PRODUCTION OF BIOPESTICIDES : SCALE UP AND QUALITY ASSURANCE

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ABSTRACT

The role of microbial insecticides has expanded considerably with the rapid development of IPM concepts and environmental legislation. Viral and fungal products represent a good potential, but their expansion on markets is slow, mainly because scaling-up of industrial production and standardisation of quality control methods still require more expertise. Mass production for Codling moth virus and *Beauveria bassiana* is now industrialised and production at a competitive cost with technical improvements are discussed.

INTRODUCTION

IPM strategies, in which reduced pesticide usage combined with use of natural enemies, (parasites, predators and pathogens) and other biotechnical tools such as semiochemicals, are one important aspect of sustainable agriculture.

Under natural conditions, entomopathogens are often responsible for drastic reduction in pest populations, thus regulating biological balance and therefore inundative sprays in order to produce massive epizootics have been the main objective of biocontrol promoters.

Although several pathogen groups : bacteria, fungi, viruses, protozoa ... have been found to be very effective in arthropod pest control, very few are presently in commercial production.

The most widely used are bacteria, mainly *Bacillus thuringiensis* isolates which are active against numerous crop insect families. On total annual world-wide biopesticide sales, *B.t.* based products represent around 90%, whilst the total biopesticide market is developing more slowly than predicted with 1% only of total pesticide world wide sales (26 billion USD).

Fungal and viral products are now under intense development because, despite their rather high selectivity, new factors must be considered : *B.t.* future, improvements in industrial production of fungi and viruses ...

B.t. situation

The main reason for *B.t.* predominance in biocontrol is that it is easy to produce by simple fermentation techniques in relatively inexpensive media for controlling a broad range of hosts. Nevertheless, *B.t.* products present a risk in the near future : because they normally act as stomach toxins, a current concern is the development of resistance to its protein toxins in target pests. Since 1985 many cases of resistance have been reported in various countries. In addition, rapid commercialisation of transgenic plants which produce *B.t.* toxin will provide new sources of resistance opportunities.

On the other hand, although *B.t.* spores may germinate and replicate in dead insects, replicating in hosts does not normally occur under field conditions.

For such reasons, other pathogens such as fungi and viruses which are able to self-replicate in hosts offer a good alternative to *B.t.*.

The key point is that industrial production has recently progressed, scaling-up for producing large cost competitive quantities has recently been achieved.

This paper describes two examples of the principal problems of industrial production : scaling up and quality assurance for Codling moth granulosis virus and *Beauveria sp.* fungal products.

I SCALE UP AND QUALITY ASSURANCE FOR ENTOMOPATHOGENIC FUNGI

Fermentation systems have been developed to produce different fungi such as : *Beauveria sp.*, *Metarhizium sp.*, *Paecilomyces sp.*, *Verticillium sp* ...

Various technologies are used, the most common being liquid fermentation, solid surface fermentation, sterile bag production on rice etc ....

In order to produce large quantities of *B. bassiana*, for treatment of hundreds of thousands of hectares, the system of mass solid fermentation has been scaled up.

The idea was to produce a solid medium colonised by sporulating mycelium and ready-to-pack as granule formulation or dry spores for liquid formulation (for example, *Metarhizium flavoviride* spores for locust control), at a cost competitive price.

1. Selection of strains

Many strains of *Beauveria* have been described for their high pathogenic power. The main parameters for mycoinsecticide production can be summarised in Figure 1.

This is the case of *B. bassiana* 147 (INRA France) for European corn borer control or *B. bassiana* (USDA strain) « Naturalis ™ » for white fly control.

2. Choice of technology

a. Laboratory work : Mostly dedicated to optimisation of C.N. ratio and components of nutrient medium and then selection of cheapest ingredients.

b. Determination of final product :
Process has been designed towards the following targets :
- Fermentation in mass forced ventilation operated system
- Non sterile process
- Finished product must be either a ready to use granule or pure spores for liquid formulation.

c. Technology developed : This technology was originally scaled up from INRA patented process to obtain clay microgranules colonised by sporulating mycelium.

Advantages

- Conidia produced on solid media are more resistant than those produced in liquid
- Forced aeration gives more rapid sporulation for B.b. 147 :
  - 7 days by static process
  - 2 days in process developed
- Mass production is high, giving better yield than surface culture
- Production and formulation of biomass is made in one sole operation
- Biomass is protected from stress inside granules
- Long storage life, see Fig 2, and no loss of pathogenicity (dried spores can lose 50% in 3 months)

3. Scale up : From Figures N° 3 and 4

The most important parameters are :

a. Building design

- Closed building with air filtration and regular prophylactic methods, decontamination of all equipment, floors and walls and measures for personnel such as showers, sterilised clothing etc.
- Forced aeration. This is the key point in the process : air flow at constant humidity and temperature regulate the entire formulation system for optimum spore production.

b. Preparation of culture medium

- Mixing inoculum, nutrient medium and clay granules : Blending system must give perfect homogeneity in order to give good colonisation and perfect air permeation
- Optimising size of batch in order to maintain minimum « sterility ».

c. Harvesting product

- Decaking granules without damaging biomass
- Protecting staff from possible allergic reactions
- Homogenising final product before packing.

d. Final pilot plant

Design of fermentation vessel

- Volume limited to 1.000 litres

- Battery of reactors : each reactor is independent and auto-regulated by computerised system. This limits losses in case of contamination or breakdown.

- Each reactor is composed of 6 plates with individual air flow system allowing a sporulation in mass up to 20 cm deep.

- Equipment constructed using food grade stainless steel and forms a closed vessel when operated.

- Air flow at 200 m^3/h is controlled by computer through electronic valves and probes.

