STUDIES ON THE FERMENTATION OF

BACILLUS THURINGIENSIS VAR *ISRAELENSIS*

by

DERMOT PEARSON

School of Biological Sciences

National Institute of Higher Education

Dublin

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SECTION 1: INTRODUCTION

ABSTRACT.

During this work the fermentation of Bacillus thuringiensis var israelensis under industrial conditions was studied with respect to the development of a process for the production of a mosquitocidal insecticide elaborated by this organism. This was done by the development of a two-stage inoculum protocol which produced a high biomass-containing inoculum of vegetative cells which were found to be preferable to free spores for use as an inoculum source. In order to optimize the production stage fermentation, a range of literature-reported media were studied with respect to cost, sporulation efficiency, biomass production, and fermentation time. The components of these media were then varied and the variant media studied with respect to the same parameters. Finally, all media were examined in relation to their bioactivity (insect toxicity) production capability, which is the most important parameter of the fermentation of B. thuringiensis. In order to be able to estimate the bioactivity of the culture broths, a mosquito rearing and bioassay facility, as well as a guinea pig colony, were established. This series of flask-scale investigations highlighted the soyabean/molasses (SM) medium as the most cost-effective medium studied. Following the flask-based experiments, the second inoculum and production stages were scaled up to laboratory-scale fermenters, and then the production stage was run on a pilot-scale.

In addition to these fermentation studies, the purification of the parasporal crystals by an aqueous biphasic separation technique was studied with a view to assessing this method for the production of an organism-free insecticide formulation.

An alternative, shorter and less labour-intensive, bioassay was also investigated.

Finally, a study of proteases produced by B. thuringiensis var israelensis was undertaken in order to determine the number, types, and time of production of these enzymes during the growth of the organism in industrial media.

1.1 PREFACE

First reports describing the use of microbes to control insects date back to 1834 when Agostino Bassi demonstrated that the fungus Beauvaria bassiana caused an infection in the silkworm (Norris, 1969; 1978). In the years that followed, infectious diseases in insects reveived some study, including an investigation by Louis Pasteur into diseasesof silkworms which were affecting the French silk industry. However, the field was considerably boosted in the early 1900's when Berliner first isolated Bacillus thuringiensis which he named after the German province, Thuringen, where he lived.

Following the original isolation of B. thuringiensis it was another 40 years before the relationship between the insect toxicity and the parasporal crystals produced by this organism was demonstrated (Angus 1954; 1956 a,b).

Since then the study of this organism, and a plethora of other bacteria, fungi and viruses causing diseases in insects, with a view to the control of insects by biological agents has been extremely vigorous.

Following the pioneering work during the 1950's and 1960's by scientists such as Tom Angus, Denis Burges, Hugette de Barjac, and Arthur Heimpel the academic and industrial interest in Biological Insect Control and Invertebrate Pathology has led to the development of a family commercially produced insecticides based on B. thuringiensis and, to a lesser extent, on a number of viruses and fungi as well as some other bacteria.

Production of insecticides based on B. thuringiensis alone in 1975 was

of the order of 2-4 million kg worldwide (Dulmage and Aizawa, 1982).

The scope for increased levels of production and use of the organism and of other biological agents is considerable. Therefore, in view of the environmental and insect resistance problems of conventional chemical insecticides it would appear that biological insecticides have a promising future.

1.2 BIOLOGICAL INSECTICIDES

Recent advances in the use of biological agents to protect a wide variety of crops against an equally wide variety of insects has led to a marked stimulation of interest, both academic and industrial, to the extent that a considerable number of organisms is in current commercial use or is being investigated with a view to commercialization at the present time.

For the purposes of this thesis, a Biological Insecticide is defined as an insecticidal material whose active ingredient is a microbial organism, where the term microbial may be extended to include viruses, protozoans, and nematodes.

The advantages of these products, which are the reasons for the high degree of current interest, are that, firstly, they exhibit a high toxicity for the host species, while being completely non-toxic for humans and other animals and plants of economic and ecological importance.

The consequences of this level of relative toxicity are that precautionary measures during field use are far less stringent than for chemical insecticides; no adverse effects on the environment are caused, predatory organisms of the pest species are not affected; and, in the USA, food crops protected using these agents can be treated up to the day of harvest, whereas chemically protected crops must not be sprayed within 30 days of harvest.

The second major advantage of these insecticides is that, to date, no resistance to them in pest species has been reported, and insects resistant to conventional chemical insecticides show no resistance to the biological agents (Briese, 1981).

As far as the bacteria and fungi which can be produced by fermentation techniques are concerned, a third advantage is the production technology is well known and the cost of production economically acceptable.

Finally, some biological insectides have the ability to cause epizootic infections (rampant infections affecting a large proportion of the population in a relatively short time) and thus need be applied less frequently than many chemical insecticides due to their marked initial effect, and prolonged residual activity. Usually it is the insect viruses which demonstrate this capacity.

However, having paid a considerable amount of attention to the advantages of Biological Insecticides, their disadvantages must also be mentioned.

One of the major problems of the selective toxicity mentioned above is that a given biological insecticide is applicable only against a small number of insect pests, and therefore if a range of insects has to be controlled, it is unlikely that a single biological agent could do the job. Differences in formulations may make different insecticides incompatible and therefore more than one spraying operation may be required, increasing the costs of insect control by these materials.

Formulation and field persistance problems have restricted the use of agents such as B. thuringiensis which has a low residual activity. However, during recent years advances have been made in formulation technology and hopefully will lead to an improvement in the situation (Couch, 1978).

Biological insecticides which must be produced in live insects such as viruses, protozoans and nematodes require labour intensive mass

insect-rearing facilities which increase production costs. But these costs are offset to a certain extent by the fact that many viral insecticides are highly virulent and can cause epizootic infections requiring lower application frequencies, thus making the overall costs of an insect control programme more attractive.

Slow target insect death is undesirable, but not uncommon with biological insectices, due to crop damage caused by hosts which may take several days to die. Even though feeding of dying insects may be much lower than healthy ones, crop producers still perfer to see dead insects soon after application.

1.2.1 Insect Classification

A short note on the classification of insects is included here to enable the reader to more thoroughly appreciate the significance of the host spectra of the biological control agents that will be mentioned.

Figures 1.2.1,2 show the taxonomic breakdown of the class Insecta with information derived from a number of sources (Barnes, 1980; Chandler and Read, 1961; Mellanby, 1963).

Of the 26 insect orders, B. thuringiensis kills larvae from only two, the Lepidoptera and Diptera. However, these two orders contain the most serious agricultural pests and human disease vectors known. It is of interest, therefore, that most of the commonly investigated entomopathogens are active against insects from these two groups, Tables 1.3.1-4,6,9.

