings in the lactation. In the genital organs, abnormal organ coefficients was not observed. Except that the rat infant survival rates of the group B and C of FO generation were significantly higher than that in the control group, the differences of the other indexes were all non-significant from those of the control groups. Thus, it was believed that the test results demonstrated that the *B.t.k.* germ powder had no apparent reproduction toxicity.

(3) **Teratogenicity Tests**

In the teratogenicity tests in vivo and the rat whole fetus culture tests *in vitro*, the *B.t.k.* germ powder showed no teratogenicity.

(4) **Metabolic Study**

On the 4th, 7th, 11th and 16th day after the intraperitoneal infections of 0.17g/kg-1.16g/kg of *B.t.*7216 spore-crystal preparation, the rats were killed in the respective stages, and the heart blood were cultured in plates, it was observed that the quantities of the *B.t.* germs decreased gradually and eliminated completely on the 17th day. The activities of the test animals were normal, but the pathological changes in liver and lungs were observed in autopsy. From the 5th days after subcutaneous injections, no *B.t.* germs was observed in the blood cultures.

The oral tests of *B.t.i.* germ powders showed that the *B.t.i* germ disappeared in the animal blood in the 72 hours after administering (Long Qishin, 1984). Giving the dose of 170 billion live spores/kg b.w. of *B.t.i.* germ powder to the ducks and geese, it was showed that there were *B.t.i.* germs existed in the intestines of the test animals in 1-3 days, but the *B.t.i.* germs was not observed on the 4th day.

The results of the metabolic study of the Thuringinsin were: The half-life time (T 1/2) in blood was 1.92±0.67 hours; the Thuringensin would not be determined in the blood in 8 hours after the intravenous injection (4.5mg/kg). 15 minutes after administering Thuringensin, there was a little high concentration of the chemical in the urine; after 3.4 hours, the concentration was at the peak, 72 hours no Thuringensin would be determined in the urine. The above results revealed that the Thuringinsin disappeared rapidly from the body, and excreted within the urine, i.e. the cumulative effect was very low.

4. **Chronic toxicity and Carcinogenicity Tests of the *Bt.* Preparations.**

The chronic toxicity tests of *Bt.*i germ powders revealed that the differences between the animals in the treatment groups and in the control group were non-significant, though the slight cloudy swelling or interstitial nephritis was observed in the animals of the test groups.

On these conditions,such as the 6-month subchronic toxicity tests of *B.t.k* spore-crystal preparations showed a negative results, the cumulative toxicity was very low, the elimination speed in the environment was fast, etc. according to the provisional regulation of the toxicity test procedure of pesticides of China in 1982, the chronic test may not be done.

The Safety of the *B.t.* Preparations of Human Beings

The oral test of Thuricide commercial preparations of Thuricides in 18 volunteers was reported by Fisher, et al (1959). In this test, 1g preparation was ingested by every volunteer per day; 5 of them also inspired additionally 100mg powder per day. The first physical examination was performed 5 days after the first dose; the second physical examination was performed 1 month later. Every indexes of all volunteers were normal. The other 8 workers exposed in the *B.t.* final products for many years were all very health. In the investigation of 23 worker who engaged in the work of the dry spraying and the packing of *B.t.* powder in f a factory in Hubei Province, they worked 8 hours a day, the longest standing was 14 years; no disease caused by *B.t.* powder was observed in the physical examinations in a hospital.

The Environmental Safety of *B.t.* and its Preparations

Though the *B.t.* has been confirmed safe to human beings and mammals, its environmental effects should be also elucidated. According to a large amount of the test and investigation data as follows, the *Bt.* was very safe to the environment.

1. The Safety of *B.t.* Preparations to the Domestic Animals and Poultry

By spraying the *Bt.* preparations on the crops, e.g., vegetables, grains, it is possible that the *Bt.* preparations will enter the bodies of domestic animals and poultry, fed on these vegetables and cereals. But the results of many tests and investigations demonstrated that these vegetables and cereals were not toxic to the domestic animals and poultry.

a. Pigs

The Health and Epidemic Prevention Station of Hubei Province (1980) mixed the germ liquid of *B.t.* 7216 (H3a3b) with the feed of pigs. To every pig 5000 billion live spores was administered per day, corresponding to 500g 7216 germ powder. The growth and development of pigs were normal. After slaughtering, no pathological changes caused by the germs were observed in the necropsy of the organs and tissues. In accordance with the three dose groups of 1,2,3.3 g/kg.b.w., the piglets weighed 5-7.5kg were fed wioth the *B.t.i.* germ powder for 3 days, observed the changes for 10 days. The body temperatures of the test piglets were normal. The test piglets in the high dose group displayed the disorders, e.g., dry feces with blood or mucus, decreased appetite, etc. These disorders recovered gradually from the 6th day without any treatments. The author sprayed the *B.t.k* germ liquid of concentration of 0.01 billion live spores/ml to the leaves of Chinese cabbage and radish, then harvested them after being dried and fed the pigs. This test lasted for half a year. In the 12 test pigs,no apparent adverse effects was observed.

b. Hens

Long Qixin, et al (1984), used the *B.t.i.* germ powder to infect the hens in accordance with the doses of 0.68, 3.3, 6.6g/kg.b.w. two times a day. The treatment groups were not different from the control group in the body weight, body temperature, spirit, behaviour, mating, etc.

c. **Ducks and Geese**

Long Qixin, et. al. (1984) performed the duck and goose tests with the *B.t.i.* germ liquid. After testing for 105 days, every duck was totally administered 21760 billion live spores of *Bt.*, every goose totally 141780 billion; no test animal died. The feed consumed, activity, feces were all normal. In the autopsy carrying out respectively at the 7th, 41th and 105th day, no pathological changes were observed.

We determiend the results of *Bt.* germs on the plants applied the *B.t.* preparations. It was concluded that after spraying *B.t.* germs onto the rice plant in the filtering stage, no *Bt.* germs were monitored in the cereal after the rice riped. At the 15th day after spraying the *B.t.* onto the chinese cabbage, *Bt.* germs were detectable on the leaves, but fewer than 0.1 million *Bt.* live spores in 1g leaves. Summarizing the results of the above three animal tests which revealed no adverse effects after being administered large doses, we considered that it is safe for domestic animals and poultry to eat the feed sprayed the *Bt.* preparations.