- Pilot plant is composed of five individual units with a total capacity of 5.000 kg production in five days, two days for sporulation, three days for drying and packing. (Fermentation parameters and temperature are not disclosed).

e. Finished product

Final product is a granule of *B. bassiana* strain147 containing minimum 4 x 10^8 spores/gram. Residual humidity is maximum 10%.

f. Final cost

For this production, final cost established for 100 MT production run (4 months) is as follows :

	FF/kg	USD	%
Raw materials :			
Clay granules	1.50		
Nutrient solution	0.30		
	1.80	0.34	30 %
Energy and prophylaxy	2.20	0.40	38 %
Manpower			
Production 4 hours/MT	0.30		
Quality assurance	0.50		
	0.80	0.15	13 %
Cost of production	**4.80**	**0.89**	
Plus depreciation	1.10	0.20	19 %
	5.90	**1.09**	

Depreciation is estimated for a plant with capacity of 500 MT/year of granules, i.e. 500.000 FF/year or 90.000 USD.

4. Quality assurance

As with laboratory production, industrial production must respect basic principles for quality of microbial pesticides.

Controls are made at each stage :

- Inoculum : purity, density, stability
- Ingredients : Specifications, purity (contaminants)
- Growing biomass : Temperature, humidity, mycelium, growth, sporulation (Fig 5).

On final product :

Lack of standardisation is a major problem. Internal procedure is :

- Sampling method : 1 sample/plate or every 100 kg
- Viability of biomass : CFU determination (requires 3 to 5 days) (Figs 6 and 7)
- Enzymatic activity : Deshydrogenase activity evaluation (24 hours).
- Determination of LT 50 activity
- Contaminants : Because of non-sterile process, even with stringent conditions of prophylaxy, contaminants (mucorales and aspergillales are present). Standard limit for contaminants has been fixed at 10^6 CFU/g. On the other hand, absence of human pathogens must be verified for each batch.
- Storage stability of *B. bassiana* : The process provides granules with good shelf-life, efficacy when stored at ambient temperatures is maintained at least a year, and at 4°C, shelf-life exceeds two years (as per fig. 2).

II SCALE UP AND QUALITY ASSURANCE FOR CODLING MOTH GRANULOSIS VIRUS PRODUCTION

In the case of mass production of insect viruses, industrial production is now more advanced.

Various companies are producing the majority at semi-industrial level and using mass rearing technologies.

Scaling up industrial production of Codling moth virus has been achieved recently (1995) in France. Industrial production at a competitive cost is mainly a problem of automisation, as cost of manpower is very high in Europe.

1. General process

This cannot be changed and the production plant has four distinct sections :

- Artificial medium quality control and preparation
- Mass rearing unit from eggs to L4 larvae
- Virus production unit (contaminated area)
- Virus extraction, purification and formulation

Scaling up had already been made and a pilot plant erected at INRA France. The main improvements achieved on this preceeding pilot plant are

- Egg production
- Egg processing
- Purification, extraction and formulation

For egg production : special rearing cages were designed in order to obtain continuous and reliable egg production on paper sheets.

For egg processing : special blister tray forming equipment was installed which moulds plastic trays, distributes nutrient medium, and seals trays after egg deposition on medium.

For purification and extraction : Raw material obtained is a mixture of insect cadavers, nutrient medium and other related debris. Extraction and purification are mechanical : sieving and centrifugation, in order to obtain centrifugates with very high virus concentration.

2. Main production parameters are listed hereafter.

a. Basic data for design

- Total area of the plant including quality assurance laboratory : 2.000 m^2
- Total cost of plant : 16.5 Million French Francs (3 Million USD)
- Capacity of production : 60.000 doses

b. Production parameters for Codling Moth GV

- Final yield : 60.000×10^{13}
- Mean centrifugate concentration : 2×10^{14}/kg
- Average number of eggs to produce 10^{13} GV : 2.100
- Average number of dead larvae to produce 10^{13} GV : 800
  i.e. 1.25×10^{10} GV/larvae
- Percentage of production used to maintain mass rearing unit : 25%

3 Scaling up of the plant

Production 60.000 doses in 350 days

a. Detailed production parameters

- Eggs : 164 million units (430.000/day)
- Moths : (60 eggs/week/female) : 100.000 moths (50.000 females)
- Insect cages : 5.000/unit : 20

- Medium produced daily : 200 kg

b. Manpower required

- Mass rearing unit and nutrient production	3.5
- Harvest, extraction, purification	1.5
- Quality control	0.5
- Maintenance	0.5
	6.0 persons

c. Cost price of operating virus plant

For annual production of 60.000 doses

	FF	USD	%
Fixed costs :			
Fluids	290.000	53.000	8.0%
Maintenance	100.000	18.000	2.7%
Salaries	960.000	175.000	26.3%
Financial costs	950.000	173.000	26.0%
Depreciation	800.000	144.000	27.0%
	3.100.000	563.000	85.0%
Variable costs :			
For 60.000 doses ex plant	550.000	100.000	15.0%
	3.650.000	663.000	100 %
Cost price/ha approximately	**61.0 FF**	**11.0 USD**	

In EEC, average price/ha ex works for product is 140 FF or 25.5 USD.
Plant must produce and sell 48.000 doses in order to reach the profit threshold.

d. Cost improvement

Very few reductions are possible. One is replacement of Agar gel by new gelifying agents which will reduce price by 5%.

4. Quality assurance

Production is under continuous scrutiny for contaminants. The maintenance of prophylaxy is the major problem.

The production is subjected to quality control at two stages :

a. Centrifugate : In order to evaluate concentration in GV, molecular biology is used : internal procedure based on a Dot Blot related technique.

b. End product : As formulation is rather sophisticated and contains pH buffer, UV screens and enhancing factors, it is important to know the titration in biologically active granules. Standardised procedure which will be GLP approved by the authorities is now quite performant. It is based on the same biological titration method as for B.t. formulation : evaluation of LD 50 on host using different dilutions (6) and compared to a standard extract of known viral activity.