FIGURE 1.2.1

Taxonomy of the class Insecta (Barnes, 1980)

CLASS	SUBCLASS	ORDER
INSECTA	Apterygota (Wingless)	1. Protrura
		2. Thysanura (Silverfish and Bristletails)
		3. Collembola (Springtails)
	Pterygota (Winged)	1. Ephemoptera (Mayflies)
		2. Odonata (Dragonflies and Damselfiels
		3. Orthophtera (Grasshoppers, Locusts etc.)
		4. Isoptera (Earwigs)
		5. Dermaptera (Earwigs)
		6. Plecoptera (Stoneflies)
		7. Embioptera (Wedspinners)
		8. Psocoptera (Booklice and Barklice)
		9. Neuroptera (Lacewings)
		10. Coleoptera (Beetles and Weevils)
		11. Strepsiptera (Beetle-like parasites)
		12. Mecoptera (Scorpion flies)
		13. Tricoptera (Caddisflies and Water moths)
		14. Zoraptera (Soft boiled insects)
		15. Mallophaga (Chewing and bird lice)
		16. Anopleura (Sucking lice)
		17. Thysanoptera (Thrips)
		18. Themiptera (True bugs)
		19. Homoptera (Leafhoppers, aphids)
		20. Lepidoptera (Butterflies, moths)
		21. Diptera (Butterflies, moths)
		22. Hymenoptera (Ants, bees, wasps, sawflies)
		23. Siphonaptera (Fleas)

FIGURE 1.2.2

Toxomy of the order Diptera (Mellanby, 1963)

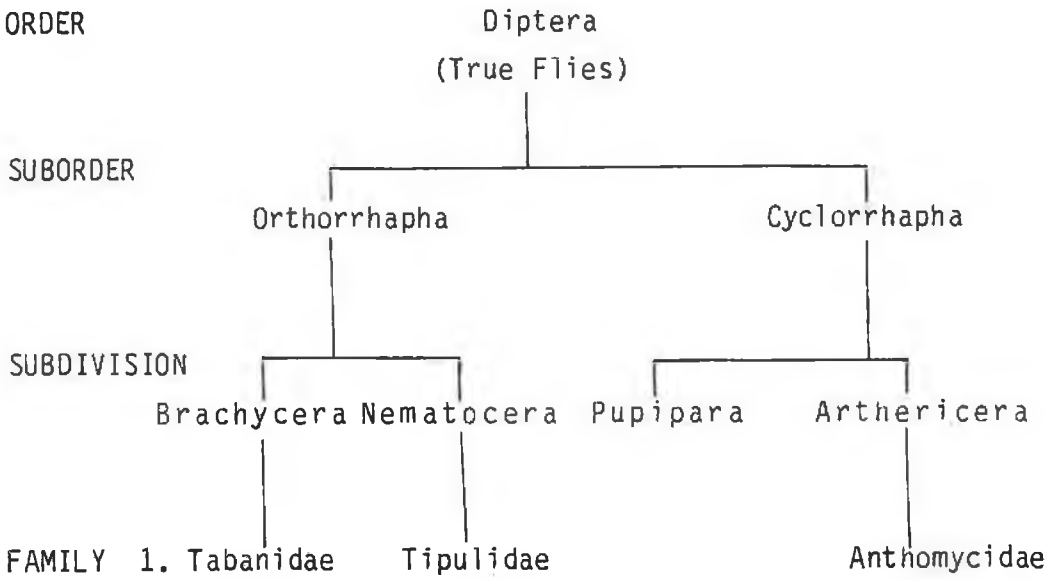
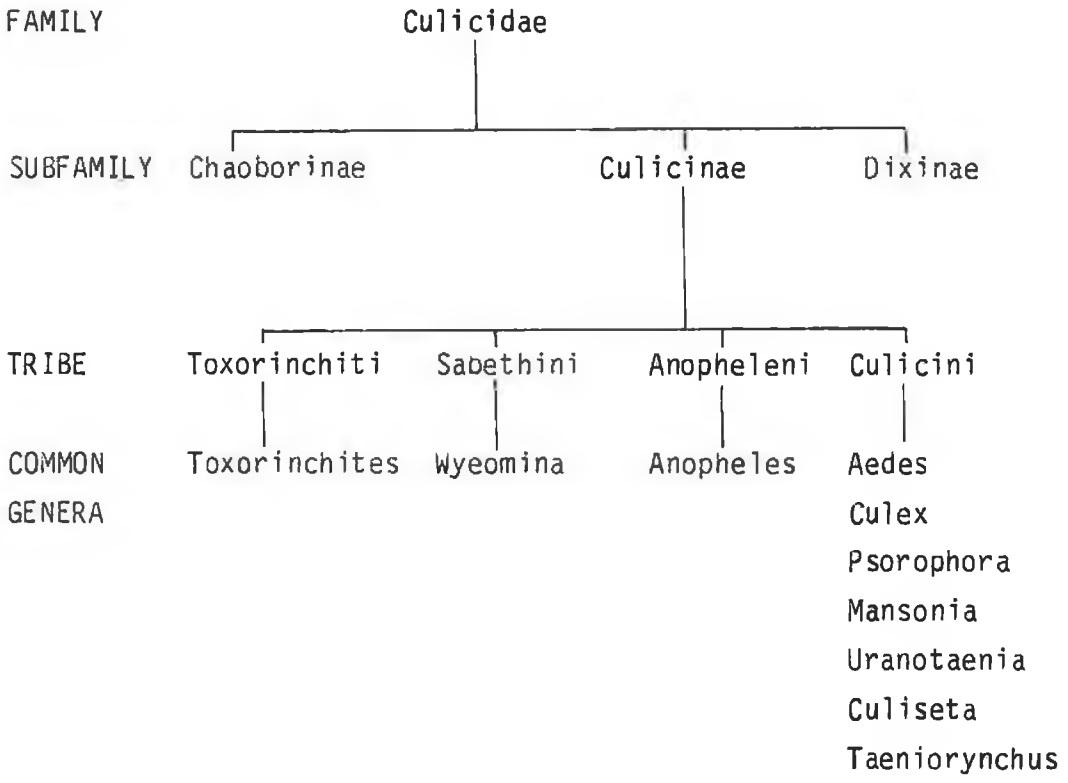


FIGURE 1.2.3

Taxonomy of the Culicidae family
(Chandler and Read, 1961; Mellanby, 1963



1.3 ORGANISMS USED IN BIOLOGICAL INSECTICIDES

The range of organisms in actual commercial use, or under investigation for their insect control potential includes viruses, bacteria, fungi, nematodes and protozoans.

1.3.1 Viruses in Insect Control

The use of viruses as agents for insect control has, until recently, been limited. This was largely due to a lack of fundamental knowledge about the viruses and to high production costs. The introduction of large-scale semi-automated insect rearing techniques (Shapiro, 1982) and the study of cell-culture systems (Hink, 1982; Stockdale and Priston, 1981) has made the economic production of viruses more attractive. These factors, coupled to encouraging field trial results, have led to the belief that the viruses, of all the organisms used in insect control, have the greatest potential for use as active ingredients in biological insecticides., (Ignoffo and Anderson, 1979; Norris, 1978).

Many insect viruses are encased, either singly or in large numbers, in water-insoluble protein crystals called polyhedra. Virus particles in polyhedra may remain stable and infective as dry powders or in a hydrated condition for periods of years (Norris, 1978). It is this property of these Occluded Viruses, as they are known, which makes them highly suitable as biological control agents.

Of the seven families of viruses associated with insects (Ignoffo and Anderson, 1979; Miller et al., 1983) it is the Baculoviridae, which are beingmost intensely investigated with a view to being used as agents of insect control.