2. **The Safety of the *B.t.* preparations to Birds and other Wild Animals.**

Smirnoff, et al (1961) administered Thuricide germ liquid to Sturnus vulgaris, Zonotrichia leucophrys and Junco hyemalis, with the dose of 8.5 x 10 8 live spores/per bird every day. Several hours later, the *Bt.* germs were detectable in the feces of the birds, which was able to be cultured to produce spores and crystals. However, no abnormality was observed after 3 months. Buchner carried out a systematic survey in the areas sprayed *B.t.* preparations in Canada. He investigated 74 species of birds of 23 families. The results in 3 days and 30 days after spraying the insecticides revealedthat there was no posoning appearance in those birds.

Li Zhannino (unpublished data) investigated the reactions of some small mammals in the spruce forestry ecological system after spraying the *B.t.* preparations by airplanes. For all observed animals, including Napaeozapus insignis, Peromyseuns maniculatus, Blarine brevicauda, Clethrionomys gapperi, Tamias striatus, there were no difference in the treatment areas from in the non-treated control areas.

3. The Safety of the *Bt.* Preparations to the Beneficial Organisms in Water.

a. *B.t.k* preparations

Adding the suspension of *B.t.k.* spore crystals(toxicity efficiency 2000IU/mg) into the pool of the goldfish bry to the final concentration of 200 ug/ml, observing continually for 15 days, the mortality of the goldfish bry was zero.

In accordance with the final concentration of 200 ug/ml, adding the above *B.t.* germ suspensions into the trough to feed the tadpoles, the mortality of the tadpoles were zero, the growth conditions was not different from this of zero control group. While the final concentration increased to 2500 ug/ml, the tadpole mortality were still zero. Raising the mature frogs, no mature frog died, the frog eggs produced by them were all incubated into tadpoles. However, the mortality of the wigglers put in the same trough was 100%.

b. *B.t.i.* preparation

When the High-eyes (Oryzias latipes) were raised for 2 weeks in the water solution made up by the *B.t.i.* germ powder of 100 g/ml, all fishes were survival. Raising the goldfishes with the suspensions of *B.t.i.* germs of the concentrations of 10,50,100 mg/l for 10 days, 5-10% of the fishes of both the treatment groups and control group were died; but no bacterial fish diseases were observed. The TLm 96 of *Bt.* i. germ powder was 380 ug/ml with *Clenopharyngodon idellus* C. etc. V. *B.t.i.* preparations showed no apparent adverse effects on the embryo development and the reproduction of *Hypophthalmichthys molitrix* C.et.V. and *Gambusia affinis.*

4. The Effects of *Bt* Preparations on the Natural Enemies of Injurious Insects.

Wilkinson, et al (1975) investigated the decreases and increases of the numbers of the natural enemies of insects on the wild areas applied the *B.t.k.* preparations. The results displayed the mortalities of 7 parasitic and rapacious natural enemies were all lower than 4%. But the average mortality of the chemical insecticides to several natural enemies were higher than 31%.

Xie Tianjian, et al (1990) and Li Xinyun, et al (in press) investigated the decreases and increases of the numbers of the natural enemies in the vegetable areas and cotton fields applied *B.t.k.* germ powders.It was observed that the numbers of the natural enemies in the *B.t.* treated areas were 5-13.7 times the numbers in the areas treated with chemical pesticides.

11. BACILLUS THURINGIENSIS BIOINSECTICIDES: PAST, PRESENT AND FUTURE

Introduction. *Bacillus thuringiensis(Bt)* is a Gram positive spore-forming bacterium that produces an insecticidal protein(s) called delta-endotoxin(s). The classification of these endotoxins is based on their biological activity and amino acid homology(Table 1). Some endotoxins have activity on Lepidoperta and Diptera or Lepidooerta and Coleoptera while other endotoxins are toxic for only Lepidoptera or Diptera or Coleoptera. Even though some endotoxins are very similar to each other with respect to amino acid homology and they may be active only on insects from a single Order, they can have different activity spectra. For example, CryIA(a) and CryIA(c) have essentially the same activity on *Manduca sexta* but CrylA(c) is muchmore active on *Heliothis virescens* (Table 2). Both endotoxins have greater than 90% amino acid identity andboth are active only on *Lepidoptera*. The current system of endotoxin classification is not perfect. One limitation is that the complete biological activity spectrum of a given endotoxin is unknown. Thus CrylB (Table 1) would not have been classified as a Cryl-type endotoxin if it's coleopteran activity had been known at the time it was discovered. In fact, CrylB is more closely related to CryV-type endotoxins than it is to Cryl-type endotoxins (Figure 1). The current system of endotoxins classification and naming is under review and will likely be changed in the near future. Biological activity and amino acid identity will continue to be important criteria in the new classification system.

Past and Present Status. Most naturally occurring strains of *Bt* produce more than one endotoxin. It is generally accepted that the primary determinant(s) of activity are the endotoxin(s) themselves (Table 3). The number of endotoxins produced, their relative amounts and the inherent capacity of the strain to produce them greatly influence toxicity. For example, Agree^{R} produces three endotoxins, each has its own activity spectrum and each is made in different amounts (Table 4). The activity of Agree^{R} is due to the combined activities of all these endotoxins. For some insects, it is also clear that spores contribute to toxicity. The spore contribution is probably due to septicemia and production of degradative enzymes and / or other toxins in the insect's gut when the spores germinate and grow. Apparently, the spores act synergisticly with the endotoxins because the spores have little or no effect by themselves.

Before 1982, all commercial *Bt* product were based on strains isolated directly from nature. These strains were selected because they had good activity on a variety of commercially important insects and they could be easily fermented and formulated. Table 5 lists the endotoxin genes present in several commercial products. Different levels

of expression of the various endotoxin genes are apparent and some of the endotoxin genes are known to be silent. A few other endotoxin genes, e.g. cryIIB, are probably also not expressed. Because the activity spectra of each endotoxin is different, a strain producing multiple endotoxins possesses a broader spectrum of activity than each individual endotoxin. Attempts to modify and improve the performance of commercial *Bt* strains has focused on (1) increasing the amount of endotoxin produced and (2) altering the types of endotoxins produced. Using the techniques of genetic engineering, it may be possible in the future to modify the endotoxin itself and increase toxicity and/or alter host range.

It has been possible to improve the insecticidal activity of some *Bt* strains by eliminating genes that code for endotoxins that are not highly active on the target pest(s) and by introducing endotoxins that have better activity. *Bt* strains can be manipulated in this way because most if not all endotoxin genes are present on plasmids that replicate as discrete circular DNA molecules. Unwanted endotoxin genes can be eliminated using standard techniques of plasmid curing and desirable endotoxin genes can be introduced by transferring a toxin-encoding plasmid from a donor strain to a recipient strain by sexual conjugation. Several commercial products have been produced using these techniques (Table 5).