At this stage, the problem remains once more stability of standard.

Contaminants are checked using classic microbial techniques, bearing in mind that legal contaminant content must be maximum 10^6 CFU/ml, and no human pathogens must be detected. *Bacillus cereus* is a common gut bacteria in insects, representing 99% of total contaminations.

Final formulations contain common microbial antagonists (sorbic acid ...) which prevent any proliferation of contaminants.

c. Storage stability : One important point : product is stored always at below 10°C and under these conditions no degradation is detectable. Industrial production of virus is now on line, as production can easily be increased on the same site or transferred to foreign partners for local production.

CONCLUSION

We have tried to show how to scale up industrial production of fungal and viral insect pathogens. Due to biological diversity of candidate microbials, perfect scaling up needs to be highly pragmatic, combining insect pathology and microbiology with molecular biology and electronic expertise.

Industrial production cannot reach the same standards as laboratory production in terms of final purity, but the target being to produce a cost competitive product with fixed standards of quality, good manufacturing procedures with continuous quality control and tracability must be strictly observed in order to obtain a reliable end product.

In the near future, many microbial products (natural or G.M.) issuing from similar technologies will be marketed. These products will be produced by specialised companies who have succeeded in scaling up their own cost competitive technology.

Figure 1 : Main sélection parameters for hyphomycete strains

Pathogenicity	Physiology	Competitivity	Technology
Adhesivity to cuticle	Germination power	- on host sites - on crops - on soil	Good sporulation growing power
Enzymatic potential for cuticle degradation	Replication from host Stress tolerance, (humidity, UV)...		Good storage stability