The Baculoviridae family contains one genus, Baculovirus, which has been extensively reviewed by Faulkner (1981). The baculoviruses are, with some exceptions, occuluded viruses and contain three major sub-groups (Faulkner, 1981). Subgroup A contains the Nuclear Polyhedrosis Viruses (NPV's) which contain many virions per polyhedron. The Granulosis Viruses (GV's) make up sub-group B and are characterized by containing a single virion per occlusion. Finally sub-group C, which has only recently been proposed, contain the non-occluded baculoviruses. All members of the Baculoviridae are found exclusively in invertebrates and are therefore ideal candidates from the point of view of safety of non-target organisms (Ignoffo and Anderson, 1979; Miller et al., 1983). Other advantages of the Baculoviridae are that they cause lethal infections in a narrow host range, and so will not affect predatory organisms; some baculoriviruses are highly pathogenic and can produce up to 10^{10} occlusions per larva, which increases the attraction of commercial insect rearing techniques (Miller et al., 1983). In addition, these viruses can cause vigourous and persistent epizootic infections and are therefore believed to have enormous potential for the control of a range of insect pests.

Baculoviruses from a range of insects have been registered in the USA with the Environmental Protection Agency (EPA) (Miller et al., 1983). These are summarized in Table 1.3.1.

Cunningham (1982) described a list of 24 forest pests (among them the spruce budworm, Choristoneura fumiferana, which is the most economically important forest pest in North America) whose control has been field tested using baculoviruses, with notable success in many cases.

In addition, an even wider range of insect pests of agricultural

importance whose, at least partial, control has been demonstrated using, almost exclusively, viruses of the Baculoviridae (Yearian and Young, 1982).

Control of the rhinocerous beetle, Orcytes rhinocerous, has been obtained using a non-occluded baculovirus (Bedford, 1981; Kelly, 1981). This insect is a serious pest of palm trees in Africa and the South-West Pacific region, and represents a threat to one of the major cash crops of these areas.

Although the Baculoviridae are the most commonly used insect control viruses they are not the only ones which have been used for this purpose.

The Cytoplasmic Polyhedrosis Virus (CPV) of the pine catterpillar Dendrolimus spectabilis has been registered as a commercial insecticide in Japan since 1974, Table 1.3.1. Another CPV, from the pine processionary catterpillar Thaumetopea pityocampa has, also been used in field trials (Katagiri, 1981).

Safety tests with CPV's have indicated no adverse affects on non-target species (Katagiri, 1981), which is extremely desirable for the success of a biological agent as an insecticide. Some non-occluded viruses have been used to control pests by the introduction of virus-infected individuals into a population and allowing an epizootic infection to occur. Tnis has been a strategy used in investigating control of the citrus red mite, Panonychus citri (Reed, 1981). However, the use of these viruses lags considerably behind that of the Baculoviridae.

In summary, it is possible to say that a large number of viruses is known which could be used in biological insecticides. Despite the

TABLE 1.3.1

Some viruses in actual use, or under investigation, as insect control agents

VIRUS TYPE	TARGET INSECTS	TRADE NAMES PRODUCERS	PROTECTED CROP	REFERENCE
NPV	*Heliothis* spp, Cotton bollworm Tobacco budworm	Elcar. Sandoz (USA) Biotrol-VZH. Nutrilite (USA)	Cotton, Corn, Tobacco, Soyabeans.	Ignoffo and Anderson, 1979 Ignoffo and Couch, 1981
	Lymantria dispar, Gypsy moth	Gypchek, USDA	Forest	Lewis, 1981
	Orgyia pseudosugata, Douglas fir tussock moth	-	Forest	Cunningham, 1982
	Choristoneura fumiferana Spruce budworm	-	Forest	Cunningham, 1982
	Neodiprion sertifer European pine sawfly	Virox. Microbial Resources (UK)	Forest	Cunningham and Entwhistle, 1981
CPV	*Dendrolimus spectabilis* Line caterpillar	Matsukemin. Japan	Forest	Katagiri, 1981 Ignoffo and Anderson, 1979
Non-occluded Virus	*Panonychus citri* Citrus Red Mite	-	Citrus	Reed, 1981

NPV: Nuclear Polyhedrosis Virus. CPV: Cytoplasmic Polyhedrosis Virus.

potential of these agents for use as highly virulent insect control tools,problems with production technology, narrow host-ranges and safety to non-target organisms have tended to ninder their widespread introduction. However, for insect-infecting viruses, it is most probable that their use will increase significantly over the next decade or so.

1.3.2 Fungi in Insect Control

As with viruses and bacteria, a large number of fungi, is known to cause infections in insects, but only a few have shown significant potential for use in biological insecticides.

Fungi can kill by the production of toxins (Somerville, 1973; Roberts, 1981; Wright et al., 1982) or by the infection of a host which is the more common method.

Often, mammalian toxicity of fungal toxins is high (Sommerville, 1973; Wright et al., 1982) so the likely value of these organisms and toxins as insecticides is questionable.

Fortunately, a wide range of fungi is known which kill insects by invasion of host tissues which would imply that biological insecticides based on such fungi would be less likely to give rise to mammalian toxicity problems.

The economically important insects which are currently, or have been, commercially controlled using entomopathogenic fungi are described in Table 1.3.2. In addition to those in actual production and use some other fungi are being thoroughly investigated on a pilot scale and extensive field trial basis.

TABLE 1.3.2

Entomopathogenic Fungi which are currently being produced for use in biological insecticides

FUNGUS	TARGET INSECT	TRADE NAME PRODUCERS	PROTECTED CROP	REFERENCE
Beauvaria bassiana	Colorado beetle Leptinotarsa decemlineata European Corn Borer Ostrina nubilalis	Boverin. Glavmikrobiprom (USSR) Biotrol-FBB. Nutrilite (USA)	Potatoes, Apples,	Ferron, 1981 Ignoffo & Anderson 1979 Miller et al., 1983
Metarhizium anisopliae	Spittlebug	Biatrol-FHA. Nutritate (USA) Metaquito. Brazil	Sugar Cane	Ferron, 1981; Miller et al., 1983; Ignoffo and Anderson, 1979.
Hirsutella thompsonii	Citrus Rust mite Phylocoptruta oleivora	ABG - 6065; Abbott Labs (USA)	Citrus Fruits	McCoy, 1981 Ignoffo and Anderson, 1979
Verticillium lecanii	Aphids and Scales	Vertalec and Mycotal. Microbial Resources (UK)	Glasshouse crops	Hall, 1981 Miller, et al; 1983

Among these experimental organisms is Nomurea Rileyi which is being used against the cabbage looper, Trichoplusia ni and the velvet bean catterpillar, Antarsica gemmatalis (Ignoffo, 1981).

Mosquito control by a number of fungi is also under study. These fungi include Lagenidium giganteum (Domnas, 1981), Coelomyces spp and Culicinomyces spp, (Domnas, 1981; Federici, 1981).

Despite the number of fungi known to be capable of causinginfections in insects the use of these organisms tends to be limited to specialized situations. In general, fungi do not seem to have the proven ability of B. thuringiensis or the potential ability of some viruses to become successful biological insecticides.

The problems facing fungi include large-scale production of a stable form which can be formulated and stored for long periods of time, often under unfavourable conditions of heat and humidity. In addition, the conditions required in the field for successful control of an insect population are highly specific. Usually a warm, humid atmosphere is necessary which is why V. lecanii is very successfully used to cause epizootic infections in greenhouses (Miller et al; 1983). However, such conditions are rarely found in much of Europe and the United States which are the largest markets for biological insecticides.