An illustration of how such a strain was constructed is shown in Figure 2 for Agree, which is marketed by Ciba-Geigy. Agree was produced by curing a plasmid from HD135 that carried a cry1A(b) gene. The resultant strain (HD135-S4) was used as a recipient in a sexual mating with HD191, which contains a cry1A(c) gene on a transmissible plasmid. The resultant strain(GC91) is the strain present in the Agree product. The biological activities of GC91 and the strains used in its construction are shown in Table 6. As a result of these genetic manipulations, GC91 possesses the most desirable activities of both HD191 and HD135. The activity of GC91 on all insects listed in Table 5 is as good or better than either parent.

Unfortunately, the techniques of plasmid curing and conjugation are of limited use for strain construction, primarily for three reasons. First, not all endotoxins are present on transmissible plasmids and, therefore, cannot be transferred at all, or at frequencies so low that the resultant transconjugants cannot be detected. Secondly, many *Bt* plasmids contain multiple endotoxins. In these cases, it is not possible to use plasmid curing to isolate a desirable endotoxin gene from an undesirable one. For example, the plasmid locations of the endotoxins present in HD1(the Dipel strain) are shown in Table 7. The cry1A(b) gene in HD1 is the only gene present on the 44 Mdal plasmid. This plasmid is

transmissible and can readily be introduced into recipient strains by conjugation. All of the other endotoxin genes in this strain are present on a 110 Mdal plasmid which can be cured but this plasmid is not transmissible by conjugation. Thus it would be impossible to use any one of the endotoxin genes on this plasmid in strain construction without using all of them. Finally, plasmid incompatibility will in some cases prevent certain endotoxin-encoding plasmids from replicating in the same cell. Another limitation of conjugation is that some transmissible plasmids carry multiple endotoxins and/or B-exotoxin. Strains for commercial use in the United States cannot produce B-exotoxin (a small molecular weight, heat stable toxin) because it is teratogenic in mice.

Current Strategies for Construction of *Bt* strains. Construction of *Bt* strains for future commercial use (at least in the United States) will undoubtedly rely on the use of recombinant DNA technology. Some general strategies, their advantages and disadvantages, for producing non-recombinant and recombinant *Bt*9r-*Bt*) strains in Table 8 and 9 respectively. The main advantages of the non-recombinant approach is that the endotoxin genes are likely to be stable and the registration process is well established. With recombinant techniques, the number, type and level of expression of all endotoxin genes present in the strain can be controlled. The ability to clone a minimum DNA fragment that onluy codes for the endotoxin gene of interest makes it possible to utilize genes that are closely linked to the genes for B-exotoxin production. A very important aspect of r-*Bt* strains is that they can always be constructed in the same recipient strain. This has the advantage that fermentation and subsequent proceessing procedures do not have to be developed for each product as they could be different for natural strains and transconjugants.

Currently, efforts to commercialize r-*Bt* strains are focussing on the development of systems to combine native, unmodified endotoxin genes into a standard, well characterized recipient strain. The properties of such an ideal recipient strain for commercial production are listed in Table 10. In the long term, r-*Bt* strains may be developed with endotoxin genes that have been engineered to increase toxicity or affect host range. Until then, efforts to isolate and characterize native endotoxins with desirable properties (high toxicity, broad host range, etc.) is a high priority. Using modern molecular tools, native genes with desirable properties will be combined to produce first generation r-*Bt* strains.

Obtaining native endotoxin genes of interest is an area of considerable research. At least two approaches can be used to identify strains that contain novel and / or desirable, known endotoxin genes (Table 11). **Approach I** uses bioassay as the primary selectin criterion. Native strains are grown and individually assayed for the desired activity. Strains

promoted to the next stage are compared in a more laborious multi-dose bioassay where activity is normalised to the amount of endotoxin protein added. Strains that have the desired level of activity are analysed furthr with molecular probes for the presence of known or novel endotoxin genes.At this stage of strain selection, an accurate method for endotoxin quantification must be used to standardise the assay. The best method currently available uses polyacrylamide gel electrophoresis in the presence of sodium dodecyl-sulfate (SDS-PAGE). The procedure and its advantages are described in Table 13. Historically, the acitvity of a strain was determined relative to an International Standard (HD1). This method gives a relative activity between the test strain and the Internatinal Standard but it does not account for differences in the amount of endotoxin present in the test strain relative to the International Standard. Also, the International Standard cannot be used to evaluate toxicity on certain insect species where it has no activity. A more complete discussion of the use of the International Standard is given in Attachment I.

The level of interest in a particular gene can only be determined by bioassay of the isolated endotoxin in the absence of other biologically active compounds. Unfortunately, the vast majority of natural *Bt* strains express several endotoxin genes and they may also produce B-exotoxin. The presence of B-exotoxin can be easily determined by bioassay because it is heat stable and can be rapidly and effectively separated from endotoxin (Table 13). In addition this test shows whether a strain producing B-exotoxin also produces an active endotoxin. Such a strain would have activity in the washed pellet as well as the supernatant.

In order to determine the activity of a single endotoxin, it is necessary to isolate it from all the other endotoxins in the producing strain. Because highly related endotoxins (which often differ greatly in their toxicity profiles) cocrystallize, they usually cannot be biochemically separated in an active form. Thus endotoxins produced i the same strain often must be separated genetically in order to determine their activity spectrum. This is done by isolating the respective endotoxin genes and independently expressing them. Classical genetics techniques of plasmid curing and conjugation can sometimes be used but in most cases the genes must be separated by gene cloning. Because gene cloning is vey labour intensive, it is desirable to decide at an early stage if a strain contains a new and desirable endotoxin gene. if a strain has a unique or a particularly high level of activity, it can be further characterised using the molecular techniques described in Approach II. Thus the presence of an unusual sized protein or an unexpected reaction with gene-specific probes indicates the presence of unique endotoxin genes. After an

endotoxin is in pure form, it is necessary to precisely determine its activity spectrum. The endotoxins with desirable activities are then analysed in detail at the molecular level to differentiate them from known endotoxin genes. Finally, the cloned genes can be introduced into a recipient *Bt* strain i the desired combinations to produce a r-*Bt* for a specific application.