Figure 2 : Storage life *Beauveria bassiana*

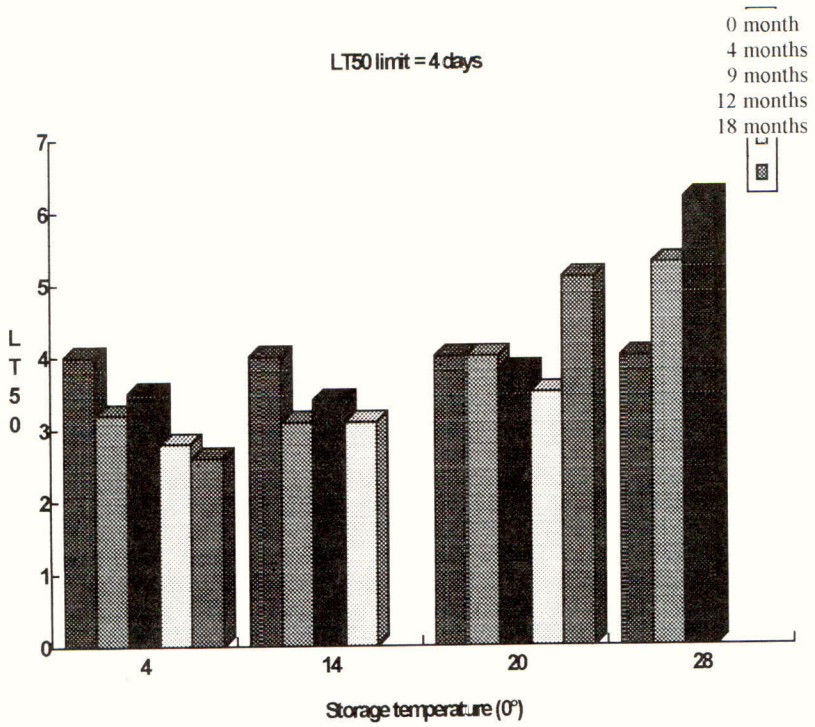


Figure 3 : *Beauveria* granule process

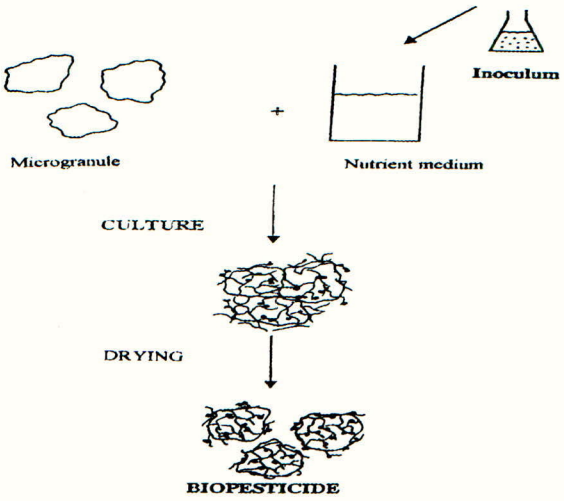


Figure 4 : Solid mass fermentation process

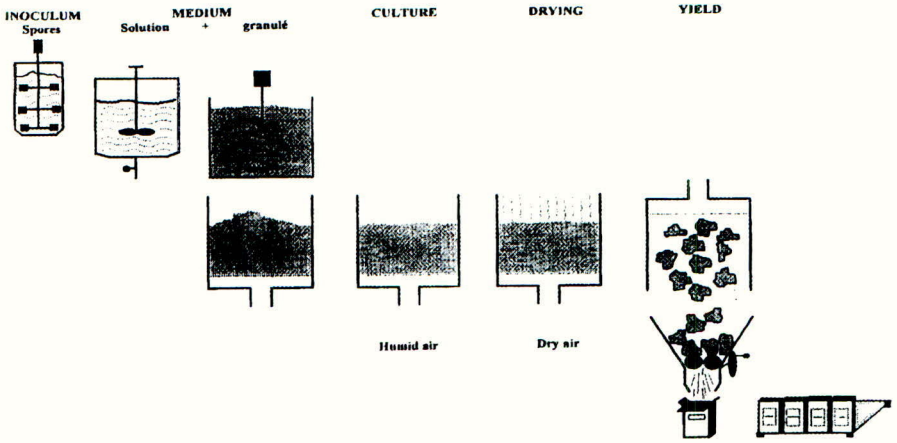
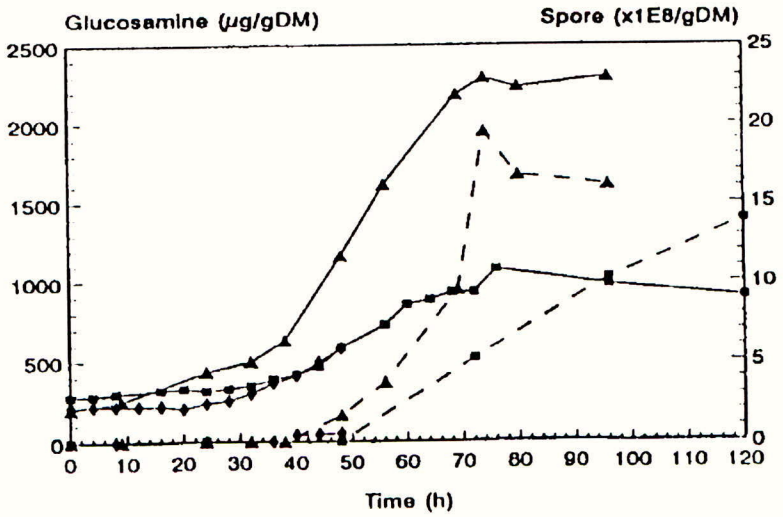


Figure 5 : Comparison of culture conditions in the three reactors

Culture condition		Reactor	
Reactor capacity (1) Reactor shape	0.5 Erlenmeyer flask	50 Cylinder 40 cm diam.	1600 parellelepiped Lxl = 2m x 0.8 m
Agitation device	No	No	Screws
Layer height (cm)	1	40	40
Aeration type	Passive	Forced	Forced
Air flow rate (ml/min.gDM)	ε	10	10
Initial water content	37.5	37.1	35.7

Figure 6 : Growth and sporulation



Growth (——) and sporulation (- - -) in R1 (■), R2 (▲) and R3 (♦).

Figure 7 : CFU variation before and after decaking

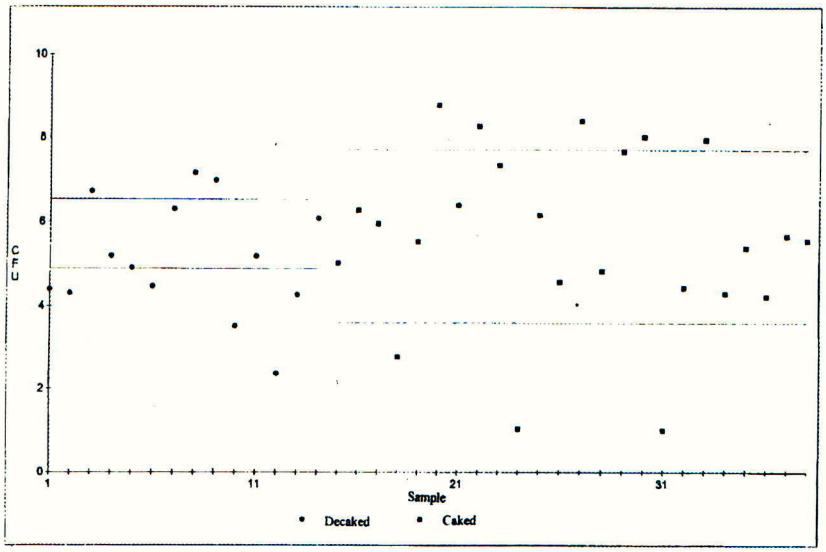
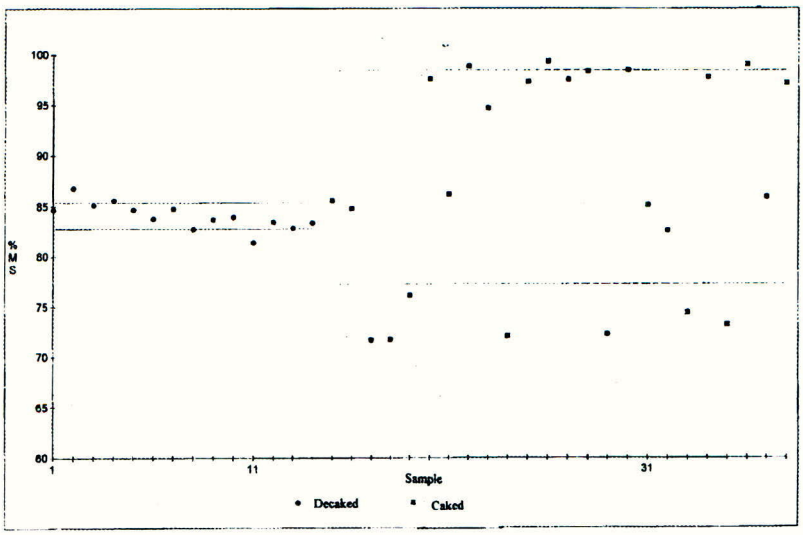


Figure 8 : % Dry matter variation



PRODUCT STABILITY: FROM EXPERIMENTAL PREPARATION TO COMMERCIAL REALITY

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ABSTRACT

Despite the innate stability of many insect pathogenic micro-organisms, the short shelf life of microbial insecticides is a limiting factor to their use. However, consideration of storage stability is often left to a late stage in product development. Shelf life can be improved through selection of species or strain, appropriate production methodology, post-production processing and formulation. The final formulated product often represents a compromise between optimising storage stability and optimising other properties required for successful and economic field use. If the right balance is to be achieved, storage properties should be studied early within a product development programme.