Fungi are slow acting patnogens in that the infection process from the time the conidium or zoospore attaches to the insect til the death of tne host takes days, and in some cases weeks (Miller et al; 1983). This represents another disadvantage of fungal insecticides. Safety of fungal entomopathogens is another major barrier to their being widely accepted as biological insecticides. Very often it is known that fungal cultures produce mycotoxins and therefore expensive safety

trials would be necessary to prove whether or not these substances are produced under conditions of commercial production. The presence of allergens in fungal formulations may pose additional safety concerns, in addition to those of toxin production (Ignoffo and Anderson, 1979).

Thus fungi, although many are known to parasitise insects, only a few have reached commercialization and very often their use is restricted to specific habitats. At the present time it is unlikely that any of the known fungal entomopathogens will become as successful as B. thuringiensis.

1.3.3 Nematodes in Insect Control

The use of nematodes as agents of insect control is at present more limited than for any of the other organism types used. Their use has been recently reviewed with respect to the potential of a small number of candidates which show possible applications in pest control (Finney, 1981). These organisms and their host species are outlined in Table 1.3.3.

The number of nematodes outlined is small and the range of affected host species is narrow. In addition, only one nematode has been used in a commercial product which is sold on a worldwide basis for general mosquito and blackfly control.

The main problems for the use of nematodes as insecticides on an industrial scale are:

1) High production, storage and transport costs due to necessity of rearing in vivo and storage under conditions of high humidity.
2) Parasite processing often causes high levels of viability loss, further increasing production costs.
3) Poor field survival due to the requirements of the parasites of being protected from dessication and the effects of UV light.
4) Frequent failure to cause infections in sufficiently high proportions of the target population and the selection of resistant sub-populations in the host.

While such problems continue to plague the use of nematodes as agents of insect control, their use as such is likely to remain restricted.

TABLE 1.3.3

Nematodes Used In Insect Control

ORGANISM	TARGET INSECTS	TRADE NAMES/ PRODUCERS	PROTECTED CROP	REFERENCE
Romanomermix culicivorax	Mosquito and blackfly larvae	Skeeter Doom. Fairfax Biological (USA) Nutrilite (USA)*	-	Finney, 1981 Ignoffo and Anderson, 1979
Diximermis peterseni	Mosquito larvae	-	-	Finney, 1981
Neoaplectana spp	Dutch Elm Disease beetle Scolytus scolytus	-	Elm trees, Sugar beet	Finney, 1981
Pristionchus uniformis	Colorado beetle Leptinotarsa decemlineata	-	Potatoes	Finney, 1981
Howardula husseyui	Mushroom phorid fly Megaselia halterata	-	Cultivated mushrooms	Finney, 1981

* Experimental product

1.3.4 Protozoans in Insect Control

Insect control by protozoans is not done on a commercial scale anywhere. However, because many insects not susceptible to other entomopathogens are parasitized by protozoans the potential for their use under industrial conditions is promising (Ignoffo and Anderson, 1979). The most likely area where protozoan control will be successful is where low pest densities can be tolerated and where the pathogen can maintain pest levels at or below the damage threshold (Henry and Oma, 1981; Wilson, 1981, 1982).

The protozoans considered most suitable as candidates for insect control are described in Table 1.3.4.

Very often, when a protozoan was used for the protection of food crops such as cabbages, tomatoes or corn, significant crop damage occured, even though the parasite colonized the hosts efficiently, (Maddox et al., 1981). This futher re-emphasizes the best niche for use of protozoans where some crop damage can be sustained and control in the longterm is desirable, such as with grasslands or forests.

The problems associated with the use of protozoans for insect control are practically identical to those for nematodes, 1.3.3. However, the protozoans which show the best potential for use in biological insecticides come from the microsporidia which produce relatively stable and environmentally resistant spores, which, once protected from UV light can be formulated as the infectious agents. The stability of these infectious particles may lead to the introduction of protozoan-based insecticides for use in longterm pest control situations (Wilson, 1982).

TABLE 1.3.4

Protozoans whose use in insect control is being investigated

ORGANISM	TARGET INSECTS	PROTECTED CROP	REFERENCE
Nosema locustae	Grasshoppers, Locusts, crickets	Grassland, Foliage crops	Henry and Oma, 1981 Wilson, 1981 Ignoffo and Anderson, 1979
Nosema fumiferana	Spruce budworm, *Choristoneura fumiferana*	Forest	Weatherston and Retnakaran, 1975 Wilson, 1982
Variomorpha necatrix	Range of lepidopteran pests	Soyabeans, Corn, cotton, Tobacco, Tomatoes	Maddox et al., 1981 Ignoffo and Anderson, 1979

1.3.5 Bacteria in Insect Control

Bacteria form the largest known group of pathogens associated with disease conditions of insects. The range of bacterial entomopathogens is, to say the least, considerable and includes representatives from the families Pseudomonaceae, Enterobacteriacece, Lactobacillaceae, Micrococcaceae and Bacillaceae (Miller et al., 1983; Faust and Bulla, 1982).

An important distinction must be made when assessing the pathogenicity of a particular bacterium isolated from a diseased host as to whether it is the actual disease causing agent, or simply an organism invading the insect after the initial infection by another bacterial species (Lysenko, 1981). Secondly, in the case of a bacterial isolate which is known to be responsible for a pathological condition in an insect host, it must be established if the cause of the disease is due to tissue invasion (septicaemia), to the production of toxins (toxicaemia), or to both (Faust and Bulla, 1982). Bacteria causing disease by each of these three methods are used in current biological insecticides.

The study of bacteria and their toxins as insecticides and of bacteria and pathological associations with insects has been reviewed recently by a number of authors (Sommerville, 1973; St. Julian et al.. Bulla et al., 1975; Lysenko, 1981; Splittstoesser and Kawanishi, 1981; Davidson. 1981; Burges, 1982; Faust and Bulla, 1982). Some of the more common bacteria isolated from diseased insects are outlined in Tables 1.3.5, 6.

Despite the large numbers of known bacterial entomopathogens only members of the genus Bacillus are used in biological insecticides,

TABLE 1.3.5

Some of the non-sporeforming bacteria commonly isolated from diseased insects

ORGANISM	SOURCE INSECTS	DISEASE TYPE	REFERENCE
Pseudomonas aeruginosa	Grasshoppers. Greater wax moth, *Galleria mellonella* Silkworm, *Bombyx mori* Tobacco hornworm, *Manduca sexta*	Septicaemia	Bulla et al., 1975 Faust and Bulla, 1982 Bulla and Yousten, 1975
Proteus spp	Grasshoppers	Septicaemia	Faust and Bulla, 1982
Serratia marcesens	Artificially-reared insects, Greater wax moth *Galleria mellonella*	Toxicaemia	Bulla et al., 1975 Lysenko, 1981
Streptococcus pluton	Honey bees	Septicaemia*	Splittstoeser and Kawanishi, 1981 Bucher, 1981