Approach II is similar to Approach I except that strains are initially screened with molecular hybridization probes to identify genes that are related to known genes with the desired activities. Table 14 shows the results of a screen with a crylB gene-specific hybridization probe. CrylB is known to be active on Colorado potato beetle and on diamondback moth (Table 2). Thus this screen might identify related genes that also have these activities and hopefully othe properties. Figure 3 describes the hybridization procedure and shows the results of screening over 5,000 native *Bt* strains with a crylB gene-specific hybridization probe. In general, DNA is transferred from native *Bt* isolates onto filter paper and hybridized with a gene-specific probe. This method can be used in a high throughput screen for large numbers of strains. Approximately 5,000 strains can be screened in two weeks by one person. Strongly bybridizing strains are then analysed by gene specific probes. Pairs of DNA primers can be made that will react specifically with each endotoxin gene and will generate a DNA fragment of a predicted size in a polymerase chain reaction (PCR). This predicted product will be formed only if a very highly related gene is present. The PCR negative strains or ones that produce a PCR product that is not predicted are of particular interest because these strains are likely to contain related and probably novel genes. The endotoxins from the strains of interest can be further characterised by SDS-AGE. Endotoxins that differ by us as little as 1 kilobase can be resolved on 4% polyacrylamide gels. Thus CrylB can be separated from all known endotoxins because it is largest of all known endotoxins (Table 15). Finally, bioassay on the target insect are used to determine if the strain has the desired level of activity. Selected strains are then used to isolate endotoxins by either classical or recombinant DNA techniques.

Endotoxins selected using Approach I and II might have increased toxicity and/or broader host range than the known endotoxins.Approach 1 is the most desirable because it relies on bioassay as the primary selection criterion. However, bioassay is very time consuming and sometimes the assays are difficult. In these cases Approach II is useful because it uses rapid, high throughout molecular screens for the initial strain selections and only in the final screen does it use labor intensive bioassays.

ATTACHMENT 1

Determing potencies of "spray-on" *Bacillus thuringiensis* bioinsecticides

Like most biological assays, assessing the insecticidal activity of endotoxins is intrinsically variable. Usually data can be compared within assay done on the same day with the same batch of insects but high day to day variability is common with the same endotoxin preparation assayed on the same insect species. Different endotoxin preparations may also show different levels of toxicity. These sources of variability can be normalised to some extent by comparison of the activity of the unknown endotoxins to the activity of a highly characterised stanard endotoxin preparation. Such a standard (HD1-S-1980) has been prepared by the USDA and is readily available. The use of this standard not only makes it possible to compare data obtained by the same researcher from day to day but it also allows different researchers to compare data obtained at different locations, at different times, by different bioassay methods. Because of the inherent variability of bioassays, it is necessary to perform repeat assays on isolated endotoxins with different preparations on different days before differences in activity can be delieved. It is clearly necessary to have a high degree of confidence in the activity spectrum of an isolated endotoxin before considerable effort is invested in its genetic isolation and characterisation. In contrast, a high degree of reliability is not required for an initial screen as described in Approach 1.

Internatinal units (IU) are often used to quantify the potency of insecticides with *Bacillus thuringiensis* (BT) as the active ingredient. IUs are defined as follows:

Potency of Test Sample (IU/mg) =

$$\frac{\text{LC50 Standard}}{\text{LC50 Test Sample}} \times \text{Potency Standard (IU/mg)}$$

Two International Standard have been prepared and their potencies are given below. These values were determined by bioassay against *Trichoplusia ni* (cabbage looper). HD1 = bacterial strain, S = Standard and the last four numbers give the year in which the standard was produced.

Standard	Potency (IU)
HD1-S-1971	18,000
HD1-S-1980	16,000

Using the latest Internatinal Standard, the potency of a BT powder would be defined as follows:

Potency of Test Sample (IU/mg) =

$$\frac{\text{LC50 HD1-S-1980}}{\text{LC50 Test Sample}} \times 16{,}000 \text{ IU/mg}$$

The primary shortcoming of this method of determining potency is that the activity is determined relative to *T.ni.* This is a problem because each endotoxin has a unique spectrum of insecticidal activity. Thus an endotoxin that has low activity on T.ni would have a low number of IUs/mg of powder bu tit could be very active on a different insect. An example of this would be an endotoxin active on Colorado potato beetle. This material would have zero IUs of activity as determined on *T.ni.* In addition, many of the endotoxins that are active on T.ni have little activity on *Spodoptera* species. Standard toxin preparations with good activity on *Spodoptera* and CPB have been prepared. The activity of test samples relative to these standards gives activity in *Spodoptera* units and CPB units, respectively. The standards for *Spodoptera* and CPB were prepared Mycogen Inc. It is not known if these standards are available for comparative analyses.

Using units of activity relative to a standard can greatly underestimate the potency of a preparation if the target insect is different from the insect used to for the standard. That is, the activity on T.ni does not predict the activity on other lepidopterian insects, e.g., *Plutella xylostella* (Diamond back moth) and *Heliothis zea* (cotton bollworm or corn earworm). One insect cannot be used to predict the activity on another because each endotoxin has different activities on different insects. The situation is complicated further because most natural isolates and all strains used in commercial products for Lepidoptera produce multiple toxins which may be present in different amounts. Thus each toxins contribution to the overall toxicity is due to its activity spectrum and the amount or proportion of it in the product.

For example, four toxin proteins are synthesized by the strain HD1, whcih is used to produce both the Internaitonal Standard and Dipel^{TM,} Abbott Laboratories Inc. These toxins are all present in different amounts and each has a different activity on cabbage looper. One or two possess good activity on cabbage looper and the others are only weakly active. Furthermore, the amount of each toxin produced can vary from batch to batch depending on the growth conditions and media components used. This could result in batches of HD1 with different potencies on *H. virescens* (for example) but having the same number of IUs.

Since most commercial BT products are used for more than one insect species, IUs are not an appropriate measure of their potency. This is particularly true for those BT products that are designed to control specific insects. These products may be very

effective on their target insects but their activity measured in IU could be very low if they have poor activity on cabbage looper.

Ideally, the activity spectrum and amount of each toxin should be determined in order toa ccurately assess the potency of a BT product. Unfortunately, the necessary analytical methods are poorly developed, time consuming and technically difficult. At present, the total amount of toxin protein (usually expressed as a weight percent) is used as a measure of the amount of active ingredient present. Although the percen toxin protein is related to the potency of the product, it does not indicate how effectively it will kill a given target insect nor does it measure the amount of active toxin present. Toxin inactivated during spray drying or formulation would still be measured by methods measuring total toxin protein.

Determining the amount of toxin protein in a technical powder or formulation can be difficult and subject to error. This is primarily due to very crude fermentation media in which the bacteria are grown. During growth, the insoluble protein present in crude fermentation media is digested by production of large amounts of bacterial proteases. When toxin crystals are solubilised for quantitative analysis, the toxin can be rapidly degraded unless special precautions are taken. In addition, formulation ingredients may also interfere with the analysis. There are various methods used throughout the industryto quantify the amount of toxin protein in formulated product. Unfortunately, these methods are not standardized. Thus the amount of toxin protein measured in a given sample may be different with two different methods. The best method described to date invovles SDS-PAGE which is summarised in Table 12. Historically, bioassay relative to an international standard was used to determine the activity of *Bt* strains (Attachment I). This method is still useful for some purposes but has clear limitations and is often of little value.