INTRODUCTION

The potential for microbes to control a number of insect pests has been reviewed by several authors including Burges (1981), Roberts *et al.* (1991) and Jones *et al.* (1993). Despite the demonstrable success of many microbial insecticides in the field, sales remain relatively small, accounting for approximately 2% of the total market. The reasons for this have been discussed by several authors and include availability, farmer acceptability and stability (Jones, 1994). Stability, either during storage prior to use or on the target following application, is a major concern with microbial pesticides and formulation plays a crucial role in helping to overcome this problem. This paper deals with the stability of the product between manufacture and eventual use. A later paper in this volume deals with stability and formulation post-application.

STORAGE REQUIREMENTS

The longest period of time in the life of a product elapses during storage. This period can range from several weeks to years. During this period the microbial agent must remain viable, with minimum loss of potency/activity. Moreover, there should be no loss or breakdown of the desired formulation properties, such as clumping and caking of powders, flocculation and settling of suspensions, or breakdown of additives that protect the microbe against environmental conditions post-application.

When initially developing a product, storage properties are often not considered until a late stage - experimental products are stored under controlled conditions, formulations can be prepared immediately prior to use and researchers are willing and able to follow difficult or awkward mixing and handling instructions. These options are rarely possible for commercial users. There are currently a number of commercially successful products which require special storage conditions, such as refrigeration, prior to use. In a few cases a more restrictive strategy has been adopted, for example, the bacterium *Serratia entomophila*, marketed as Invade®, is produced by small batch culture and the product is transported to the field in refrigerated containers by the manufacturer (Jackson, 1994). However, such approaches are likely to be limited to small markets (Rhodes, 1993). Special storage, transport and use conditions should not be regarded as an alternative to appropriate formulation (Jones & Burges, 1997).

Most currently available commercial chemical pesticides require a shelf life of two years minimum, and Rhodes (1993) indicates that up to four years is desirable. This should be achievable at temperatures at which the product may be stored. In the tropics it is not unusual for temperatures in pesticide stores to reach 40°C or more for extended periods. Microbial insecticides are usually live, albeit often in a dormant stage, so are generally less stable than chemicals and, unlike chemical insecticides, cannot easily be altered chemically to improve stability.

The innate stability of microbial pesticides depends on the organisms. The most widely used - spore forming bacteria, deuteromycete fungi and baculoviruses are, in the absence of external, inactivating factors, very stable. For example, suspensions of purified nuclear polyhedrosis virus (NPV) can be kept at room temperature, in the dark for several years (McKinley, 1985); NPV also persists in soil and cadavers for several years (e.g., Thompson et al., 1981). *Bacillus popilliae* remained viable for more than 30 years when stored under laboratory conditions (Dunbar & Beard, 1975). Eggs of the nematode, *Romanomermis culicivorax*, remain viable for several months in moist sand at room temperature (Petersen, 1984). Any poor stability of these agents noted in storage is mostly a result of the action of contaminants, formulation materials or external conditions, such as high temperature. In contrast some agents, such as non-spore forming bacteria, are relatively unstable and remain viable for only a few weeks or months at best. These delicate agents may also require certain conditions or treatment to remain viable for any length of time. Some nematodes, for example, require air or free oxygen (Poinar, 1979) to remain viable. Also, restriction of movement of the infective juveniles in storage, through entrapment or partial desiccation, improves shelf-life (Parwinder & Georgis, 1994).

The stability of the product can be maximised through a) selection of microbe species or strain, b) appropriate production conditions, c) post-production processing and d) addition of formulation additives.

SELECTION OF MICROBIAL SPECIES OR STRAIN

It is possible to select strains of microbes that are more tolerant to external factors or are less prone to breakdown. Thus, most secies of *Metarhizium* produce blastospores in submerged liquid cultures, whereas a number of isolates of *M. flavoviride* will produce the more robust conidia (see below). There is marked variation in interstitial proteinases between strains of *Bacillus thuringiensis* (Burges & Jones, 1997), which may play a role in enzymatic breakdown of the toxin crystals. In general, the shelf life of heterorhabtid nematodes is shorter than that of steinernematids nematodes (Wijbenga & Rodgers, 1994), which may be partly related to differences in activity of the nematodes in storage. Differences in activity have also been noted between different species of steinernematids. Selection of microbes tolerant to high temperatures may improve storage properties, particularly in the tropics.

APPROPRIATE PRODUCTION

Production methodology can affect subsequent storage stability. This has been shown with *Trichoderma*, which is used to control fungal pathogens of plants, where decreasing the water potential of the culture medium results in conidia with increased desiccation tolerance (Whipps & McQuilken, 1993). Submerged culture *of M. flavoviride* will normally result in formation of blastospores, whereas culture on solid media results in the production of aerial conidia. Under specific conditions, true conidia can be formed with submerged fermentation (Jenkins & Prior 1993), although these are believed to be less resilient than aerially formed conidia (Jenkins pers. comm.). Culturing *M. flavoviride* at 30°C instead of 26°C resulted in improved high temperature storage (Moore et al., 1995). Storage was also improved with older conidia and when produced under high C:N ratio. With *in vivo*-produced baculoviruses the presence of contaminating micro-organisms, as well as insect proteins and debris, may present a problem. Contaminant micro-organisms can be minimised through use of purified inoculants, the enforcement of strict hygiene procedures in the production process, including the use of food grade ingredients in insect diets, and harvesting of the virus-infected insects whilst they are still alive (McKinley *et al.*, 1989). Contaminants may also present a problem with other microbial insecticides. Holding large volumes of nematode suspensions in tanks has met with contamination problems (Parwinder & Georgis, 1994). Small numbers of contaminant bacteria and fungi can be present in the harvested fermentation liquor of *Bacillus thuringiensis*. The growth of these can be minimised, along with any remaining vegetative bacteria, by cooling and lowering the pH of the liquor; this also curbs the action of proteases and autolysis (Burges & Jones, 1997). The storage characteristics of the nematode, *Heterorhabditis megidis*, is partly dependent on the lipid levels of the nematodes (Parwinder & Georgis, 1994), which may be influenced by culture conditions.