* This organism is the causative agent of European foulbrood disease

TABLE 1.3.6

Some of the sporeforming bacteria commonly isolated from diseased insects

ORGANISM	SOURCE INSECTS	DISEASE TYPE	REFERENCE
Clostridium spp	Tent catterpillars, *Malcasoma* spp Greater wax moth, *Galleria mellonella*	Gut lumen invasion; Toxicaemia	Bucher, 1981 Davidson, 1981 b Bulla et al., 1975
B. cereus	Coleoptera, Hymenoptera, Lepidoptera	Septicaemia and toxicaemia	Bulla et al., 1975 Davidson, 1981 b St. Julian et al., 1973
B. popillae	Japanese beetle *Popilla japonica*	Septicaemia	Klein, 1981 Faust and Bulla, 1982 Bulla et al., 1978
B. lentimorbus	Japanese beetle *Popilla japonica*	Septicaemia	Klein, 1981 Splittstoeser and Kawanishi, 1981
B. alvei/ *B. circulans*	Blackfly larvae *Simulium* spp	Toxicaemia	Singer, 1981
B. larvae	Honey bees	Septicaemia	Splittstoeser and Kawanishi, 1981
B. sphaericus	Mosquito larvae	Toxicaemia	Singer, 1981 Davidson, 1981 c
B. thuringiensis	Range of lepidopteran and dipteran larvae	Toxicaemia	Faust and Bulla, 1982 Burges, 1982 Luthy et al., 1982

(Burges, 1982). This has been due to the fact that the disease causing agent, produced by the bacilli is stable to a range of environmental conditions and can be produced, formulated and stored for extended periods, all at an economic cost.

The entomopathogenic bacilli most commonly isolated from diseased insects are Bacillus thuringiensis, B. sphaericus, B. popilliae, B. lentimorbus, B. larvae, and B. cereus. Of these only the first three are in current use as commercial or experimental microbial insecticides, Table 1.3.7, 8.

1.3.5.1 Bacillus popilliae as a microbial insecticide.

Bacillus popilliae and its close relative B. lentimorbus are the causative agents of the well-documented milky disease of the Japanese beetle, Popilla japonica (Klein, 1981). The use of these bacteria has been dramatic in the control of the pest with the result that 25 to 30 years after the introduction of the disease in New Jersey and Delaware, USA, the pathogen was still present at colonization sites and had spread into nearby pastures and areas of cultivation (Klein, 1981).

B. popilliae spores invade the larval host ater being ingested and germinating in the insect gut, whereupon they penetrate through tne gut wall and establish a septicaemia. Once vegetative growth has finished the organism sporulates, and it is the formation of the spores which turns the dead insect into a whitish colour, hence the name of the disease. The bodies of deceased insects contaminate the soil habitat of the beetle and infect the next generation (Burges, 1982). Thus this pathogen is an important example of one which causes host death only by invasion of the haemocoel and the establishment of

TABLE 1.3.7

Commercial microbial insecticides based on members of the *Bacillus* genus other than *B. thuringiensis*.

BACILLUS SPECIES	TRADE NAMES	PRODUCERS AND COUNTRY OF ORIGIN	REFERENCE
B. popillae	Doom, Japidemic Milky spore	Fairfax Biological (USA) Reuter Labs. (USA)	Bulla and Yousten, 1979 Ignoffo and Anderson, 1979 Klein, 1981
B. sphaericus	Experimental powders	Abbott Labs. (USA) Staufer Chemical Co. (USA)	Lacey, 1984 Singer, 1980
B. moritai	Lavillus M.	Sumitomo Chemical Co. (Japan)	Faust and Bulla, 1982

TABLE 1.3.8

Commercial microbial insecticides based on *B. thuringiensis* products

ACTIVE PRODUCT	INSECT TARGET	TRADE NAMES	PRODUCERS AND COUNTRY OF ORIGIN	REFERENCE
B-exotoxin	Wide range	Biotoksybacillin	All-Union Institute of Agricultural Microbiology (USSR)	Faust and Bulla 1982
		Eksotoksin	Glavmikrobioprom	Ignoffo &
		Toxsobacterin	(USSR)	Anderson, 1979
Parasporal Crystal	Lepidopteran insects	Agitrol	Merck & Co. (USA)	Bulla and
		Bacthane	Rohm & Haas (USA)	Yousten,
		Bactospeine	Roger Bellon (France)	1979
		Bactur	Soplana (Yugoslavia)	Faust and
		Bakuthal	"	Bulla 1982
		Bathurin	Chemapol-Biokrma (Czechoslovakia)	Ignoffo & Anderson,
		Biobit	Microbial Resources (UK)	
		Biospor	Hoechst (West Germany)	
		Biotrol	Nutrilite (USA)	
		Dendrobacillin	Glavmikrobioprom (USSR)	
		Dipel	Abbott Labs. (USA)	
		Entobactericin	Glavmikrobioprom (USA)	
		Insektin	"	
		Parasporin	Grain. Proc. Lab (USA)	
		Sporeine	LIBEC Lab (France)	
		Thuricide	Sandoz (USA)	
	Dipteran Insects	Bactimos	Biochem Products (USA)	Commercial
		BMC	Reuter Labs (USA)	literature
		Skeetal	Microbial Resources (UK)	
		Teknar	Sandoz (USA)	Klausner
		Vectobac	Abbott Labs. (USA	1984

a septicaemia.

Widespread use of this pathogen does not occur for a number of reasons. Firstly, it can only be produced *in vivo* and this difficulty is compounded by the fact that the larvae are difficult to rear artificially. Vegetative cells can be produced *in vitro* but efficient sporulation under these conditions has never been observed (Klein, 1981; Burges, 1982; Bulla et al., 1978).

Secondly, due to the success of colonization of pest populations, single applications of the organism are usually all that is required to achieve satisfactory pest control. Thirdly, the organism has a narrow host range (Burges, 1982). Lastly, in recent times in certain parts of the USA resurgence of the Japanese beetle has been observed suggesting that the host may develop a resistance, at least to some strains of *B. popillae* (Klein, 1981).

Thus *B. popilliae*, although not widely used in biological pest control, is important in that it provides a classical example of pathogenicity by infection. Modern techniques of tissue culture and genetic manipulation may enhance the use of this organism by the development of a virulent, more easily produced strain whose host range might be expanded to include a greater number of the scarabeid beetle pests.

1.3.5.2 *Bacillus sphaericus* as a microbial insecticide.

Recently, *B. sphaericus* has aroused considerable interest as a mosquito control agent (Singer, 1981 a,b). This organism has a well-documented toxicity to a variety of mosquito species including members of the *Culex*, *Anopheles* and *Aedes* genera (Davidson, 1981 a, 1982a). It is commonly isolated from soil and diseased mosquito larvae. A

large number of isolates has been reported, among which insecticidal activity varies from significant to none, with only those originally derived from mosquitoes being insecticidal (Davidson 1982, 1984). The strains showing the highest insecticidal activity include strain 1593 (Davidson, 1981 b), strains 2297, 2173 (Davidson, 1984) and strain 2362 (Mulla et al., 1984; Yousten, 1984).

Pathogenicity in the most insecticidal strains is fully expressed only during sporulation (Davidson, 1984) and is caused by a toxin located in the spores (Davidson, 1982b, 1983;Tinelli and Bourgouin, 1982; Myers et al., 1979; Myers and Yousten, 1980), and/or in the parasporal inclusions produced by certain strains (de Barjac and Charles, 1983; Kalfon et al., 1984; Yousten and Davidson, 1982). However, a soluble, cytoplasmic toxin has also been isolated from sporulating cells of B.sphaericus 1593, but only small amounts were recovered, and it is probable that this entity is of less importance than the spore or parasporal inclusion - associated toxins (Davidson, 1982b).