Table 1. Criteria for classification of Bt endotoxin genes

Endodotoxins Grouped by Amino Acid Identity[1]	Biological Activity
CryIA(a), CryIA(b), CryIA(c)	Lep
CryIB	Lep, Col
CryIC	Lep
CryID	Lep
CryIE(a), CryIE(b), CryIE(c)	Lep
CryIF	Lep
CryIG	Lep
CryIH	Lep
CryIIA, CryIIB, CryIIC	Lep, Dip
CryIIIA	Col
CryIIIB, CryIIIB2	Col
CryIIIC(a), CryIIIC(b)	Col
CryIIID	Col
CryIVA	Dip
CryIVB	Dip
CryIVC	Dip
CryIVD	Dip
CryV	Lep, Col

Lep = Lepidoptera active
Col = Coleopteran active
Dip = Diptera active

[1]Genes listed horizontally are very highly related. Genes grouped within a given primary class differ by more than 10%. Genes within a primary group, e.g., CryI-type, are less related and genes in different primary groups, e.g., CryI-type compared to CryII-type are generally less related.

Table 2. Relative activities of -endotoxin gene types on selected insects.

Endotoxin	Px sen	Px res	Se	Sl	Tn	Hv	Hz	On	Ms	Ldis	Pb	Ld
cryIA(a)	+++	.	-	-	+	++	.	++	+++	.	+++	.
cryIA(b)	+++	-	-	-	+++	++	++	++	+++	+++	+++	.
cryIA(c)	+++	.	-	-	+++	+++	+	++	+++	+++	+++	.
cryIB	+++	+++	.	-	.	++	.	[++]	-	.	+++	.
cryIC	++	++	[++]	++	.	-	.	.	++	.	+++	.
cryID	-	-	.	-	.	≤+	.	.	+++	.	≤++	.
cryIE	-	-	(++)	++	.	-	.	.	++	.	.	.
cryIF	++	.	-	.	.	++	.	++	.	.	.	.
cryIIA	.	.	.	.	++	++	++	++	+++	+++	.	.
cryIIB	.	.	.	.	++	++	++	+	.	+++	.	.
cryIIC	.	.	.	.	.	.	.	.	.	.	.	.
cryIIIA	.	.	.	.	.	.	.	.	.	.	.	++
cryIIIB	.	.	.	.	.	.	.	.	.	.	.	+
cryIIIB2	.	.	.	.	.	.	.	.	.	.	.	++
cryIIIC(a)	.	.	.	.	.	.	.	.	.	.	.	++
cryIIIC(b)	.	.	.	.	.	.	.	.	.	.	.	+
cryIIID	.	.	.	.	.	.	.	.	.	.	.	(+++)
cryV	.	.	.	.	.	.	.	++	.	.	.	++

blank = data not available
Px sen = *Plutella xylostella* (diamond back moth) sensitive to Dipel
Px res = *Plutella xylostella* (diamondback moth)resistant to Dipel
Se = *Spodoptera exigua* (beet armyworm)
Sl = *Spodoptera litoralis* (cotton leafworm)
Tn = *Trichoplusia ni* (cabbage looper)
Hv = *Heliothis virescens* (tobacco budworm)
Hz = *Helicoverpa zea* (cotton bollworm, corn earworm)
On = *Ostrinia nubilalis* (corn borer)
Ms = *Manduca sexta* (tobacco hornworm)
Ldis = *Lymantria dispar* (gypsy moth)
Pb = *Pieris brassicae* (European cabbageworm)
Ld = *Leptinotarsa decemlineata* (Colorado potato beetle)

[a]Relatives activities are estimates made from published values or from our unpublished data [data in brackets]. The activities are defined as follows:

+++ = $LC50 \leq 10$ ng/cm^2 or for Pb < 1 g/ml
++ = 10 ng/cm$^2 \leq LC50 \leq 100$ ng/cm^2 or for Pb $1 \leq LC50 \leq 10$ g/ml
+ = 100 ng/cm^2 < LC50 < 1000 ng/cm^2 or for Pb 10 < LC50 < 100 g/ml

Table3. Endotoxins in Agree (GC91)

Endotoxin	[a]Insecticidal Activity	MW (Mdal)	Proportion
cryIA(c)	^{1}Px(s), ^{1}Tn, ^{1}On, ^{1}Hv, ^{2}Hz	131	50%
cryIC	^{1}Se, ^{1}Tn, ^{3}Hv, ^{3}Px(s), ^{3}Px(r).	132	40%
cryID	^{1}Se	129	10%
cryIA(a)	^{1}Px(s), ^{1}Tn, ^{1}On, ^{2}Hv	131	silent

[a]Px (s) = *Plutella xylostella* (diamond back moth) sensitive to Dipel
Px (r) = *Plutella xylostella* (diamondback moth)resistant to Dipel
Se = *Spodoptera exigua* (beet armyworm)
Tn = *Trichoplusia ni* (cabbage looper)
Hv = *Heliothis virescens* (tobacco budworm)
Hz = *Helicoverpa zea* (cotton bollworm, corn earworm)
On = *Ostrinia nubilalis* (corn borer)

1 = $LC_{50} < 1$ ng/cm^2
2 = $1 < LC_{50} > 10$ ng/cm^2
3 = $10 < LC_{50} > 50$ ng/cm^2

Table 4. Toxicity determinants in Bt strains

Endotoxins:
Number Of Endotoxins Produced
Activity Of Endotoxins
Amount Of Endotoxin Made
Effects May Be Additive Of Synergistic
Spores:
Septicemia
Production Of Lipases, Proteases Etc.

Table 5. Endotoxins present in commercial products and their proportional amounts.