PROCESSING

Microbial pesticides can be stabilised as dry powders/granules or liquid suspensions. Moreover they may or may not have been purified in order to reduce contaminant

material. The methods used in these processes, as well as the final form of the product itself, can influence storage stability.

Purification

This is mostly an option with *in vivo* produced viruses, most other agents being produced in pure culture. There are several methods that can be used for purification, including centrifugation, filtration and acetone-lactose co-precipitation. Density-gradient centrifugation (Harrap *et al.*, 1977), produces a highly purified suspension although not axenic that can remain stable for several years at room temperature, but is costly (Burges & Jones, 1997). Filtration and low speed centrifugation, removes some contaminants, but not all. Acetone-lactose co-precipitation, is carried out only on a small scale and results in a product of reduced stability (Burges & Jones, 1997). Due to the cost factor, many products are not highly purified and rely on subsequent treatment or formulation to counteract harmful effects of contaminants.

Drying

Suspensions are dried by spray-drying, freeze-drying or air-drying. With spray-drying the suspension is sprayed into a hot air stream in an expansion chamber. Although the airstream can be as hot as 120°C, the micro-organism is exposed for only a very short duration and is kept cool by evaporation of the water. However, air inlet and outlet temperatures must be carefully controlled and the microbes must not be allowed to remain in a hot condition in the collection vessel for a long period. This method is unsuitable for delicate micro-organisms, but is good for spore-forming bacteria, some fungi and baculoviruses. It has the additional advantage of killing some contaminant micro-organisms that may be present (Huber, 1986; Cherry pers. comm.). Inclusion of stabilisers, such as lactose, can protect the active ingredient against the heat (Lisansky *et al.*, 1993). Freeze-drying, requires that the product be frozen and the water evaporated under vacuum. This method is suitable for bacteria, viruses and, under appropriate conditions, fungi (Burges & Jones, 1997; Burges, 1997). Air-drying of suspensions has also been employed, e.g. with *Anticarsia gemmatalis* NPV in Brazil (Jones *et al.*, 1993), but runs the risk of allowing invasion and growth of contaminant micro-organisms. Freeze or spray-drying are therefore the most commonly used methods. Comparison of resulting product stability between the methods has led to different conclusions with different micro-organisms. Spray-drying reduced shelf life of *Cydia pomonella* GV (Huber, 1986). In contrast, Cherry (pers. comm.) found no difference between the storage stability of freeze-dried and spray-dried samples of highly purified NPV, however, it does appear that unpurified samples stored less well when spray-dried. With *B. thuringiensis,* dry formulations (most commonly spray dried) store better than liquid suspensions. Conidia of *M. flavoviride* stored better as dry powders than as oil suspensions (Moore *et al.*, 1995).

A number of authors highlight the importance of moisture content of powders. It is generally concluded that the moisture content should not be higher than 5% (Burges & Jones, 1997; Burges, 1997). It is intriguing to note that also with oil-formulations fungi storage is poor when the moisture content of the oil also exceeds 5%. This means that this

product should be stored within airtight containers to avoid absorption of moisture from the air, and if possible, highly hygroscopic ingredients should be avoided. Inclusion of silica gel in packages has also been employed. A comparison between liquid suspensions and dry powders shows that stability can be exceptional with some organisms in the former, for example, purified suspensions of baculoviruses. David (1978) reported that *Pieris brassicae* GV survived better as aqueous suspension than dry powder. Thus, the presence of free water is not necessarily the problem. The poor stability of some samples may be related to the presence of contaminants. In general the storage stability of purified NPV is superior to that of unpurified NPV. Absorption of water by unpurified dried samples will allow growth of contaminant micro-organisms (see below) and will accelerate any detrimental action of soluble metabolites that may be present. Possibly, inactivation of the active ingredient may also be due to oxidation of other contaminants and the formation of free oxygen radicles. Storage under anaerobic conditions may counter this (Cherry pers. comm.).

Suspensions

With suspensions the carrier used is normally either water or oil. In the absence of contaminants, micro-organisms are generally stable in water, but stability in oils varies according to the oil. Comparison of storage in different oils reveals that some mineral oils can inactivate micro-organisms fairly rapidly, whereas storage in vegetable oils, at least in the medium term, is more stable (e.g. Cherry *et al.*, 1994; Daoust *et al.*, 1983). However, the purity of the vegetable oil may be important, as well as its exposure to air (Cherry pers. comm.). As mentioned above the water content of oil is also important; water can be removed through prior heating or addition of silica gel to the oil.

Growth of contaminant micro-organisms is a major problem when water is used as the carrier, but may also be a problem with vegetable oils. Contaminants may include primary or facultative pathogens of mammals, or antagonists of the agent, or they may cause pH changes due to accumulation of waste products, or they may produce enzymes harmful to the agent. Gases from microbial activity can cause explosive release of the product prior to, or at, opening. Initial levels of contaminants and the range of species present may be limited by appropriate conditions or treatment during production and timing, as well as handling, of harvest (Lisansky *et al.*, 1993; McKinley *et al.*, 1989 - see above); alternatively additives may be incorporated to prevent growth. Quality control measures prevent the presence of hazardous organisms.