Production of B. sphaericus by cultivation under industrial conditions has been achieved (Lacey, 1984; Yousten, 1984; Yousten et al; 1984 a,b). It has been found that the organism does not utilize carbohydrates and requires biotin and thiamine for growth (Lacey, 1984). High levels of aeration are required for optimal bioactivity production, but the use of pure oxygen does not increase levels of insect toxin in the fermentation broth (Yousten et al., 1984 a,b). Furthermore, insect toxin titres are maximized by maintaining the culture broth near neutral pH and controlling the temperature at 30^{o}C (Lacey, 1984; Yousten et al., 1984 a,b).

The toxicity of B. sphaericus appears to be limited exclusively to mosquitoes with no known reports showing significant activity toward the closely related blackflies, or indeed to any other insects (WHO,

1980). Within the mosquito group, B. sphaericus seems to be most toxic to Culex spp, Psorophora spp and Anopheles spp larvae, and less toxic to Aedes spp (Burges, 1982; Faust and Bulla, 1982; Ramoska et al., 1977).

Safety studies carried out thus far with B. sphaericus have revealed no adverse effects in rats, mice and guinea pigs (Burges, 1981). By comparison to B. thuringiensis, less work has been done on the effects of B. sphaericus on non-target organisms, but the results available indicate that the environmental impact of this organism should be minimal (Burges, 1982; WHO, 1980).

One of the most important advantages B. sphaericus has in terms of its use as a microbial insecticide is the reported ability to not only survive, but recycle in polluted environments where infestations of mosquito larvae are likely to occur (Des Rochers and Garcia, 1984).

The length of time over which B. sphaericus was reported to multiply and maintain toxicity varied from 6-9 months in one study (Silapanuntakul et al., 1983) to a matter of about 4 weeks in others (Mulla et al, 1984). The activity of the organism was significantly decreased in very heavily polluted waters due to the high level of particles (Mulla et al, 1984). Where the spores had sunk to the bottom silt layer of a street catch basin, activity was retained over an extended period, but reinfesting larvae were not affected, reportedly because the new larvae did not feed at the bottom of the basin (Mulligan et al., 1980).

Thus, in recent years B. sphaericus has been considered as a candidate for the control of mosquitoes, which are vectors of a number of serious diseases of man and animals worldwide. This organism is easily and cheaply produced on an industrial scale, is highly toxic

for many mosquito species and has the ability to survive for extended periods of time in mosquito breeding habitats. The main reason why B. sphaericus has not found greater use as a microbial insecticide is that most of the advantages which it has are duplicated by the superior mosquito pathogen Bacillus thuringiensis var israelensis, which grows and sporulates better in industrial fermenters, and produces larger amounts of a toxin which is more toxic that than of B. sphaericus. In addition, the toxin of B. thuringiensis var israelensis is lethal to a wider variety of insects than is the toxin which is produced by B. sphaericus.

1.3.5.3 Bacillus thuringiensis as a microbial insecticide.

Bacillus thuringiensis is the most successful biological agent (in terms of annual production rates) in current use as a commercial insecticide.

Its production has been in progress on a large scale for at least 20 years in many countries, including the USA, USSR, France, West Germany, Yugoslavia, Czechoslavia, China and the UK, Table 1.3.8 (Ignoffo and Anderson, 1979; Bulla and Yousten, 1979; Norris 1978). At the moment, of the order of 2-4 million kilograms are produced annually on a worldwide basis (Burges, 1982; Dulmage and Aizawa, 1982).

The reasons B. thuringiensis is used on such a large scale are that it demonstrates high toxicity to a wide range of lepidopteran and dipteran pests, it is easily and economically produced on an industrial scale by submerged fermentation, the insecticidal material is stable to a wide range of storage and processing conditions and can be formulated into a range of forms which can be stored for up to 2-3

years under cool, dry conditions. Extensive studies on the safety of B. thuringiensis have demonstrated that the spore/parasporal crystal (see below) preparations used in insecticides are completely harmless to non-target forms of life (Burges, 1981).

In addition, B. thuringiensis was the first microorganism discovered which could be easily produced on a large scale and thus a considerable degree of attention was focussed on it before other biological agents with potential for insect control were studied in depth.

Some of the registered uses of B. thuringiensis are mentioned in Table 1.3.9.

The pathogenicity of B. thuringiensis is due, mainly, to the production of, at most, two toxins. The beta-exotoxin, or thuringiensin (Sebesta et al., 1981) is an ATP-analogue and is produced only by certain strains. The use of thuringiensin in insecticides is banned outside the Eastern bloc due to it demonstrated toxicity in higher animals (Linnainman et al., 1977; Mehrotra et al, 1977; Sebesta et al., 1981).

The most important insecticidal toxin produced by B. thuringiensis is the proteinaceious parasporal crystal which is synthesized at the end of log phase growth at the same time as the spore (Fast, 1981).

Because B. thuringiensis is the main topic of the remainder of this thesis, further discussion of it will be left to later sections.

TABLE 1.3.9

Some of the USA-registered uses of *B. thuringiensis*

PLANT OR CROP	PEST INSECT
-	Mosquitoes and Blackflies
Cabbage, broccoli, cauliflower, spinach	Cabbage looper, Diamond back moth
Tobacco	Tobacco budworm, cabbage looper, tobacco hornworm, tobacco moth
Tomatoes	Cabbage looper, tomato hornworm
Alfalfa	Alfalfa catterpillar
Chrysanthemums	Cabbage looper
Soya beans	Velvet bean catterpillar, soyabean looper, cabbage looper
Cotton	Tobacco budworm, cotton bollworm, cabbage looper
Forest, ornamental, and shade trees	Gypsy moth, red-numped catterpillar, spring and fall cankerworm, fall webworm, tent catterpillar, California oak moth, pine butterfly, spruce budworm, western and Douglas-fir tussock moths, cabbage looper, tobacco budworm, diamondback moth
Avocados	Amorbia moth, omnivorous leaf roller
Oranges	Fruit tree leafroller, orangedog

1.4 BACILLUS THURINGIENSIS

1.4.1 Bacteriology

The bacteriology of B. thuringiensis has been reviewed by Rogoff and Yousten (1969) and only a summary will be given here. This bacterium is a facultatively anaerobic, spore-forming rod which is gram-positive in its early stages of growth, but tends to be gram-variable later on. The species is distinguished from the closely related B. cereus by the intracellular formation of a proteinaceous crystal during the phase of spore formation which is toxic to certain insects. This proteinaceous crystal is more commonly referred to as the parasporal crystal and can comprise up to 30% of the dry weight of the sporangium (Rogoff and Yousten, 1969).

A number of strains which are differentiated on the basis of flagellar antigens is known and among these strains, variation in properties such as the production of acetoin, lecithinase, and urease have been documented (Buchanan and Gibbons, 1974).

Acid is produced from ribose, glucose, fructose, glycerol, soluble starch, maltose, and trehalose, but not from arabinose, galactose, xylose, rhamnose, sorbose, erythritol, dulcitol, mannitol, sorbitol, meso-inositol, lactose, raffinose or inulin; gas is not produced. Citrate is not used as a sole source of carbon and energy; indole is not produced; haemolysis is observed in horse blood agar (Rogoff and Yousten, 1969).