ENDOTOXINS PRESENT IN COMMERCIAL PRODUCTS			
Product	Company	Endotoxin Gene	% Protein
[b]Agree	Ciba-Geigy	cryIA(a)	ND
		cryIA(c)	50
		cryIC	40
		cryID	10
Xentari	Abbott	CryIC	50
		CryIA(a) + CryID	50
		CryIA(b)	ND
Dipel 2X	Abbott	CryIA(a) + CryIA(c)	90
		CryIA(b)	ND
		CryIIA/[a]B	10
Javelin	Sandoz	CryIA(a) + CryIA(c)	30
		CryIA(b)	60
		CryIIA/[a]B	10
[b]Cutlass	Ecogen	CryIA(a) + CryIA(c)	85
		CryIA(b)	5
		CryIIA/[a]B	10
[b]Condor	Ecogen	CryIA(a) + CryIA(c)	90
		CryIIA/[a]B	10
Biobit	Novo	CryIA(a) + CryIA(c)	90
		CryIA(b)	ND
		CryIIA/[a]B	10

Table 6. Insecticidal activity of GC91 and its parents

Insect Species	LC50 (ug bacteria per gram diet)				
	HD1	HD191	HD135	HD135-S4	GC91
Galeria mellonella	2,600	3,500	20	64	18
Heliothis armigera	42	48	230	850	44
Heliothis virescens	9	6	200	>2,000	5
Spodoptera littoralis	5,700	10,000	450	690	330
Pieris brassicae	1	1	1	>100	1
Mamertra brssicae	1,500	>10,000	190	280	160

Table 7. Plasmid location of endotoxin-encoding genes in HD1

Plasmid (Md)	Toxin
120	
110	.....Cry IAa, Cry IAc, Cry IIA, Cry IIB (silent)
52	
44	.....Cry IAb
29	
10	.....Linear DNA element
9.3	
5.4	
5.2	
4.9	
1.4	

Table 8. Non-recombinant Strain Construction.

Non-Recombinant Strain Construction
*identify highly active, unmodified strain from nature *use plasmid curing and conjugation
Advantages: *Genes are likely to be stable in the absence of selection *Registration process is well established
Disadvantages: *Fermentation and formulation procedures will be strain specific *Limited capabilities for construction highly active strains

Table 9. R-DNA Approaches for Strain Construction.

Recombinant Strain Construction
*clone desired endotoxin genes on plasmid vectors *Introduce cloned genes into existing plasmids or the chromosome *Introduce cloned genes into a universal fermentation strain *Strains cannot contain antibiotic resistance markers
Advantages: *Any combination of genes is possible *Level of gene expression can be controlled *Toxin itself may be altered to increase toxicity or alter host range *Consistent fermentation is possible *Exotoxin can be eliminated
Disadvantages: *Genes may be lost from strain in the absence of selection *Unknown requirements for registration of recombinant Bts

Table 10. Properties of a universal recipient strain.

*Transformability
*Good donor and recipient in matings
*Absence of and resistance to phage
*Good toxin expression
Laboratory
Commercial conditions

Table 11. Strategies for strain selection and construction.

I. Select Strains With Activity > Standard
Molecular Analysis Of Endotoxins And Genes Present
II. Molecular Based Screen
Probe For Presence Of Genes Similar To Known Types
PCR To Identify Novel Genes
Determine Activity Of Strain
Clone Novel Genes

Table 12. Quantification of endotoxin by SDS-PAGE

- Suspend powders or cell paste suspended in dH20 at 10 mg/ml or 20 mg/ml, respectively
- Adjust to 0.1 N NaOH (Inhibits proteases)
- Incubate 5 minutes at room temp.
- Add 65 ul of 3X Laemmli buffer
- Remove 50 ul, heat 100C, 5 minutes
- Centrifuge, load 10 and 5 ul.
- Electrophoresis at 125V, 90 minutes (SDS, TRIS-Glycine buffer)

Advantages of SDS-PAGE method for endotoxin quantification.

Toxin must be solubilized for all existing assays.

Proteases must be inhibited or removed

*Washing with high salt. (loss of material, not effective)

*Protease inhibitors. (Not effective)

*High pH. (Probably best)

SDS-PAGE seems to be the most reliable.

*Minimal sample manipulation

*Effectively inhibits proteases

*Protein LC50 is same for crude samples and purified crystals

*Protein LC50 is same for purified crystals using BioRad protein

Table 13. Detection of exotoxin in culture broths of Bt cultures.

*GROW CULTURE TO STATIONARY PHASE			
*CENTRIFUGE	SUPERNATANT	BIOASSAY HEATED NOT HEATED	HPLC
	PELLET WASH 2X CULTURE VOLUME	BIOASSAY HEATED NOT HEATED	

Table 14. Molecular screening of native based Bt strains for cryIB-like genes.

ANALYSIS	PCR SCREEN for cryIB	
	PCR -	PCR +
*Hybridization +	58	12
*SDS - PAGE		
138 kDa	34	
slightly < than 138 kDa	2	
slightly > than 138 kDa	4	
*No protein at 136-142 kDa protein	18	None
*Activity ≥ Agree (Colorado potato beetle)	4	6
*Exo. Neg.	1	3

Table 15. Endotoxin molecular weights

TOXIN	MOLECULAR WEIGHT (kD)
cryIAa	130.6
cryIAb	128.3
cryIAc	130.9
cryIB	136.4
cryIC	132.1
cryID	129.4
cryIEa	130.2
cryIEb	130.6
cryIF	130.5
cryIG	128.6
CryIIA/B	65
CryIIIA	70
CryIIIB/IIID	74
CryIIIB2	74
CryIIIC	133
CryIIID2	72
CryV	81