FORMULATION ADDITIVES

Additives may affect shelf stability of a product in two ways. Firstly they may be included for reasons other than storage - for example to aid spray application or to protect the micro-organism in the environment; these additives may be detrimental to the storage stability of the micro-organism, e.g. addition of some surfactants (Burges & Jones, 1997). A balance is required between the advantage of including the additive and any negative effect on the micro-organism itself. The formulator has the option here of not including

the additive in the shelf product and recommending its addition to the tank mix (although this leaves the user the option of not including the additive).

Secondly, an additive may be included specifically to enhance the storage properties of the product. With microbes that are stored in a resting stage, such as fungal and bacterial spores, additives that prevent premature growth may be needed or those with a nutritive value avoided. With powders, additives such as silica may be included to improve flowability and prevent caking of the product (McKinley *et al.*, 1989). The problem of caking can be solved by formulating the product as granules or briquettes; production of these will, of course, need binders to maintain their integrity over a period of time.

The importance of maintaining low water content of dry formulations, is referred to above; this also prevents growth of contaminant micro-organisms and so, as long as the product does not absorb water, additional additives are not necessary to suppress these.

With suspensions the growth of contaminants must be prevented. Most contaminant micro-organisms have growth optima at near-neutral or alkaline pH values. Thus growth can be minimised by maintaining the pH of the suspension at a value outside the optima. However, very high and very low pH conditions will normally inactivate or inhibit growth of the active agent of the product (e.g. Griffiths, 1982; Salama and Morris, 1993). Maintenance of pH between 4 and 6 is likely to offer the best compromise. The alternative is to include an additive that suppresses the growth of contaminant micro-organisms, e.g. sugar concentrates, antibiotics, sorbic acid, sodium benzoate, benzalkonium and proprionate (Burges and Jones, 1997). A caution here, however, is against the use of medically important antibiotics which, by wide-scale application in the environment, might promote resistance to develop in micro-organisms that are pathogenic to humans and other vertebrates.

Additives may also be included to maintain a microbial agent in suspension, or to aid resuspension after sedimentation. The products used are thickeners such as vegetable gums, thixotropic agents such as algins, which are gels when stationary but become flowable on agitation, and surfactants, which aid resuspension. A range of additives used to maintain suspensions of microbial insecticides is listed by Burges (1997) and Burges & Jones (1997). Sedimentation in oil formulations is generally faster than with water due to the low density of the oil carrier, this can be partly counteracted by milling the product into particles fine enough for colloid formation. Formulation as emulsions reduces sedimentation because the buoyancy of the oil counteracts the high relative density of the particles. Production and maintenance of an emulsion will require the inclusion of appropriate surfactants.

Arguably the ultimate stable 'formulation' is the insertion of microbe genes into crop plants. Length of storage is dependent on seed viability. Although presently limited to *B. thuringiensis* toxin genes (see review, Ely, 1993), it has been suggested that such an approach can be adopted with other insect pathogenic microbes, such as insect stunt viruses (Hanzlik et al., 1995)

CONCLUSIONS

Widespread commercial use of microbial insecticides will require storage characteristics comparable to those of chemical insecticides. This requires appropriate choice of species or strains, production methodology, processing and formulation. Optimisation of these parameters for storage may be detrimental to other important factors that influence the biological and/or commercial success of the microbe. For example, the most productive or infective strain of a microbe may not exhibit the best storage properties. With microbes that infect through ingestion, additives must not be strongly antifeedant. With fungi, additives must not prevent germination of the spore on the insect cuticle. Thus, a compromise must normally be made. Tests on stability should be carried out early in the development of the product if the best compromise is to be found.

Several formulation types are currently available. With the exception of transgenic plants, it is likely that formulation trends with microbial pesticides will follow those of chemical pesticides, at least in the near future. At present the trend is toward the development of suspension concentrates, oil-in-water emulsions and water-dispersible granules (Seaman, 1990). With all these formulations, additives are necessary to preserve the activity of the active ingredient, as well as the characteristics - and hence ease of use - of the formulation.

REFERENCES

Burges, H D (1981) *Microbial Control of Pests and Plant Diseases*, 1970-1980, London: Academic Press, 949 pp.

Burges, H D (1997 *in press*) Formulation of mycoinsecticides. In: *Formulation of Microbial Biopesticides, Beneficial Microorganisms and Nematodes,* H.D. Burges (ed), London: Chapman & Hall.

Burges, H D; Jones, K A (1997 *in press*) Formulation of bacteria, viruses and Protozoa to control insects. In: *Formulation of Microbial Biopesticides, Beneficial Microorganisms and Nematodes*, H.D. Burges (ed), London: Chapman & Hall.

Cherry, A J; Parnell, M A; Smith, D; Jones, K A (1994) Oil formulation of insect viruses, *IOBC Bulletin*, 17(3), 254-257.

Daoust, R A; Ward, M G; Roberts, D W (1983) Effect of formulation on the viability of *Metarhizium anisopliae. Journal of Invertebrate Pathology*, 41, 151-160.

David, W A L (1978) The granulosis virus of *Pieris brassicae* L. and its relationship with its host, In: *Advances in Virus Research*, M.A. Lauffer, F.R. Bang, K. Maramosch & K.M. Smith (eds.), New York: Academic Press, pp. 111-61.

Dunbar, D M; Beard, R L (1975) Present status of milky disease of Japanese and oriental beetles in Connecticut. *Journal of Economic Entomology*, 68, 453-457.

Ely, S (1993) The engineering of plants to express *Bacillus thuringiensis* δ-endotoxins, In: Bacillus thuringiensis, *an Environmental Biopesticide*: *Theory and Practice,* P.F, Entwistle, J.S. Cory, M.J. Bailey & S. Higgs (eds.), Chichester: Wiley, pp. 105-124.

Griffiths, I P (1982) A new approach to the problem of identifying baculoviruses, In: *Microbial and Viral Pesticides*, E. Kurstak (ed), New York: Marcel Dekker, pp. 527-583.