The spore is oval, subterminal and does not cause distortion of the sporangium. During the early stages of vegetative growth the cells are motile by means of peritrichous flagella whose antigens are used to identify the different strains, varieties or subspecies of the group

(Buchanan and Gibbons, 1974).

Despite their close relationship B. thuringiensis and B. cereus do have a number of biochemical differences besides the production of the parasporal crystal by the former. The formation of acetylmethylcarbinol is variable in B. thuringiensis strains, but always is a prime characteristic in B. cereus. The inability of B. thuringiensis to use citrate as a sole carbon and energy source, and residual phosphate levels in its spores X10 as high as in B. cereus are useful biochemical criteria for distinguishing acrystalliferous mutants of B. thuringiensis from B. cereus (Rogoff and Yousten, 1969). In the opinion of those authors the formation of a parasporal crystal was of major taxonomic importance in distinguishing the two species, despite the fact that this ability can be spontaneously lost in laboratory cultures of B. thuringiensis.

In addition, acrystalliferous mutants of B. thuringiensis can be distinguished from B. cereus by a serological comparison of the flagellar antigens and, thus, there appears to be very little evidence which would mitigate strongly toward having both these bacilli included in the one species (de Barjac, 1981; Burges, 1984).

1.4.2 Classification and Taxonomy

Since the initial description of B. thuringiensis by Berliner in the early part of this century, a large number of isolates of this organism has been found. Characterization of these isolates has been an extremely important part of the study of this bacterium as it has allowed the division of the species into a number of sub-groups. Variations between sub-groups or insect host ranges has been the most important revelation of its classification. An explanation of the

methods of taxonomic differentiation and the nomenclature of B. thuringiensis is therefore necessary.

1.4.2.1 Techniques used in the taxonomy of B. thuringiensis, and nomenclature recommendations.

Since the pioneering work of de Barjac and Bonnefoi (1962, 1968, 1973) the classification of B. thuringiensis according to serological analysis of the H, or flagellar, antigens has become the primary tool for distinguishing between subgroups of this species (Burges, 1984). This was because this easily performed technique gave good agreements with other, more laborious, methods such as:

1) electrophoretic patterns of esterases from vegetative cells (Norris and Burges, 1963, 1965).
2) Serological analysis of parasporal crystal antigens (Krywienczyk, in Dulmage and Co operators, 1981).

The flagellar antigen analysis technique has turned out to be the most sensitive, specific, rapid and reliable method of identification which has encouraged its adoption as the main classification tool for B. thuringiensis isolates (de Barjac, 1981). The protocol used for this system is described in detail by de Barjac (1981).

The subgroups defined by the serological analysis of the flagellar antigens have been called varieties, subspecies and H-serotypes. According to recommendations for the purpose of standardized nomenclature, these subgroups are to be considered the most important subdivision of the species and are to be called varieties, abbreviated var (Burges, 1984). This convention has been adopted during this work.

The method of classifying sub-groups of B. thuringiensis according to the electrophoretic pattens of esterases from vegetative cells was developed in the early 1960's (Norris and Burges, 1963, 1965). This

technique is no longer in use because the results it gave were similar to those provided by the serological H-antigen analysis (described above) while requiring a greater work input.

A newer technique used for classification below the level of varieties is the serological analysis of the parasporal crystals, (Burges, 1984). Using this methopd Krywienczyk et al. (1978) were able to to divide H-serotype 3 or B. thuringiensis var kurtaki into two crystal serotypes. This crystal serotyping distinction was reinforced by differences in the toxicity spectrum of the two groups revealed in a later study (Dulmage and Co operators, 1981). According to the nomenclature recommendations of Burges (1984) the crystal serotype, if known, or the culture collection code number should be given, in addition to the variety name, when describing an isolate of B. thuringiensis.

The classification of B. thuringiensis by phagetyping has received little attention, but some workers have been able to use bacteriophages to distinguish divisions in given varieties of B. thuringiensis (Jones et al., 1983).

Classification of B. thuringiensis according to plasmid profiles has also been studied, but to a very limited degree (Jarrett, 1983). However, plasmids of B. thuringiensis are too easily lost and gained to be of useful benefit in terms of a long-term classification scheme, (Burges, 1984).

1.4.2.2 Sub-groups of B. thuringiensis based on flagellar antigen analysis.

New varieties (see 1.4.2.1 for definition) of B. thuringiensis are continually being found but at the time of writing there were at least

30 known, Burges (1984). These varieties are listed in Table 1.4.1. The information presented in this table was obtained from Dr. J.F Charles of the International Laboratory for Serotyping B. thuringiensis isolates, located at the Pasteur Institute in Paris, and was valid for April 1985.

1.4.3 Toxins Produced by B. thuringiensis

B. thuringiensis is known to produce a range of secondary metabolites which are toxic to insects. However, there are only two which are of current interest with regard to biological insecticides. These are the beta-exotoxin, thuringiensin, and the delta-endotoxin, or more correctly, the parasporal cyrstal.

The range of toxins produced varies from strain to strain and can also be variable within a given variety. Thus, the toxins produced by B. thuringiensis are not considered useful criteria for the classifications of the organism, but nonehtless, are of considerable fundamental interest (Bulla et al., 1980; Rogoff and Yousten, 1969).

A description of each of the known B. thuringiensis toxins follows.

1.4.3.1 Note on the classification of toxins produced by bacteria.

The terms endotoxin and exotoxin classically refer to mammalian-toxic materials and their definitions are described by Barry (1977) and Bonventure (1970). The toxins of B. thuringiensis do not fall into the classical divisions which have been used to categorisethe mammalian-toxic materials produced by other bacteria. A number of authors have, therefore, recommended that the terminology of exotoxin and endotoxin not be used when discussing the toxins of B. thuringiensis (Bulla et

TABLE 1.4.1

The known varieties of *B. thuringiensis*

VARIETAL NAME	H-SEROTYPE NUMBER	PARASPORAL CRYSTAL PRODUCTION	B-EXOTOXIN PRODUCTION
thuringiensis	1	+	+
finitimus	2	+	-
alesti	3a	+	
kurstaki	3a,3b	+	-
sotto	4a,4b	+	-
dendrolimus	4a,4b	+	-
kenyae	4a,4c	+	+
galleriae	5a,5b	+	-
canadensis	5a,5c	+	-
subtoxicus	6	+	-
entomodicus	6	+	-
aizawai	7	+	-
morrisoni	8a,8b	+	+
ostriniae	8a,8c	+	-
nigeriensis	8b,8d	+	
tolworthi	9	+	+
darmstadtiensis	10	+	+
toumanoffi	11a,11b	+	-
kyushensis	11a,11c	+	-
thompsoni	12	+	-
pakistani	13	+	-
israelensis	14	+	-
dakota	15	+	ND
indiana	16	+	ND
tohokuensis	17	+	+
kumatotoensis	18	+	+
tochigiensis	19	+	-
yunnanensis	20	ND	ND
pondicheriensis	21	ND	ND
colmeri	21	ND	ND
shandongiensis	22	ND	ND
wuhaniensis	-*	+	+
fowleri	-*	+	-

ND: not determined

* no flagella

al., 1980; Sebesta et al., 1981). This approach will be used as far as possible in this work, except when introducing the toxins for the first time where the range of names used for each will be given.