Figure 1. Amino acid homologies between most known endotoxins

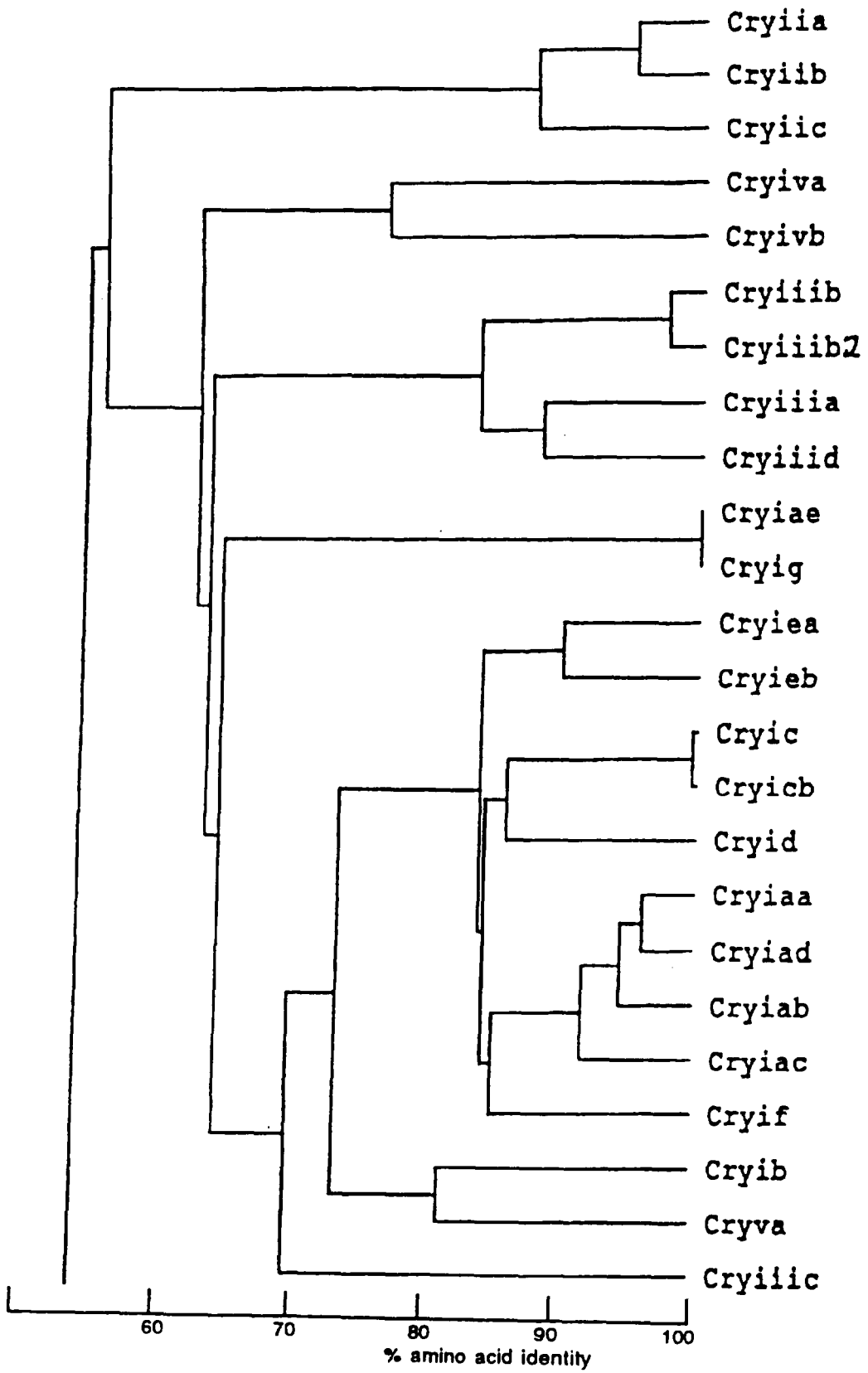
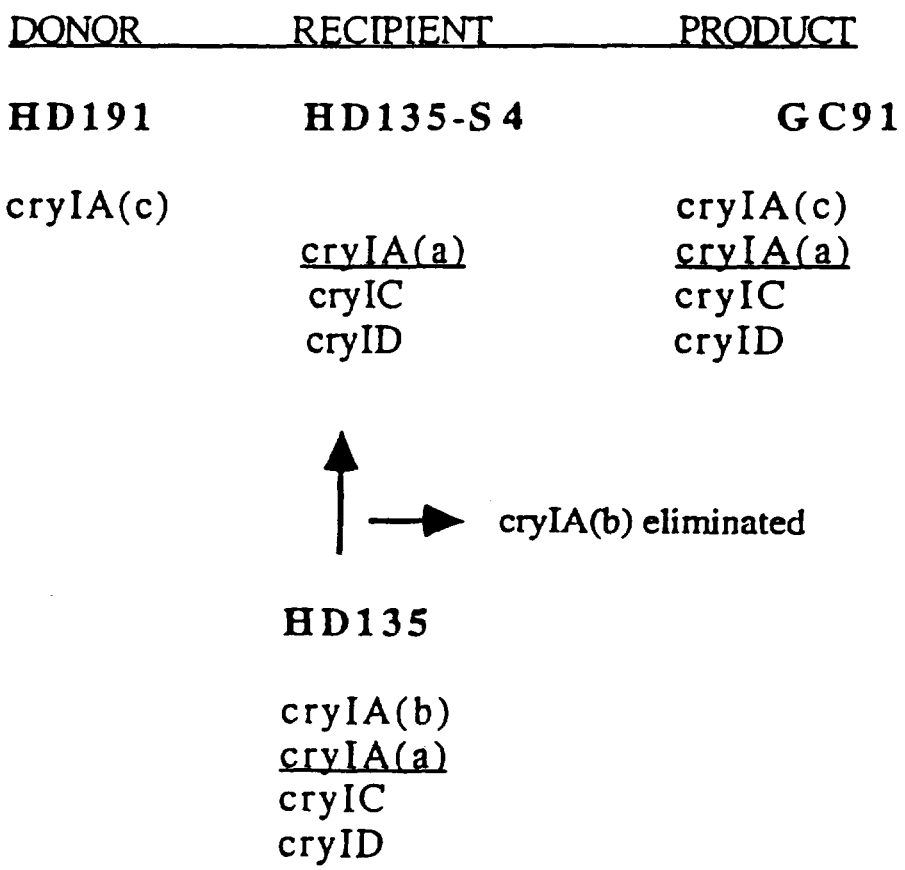


Figure 2. Construction of GC91.



GENE	MW	HOMOLOGY DNA	PRO	CPB ACT	COMMENTS
cryIIIA	70	100	100	++++	Current products
cryIIIB/IID	74	75	81	++	Ecogen
cryIIIB2	74	73	81	++++	Ecogen, SCRW
cryIIIC	133	42	58	++	PGS, Protease treatment
cryIIID2	72	81	86	+++++	PGS, Protease treatment
cryV	81	55	59	++	Lep and Col Activity
cryIB	136	39	58	?	Protease treatment