Hanzlik, T N; Bawden, A; Brooks, E M, Dorian, S J; Lincoln, M R; Gordon, K H J (1995) Producing an insect virus in cells of plants and other non-hosts, In: *Proceedings, Vth International Congress on the Impact of Viral Diseases in the Developing World, Johannesburg, South Africa*, 9-14 July, 1995, Montreal: International Comparative Virology Organisation, p. 36.

Harrap, K A; Payne, C C; Robertson, J S (1977) The properties of three baculoviruses from closely related hosts. *Journal of General Virology*, 3, 221-225.

Huber, J (1986) In vivo production and standardisation, In*: Fundamental and Applied Aspects of Invertebrate Pathology*, A, Samson, J.M. Vlak & D. Peters (eds.), Wageningen: Fourth International Colloquium of Invertebrate Pathology, pp. 87-89.

Jackson, T A (1994) Progress in bacterial control of grass grub using Invade®, a commercial biocontrol. In: *Proceedings VIth International Colloquium on Invertebrate Pathology and Microbial Control, Montpellier, France 28 August - 2 September, 1994*, Society for Invertebrate Pathology, pp. 401-406.

Jenkins, N E; Prior, C (1993) Growth and formation of true conidia *by Metarhizium flavoviride* in simple liquid medium. *Mycological Research,* 97, 1489-1494.

Jones, K A (1994) Registration and use of microbial insecticides in developing countries. In: *Proceedings VIth International Colloquium on Invertebrate Pathology and Microbial Control, Montpellier, France 28 August - 2 September, 1994*, Society for Invertebrate Pathology, pp. 82-88.

Jones, K A; Burges, H D (1997 *in press*) Principles of formulation and application. In: *Formulation of Microbial Biopesticides, Beneficial Microorganisms and Nematodes*, H.D. Burges (ed), London: Chapman & Hall.

Jones, K A; Westby, A; Reilly, P J A; Jeger, M J (1993) The exploitation of micro-organisms in the developing countries of the tropics. In: *Exploitation of Microorganisms*, D.G. Jones (ed), London: Chapman & Hall, pp. 343-370.

Lisansky, S G; Quinlan, R J; Tassoni, G (1993) *The* Bacillus thuringiensis *Production Handbook*, Newbury: CPL Press, 124 pp.

McKinley, D J (1985) Nuclear polyhedrosis virus of *Spodoptera littoralis* Boisd. (Lepidoptera, Noctuidae) as an infective agent in its host and related insects, PhD Thesis, University of London, 250 pp.

McKinley, D J; Moawad, G; Jones K A; Grzywacz, D; Turner, C (1989) The development of nuclear polyhedrosis virus for the control of *Spodoptera littoralis* (Boisd.) in cotton. In: *Pest Management in Cotton*, M.B. Green & D.J. de B. Lyon (eds.), Chichester: Ellis Horwood, pp. 93-100.

Moore, D; Hedgecock, S; McClatchie, G V; Morley-Davis, J (1995) Altering the tolerance of *Metarhizium flavoviride* conidia to environmental conditions by manipulation of culturing. In: *Abstracts, 28th Annual Meeting of the Society for Invertebrate Pathology, Cornell University, Ithaca, New York, 16-21 July* 1995, Society for Invertebrate Pathology, p. 45.

Parwinder, G; Georgis, R (1994) Fundamental research on entomopathogenic nematodes: an industrial perspective, In: Proceedings *VIth International Colloquium on*

Invertebrate Pathology and Microbial Control, Montpellier, France 28 August - 2 September, 1994, Society for Invertebrate Pathology, pp. 126-130.

Petersen, J J (1984) Nematode parasites of mosquitoes, In: *Plant and Insect Nematodes*, W.R. Nickle (ed.), New York: Marcel Dekker, pp. 797-820.

Poinar, G O Jnr (1979) *Nematodes for Biological Control of Insects*, Boca Raton: CRC Press, 277pp.

Rhodes, D J (1993) Formulation of biological control agents. In: *Exploitation of Micro-organisms*, D.G. Jones (ed), London: Chapman & Hall, pp. 411-439.

Roberts, D W; Fuxa, J R; Gaugler, R; Goettel, M; Jaques R; Maddox J (1991) Use of pathogens in insect control. In: *CRC Handbook of Pest Management in Agriculture vol.* 2, D. Pimentel (ed), Boca Raton: CRC Press, pp. 243-278.

Salama, H S ; Morris O N (1993) The use of *Bacillus thuringiensis* in developing countries, In: Bacillus thuringiensis, *an Environmental Biopesticide: Theory and Practice*, P.F Entwistle, J.S. Cory, M.J. Bailey & S. Higgs (eds.), Chichester: Wiley, pp. 237-253.

Seaman, D (1990) Trends in the formulation of pesticides - an overview. *Pesticide Science,* 29, 437-449.

Thompson, C G; Scott, D W; Wickman, B E (1981) Long term persistence of the nuclear polyhedrosis virus of the douglas fir tussock moth *Orgyia pseudotsugata* (Lepidoptera: Lymantriidae) in forest soil. *Environmental Entomology*, 10, 254-255.

Whipps, J M; McQuilken, M P (1993) Aspects of biocontrol of fungal pathogens. In: *Exploitation of Micro-organisms,* D.G. Jones (ed), London: Chapman & Hall, pp. 45-79.

Wijbenga, J; Rodgers, P B (1994) Storage of *Heterorhabditis megidis* UK211: Effect on performance and the physiological state of the nematode. In: *Abstracts, VIth International Colloquium on Invertebrate Pathology and Microbial Control, Montpellier, France 28 August - 2 September, 1994*, Society for Invertebrate Pathology, p. 265.