Figure 3. Hybridization of cryIB probe to native Bt isolates

CHARACTERIZATION OF STRAIN COLLECTION

Inoculate Strains Into 96 Well MICROTITER Dishes

Grow to Sporulation and Store at 4C

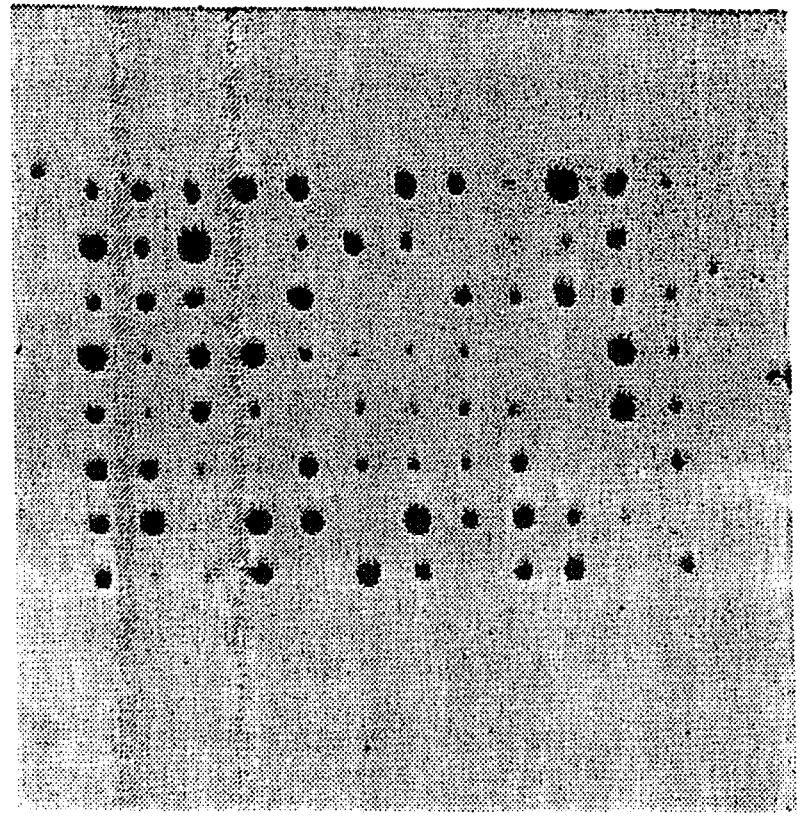
Use Master Plates to Inoculate Agar Plates

Grow Overnight

Transfer DNA from Colonies to Membranes

Hybridize with Gene Specific DNA Probes

Characterize Positives
- PCR Analysis
- SDS-PAGE
- Bioassay



VII. CONCLUSION

The delegates from the member countries of the network appreciated well the proceedings of the Workshop on Production and Quality Control of Bio-pesticides (*Bacillus thuringiensis*) covering practically all aspects including biological properties, molecular biology, management of insect resistance to Bt, fermentation technology, quality control of Bt products, industrial production, application of Bt for control of agriculture, forest and public health pests, mechanisms of action of Bt etc. The delegates particularly appreciated the excellent mix of lectures and laboratory practical and field testing which gave them an opportunity to understand the intricacies involved in the production and quality control of Bt based pesticides. The significant role that Bt can play in the Integrated Pest Management Programme in the member countries of the network was endorsed by the delegates.

VIII. RECOMMENDATIONS

1. Having discussed at length the various aspects of Bt development, production and usage and recognising the need for pilot scale production and quality control of Biopesticides (*Bacillus thuringiensis*) the workshop

   recommends UNDP/ UNIDO/ RENPAP through Bt R&D Center, Wuhan extend all technical assistance to member countries to create pilot scale & industrial scale facilities for the commercial production of biopesticides in the member countries.

2. Having been offered excellent facilities and expertise of Bt Research & Development Centre, Hubei Academy of Agricultural Sciences, Wuhan, the workshop

   recommends that interested network member countries may set up joint venture with the Hubei Academy of Agricultural Sciences for transfer of technology for commercial scale production of Bt and its usage in IPM programme through the assistance of UNDP/ UNIDO/ RENPAP.

3. Having recognised the need for improved formulations of Bt based pesticides for the better efficacy and shelf life and having considered the offer of Govt. of P.R. China through country project "Sustainable Pest Control & Soil Fertility Programme" and National Coordinating Unit of RENPAP, the workshop

   recommends that newer formulations of Bt based formulations may be

developed and made available to the member countries to improve the field performance of biopesticides.

4. Recognising the need of protocols and guidelines for testing the bioeffectiveness and data generation for registration purposes of Biopesticides, the workshop

recommends that UNIDO/ RENPAP extend assistance and develop protocols and guidelines for large scale field demonstration of Bt pesticides for the member countries of the network.

5. Having noted the availability of different strains of Bt in the member countries and the need for collection and exchange of information for promoting Bt based pesticides, the workshop

recommends that UNDP/UNIDO/ RENPAP extend assistance in screening of various strains of Bt and strengthen exchange of information on production and quality control of Biopesticides through establishment of a Databank and regular publication of bulletins for the benefit of the member countries of the network.

IX EVALUATION OF THE WORKSHOP

On the last date of the workshop an evaluation was carried out. The participants evaluated the workshop to be quite informative and useful. They felt that the aspects of production and quality control of bio-pesticides were very well covered and adequate "hands-on" training has been provided in course of the workshop. The participants felt that they were not only benefited but also would be in a position to take lead roles in their respective countries for promoting production and use of Bt based pesticides.

Annexure - I

LIST OF PARTICIPANTS

Country Delegates

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Annexure - II

Workshop on Production and Quality Contorl of Bio-pesticides (B. *thuringiensis*), Wuhan, P.R.China

Programme

October 31, 1995	Registration Inaugural Session - The Opening Ceremony Tea/Coffee Break Country Papers Lunch Technical Session Biological Property of Bt
November 1, 1995	Technical Session Molecular Biology of Bt Lunch Management of Insect Resistance to Bt
November 2, 1995	Technical Session Fermentation Technology of Bt Lunch The Assay of Quality Control of Bt products
November 3, 1995	Technical Session Industrial Production of Bt Lunch Application of Bt in controlling insects
November 4, 1995	Technical Session Experimentations * Isolation * Shake-Flask Fermentation, * Bioassay * Field Test
November 5, 1995	Site seeing (Sunday)
November 6, 1995	Technical Session Mechanisms of Action of Bt Lunch Checking Results and Discussions
November 7, 1995	Technical Session Bt for Mosquitoe Control Lunch Checking Results and Discussion
November 8, 1995	Visiting
November 9, 1995	Evaluation of Workshop Recommendations Closing Ceremony

UNIDO Comments

The document,prepared based on the proceedings of a workshop organised in China in collaboration with Bt Research & Development Centre, Wuhan, China, only one of its kind in Asia, devoted fully for the development of Bt. The workshop is the result of concerted efforts between the Centre with its excellent staff and the industry in the West and RENPAP supported by UNDP and UNIDO.

The hands-on training clearly brought about the research side, bio-assay, quality control, production, formulation and packaging. To have all this in place in a developing country is unique. On account of its importance, the participating countries paid their own travel costs and the hosting country took care of the training cost. The response from the country is clearly indicative of the importance given to Bt and the awareness to combine bio-pesticides and synthetic ones complementing IPM. The recommendations clearly show that the countries of RENPAP need additional resources in capacity building to promote the use of bio-pesticides. Bt gives an ideal opportunity since this is the only bio-pesticide making a great impact and future modifications of the genes will bring great dividends for safe crop protection both in the developed and developing World.