Bulla et al. (1980) recommended the use of the term parasporal crystal when referring to the actual inclusion particle, and parasporal or proteinaceous crystalline protoxin when discussing the moiety which comprises the inclusion. Thuringinesin has been suggested as a more appropriate term for the beta-exotoxin (Sebesta et al., 1981). These proposals have been adopted during this work.

1.4.3.2 Alpha-exotoxin, phospholipase C, lecithinase

This type of toxin is widely distributed in bacteria and is also found in B. cereus (Davidson, 1981). The enzyme mainly affects the plasma membrane causing lysis of the cell. The role of this toxic factor in the pathogenesis of B. thuringiensin is not known. (Faust and Bulla, 1982).

1.4.3.3 Beta-exotoxin or thurigiensin

This is a heat-stable, extracellular nucleotide derived toxin which affects the terminal stages of RNA biosynthesis (Sebesta et al., 1981). This toxin affects a wide variety of insects, much wider than the parasporal cyrstal, and also has toxicity for higher animals which is the reason that its use as an insecticide is not permitted in Western bloc nations (Lecadet and de Barjac, 1981). Only a small number of varieties of B. thuringiensis produce the toxin, Table 1.4.1 which means that by simply avoiding these strains an insecticide free of this substance can be produced. Serotype 1 is the variety

which produces the toxin in largest amounts where accumulation in the culture broth increases during log phase growth (Sebesta et al., 1981). Production of the toxin is not related to parosporal crystal and spore formation (Vankova, 1978). The toxin has been purified and artifically synthesized and its structure is shown in Figure 1.4.1. The artificial synthesis of the compound has paved the way for the production of analogues whose ratio of mammalian toxicity/insect toxicity may be altered to make the use of this compound or one of its derivatives more attractive. Such an advance would be of considerable importance as thuringiensin is known to kill insects of the orders Lepidoptera, Diptera, Coleoptera, Hymenoptera, Isoptera and Orthoptera and if a derivative lacking mammalian toxicity were found this would make possible the introduction of a chemical insecticide produced by B. thuringiensis. However, given the nature of chemical insecticides with regard to toxicity in non-target organisms and resistance in target pests it is likely that such an analogue would eventually become displaced by the biological insecticies of B. thuringiensis.

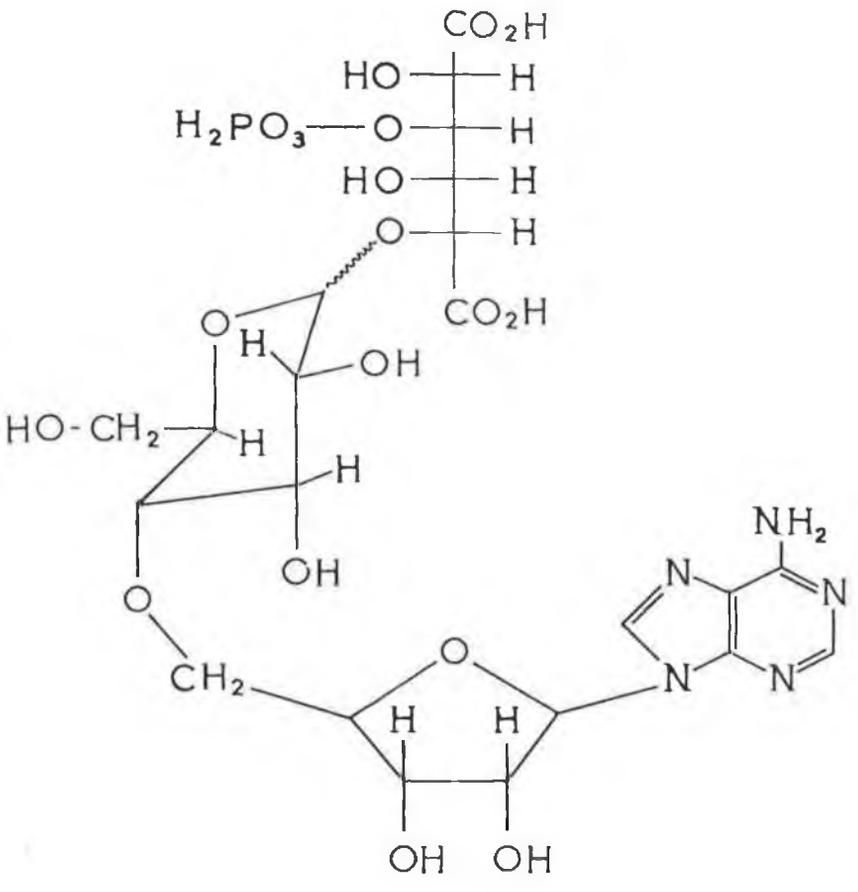
The mode of biosynthesis of thuringiensin is not known (Sebesta et al., 1981; Lecadet and de Barjac, 1981).

1.4.3.4 Gamma exotoxin

This is an unidentified enzyme(s) which causes clearing in egg yolk agar; no proof of its toxicity has been reported and its exact nature has yet to be discovered (Faust and Bulla, 1982).

FIGURE 1.4.1

Structure of thuringiensin, or the beta-exotoxin of B. thuringiensis



1.4.3.5 Parasporal crystal toxin

The parasporal crystal synthesized at the end of log phase at the same time of the spore will be the subject of 1.5 and will not be discussed in detail here.

1.4.4 Genetics of B. thuringiensis

Up until about 1980 knowledge of the genetics of B. thuringiensis was extremely limited, but since then a considerable body of work has been done in this area. A thorough review of this field is beyond the scope of this dissertation and the reader is referred to a number of recent reviews on the subject (Andrews et al., 1982; Lereclus, 1984; Martin and Dean, 1981; Whiteley et al, 1982). Instead, a limited number of important topics where significant advances have been made will be briefly discussed.

1.4.4.1 Plasmid analysis

Recently, a number of groups have studied the plasmid patterns in wild-type and acrystalliferous mutants with a view to determining if plasmid loss can be correlated with the loss of the ability of an isolate to produce a parasporal crystal.

Plasmid sizes have varied from less than five megadaltons to over 100 megadaltons (Gonzalez and Carlton, 1980, 1984). Using leptox strains, the loss of cyrstal production was associated with the loss of a different sized plasmid, depending on the actual isolate used (Gonzalez et al., 1981). Different isolates of a single serotype were found to have varying plasmid numbers and molecular weights; the loss of crystal producing capacity could not be associated with the absence

of any particular plasmid, but isolates lacking plasmids of greater than 33 megadaltons produced inactive parasporal crystals (Jarrett, 1983).

Similar studies done on B. thuringiensis var israelensis have indicated the presence of 6-9 plasmids of molecular weight from 4 to 135 megadaltons (Faust et al., 1983; Gonzalez and Carlton, 1984; Ward and Ellar, 1983). Three studies implicated the loss of a high molecular weight plasmid being responsible for the loss in crystal producing capacity (Gonzalez and Carlton, 1984; Kamdar and Jayaraman, 1983; Ward and Ellar, 1983, 1984), while a low molecular weight plasmid was suggested by another report (Faust et al., 1983). These investigations would seem to indicate conflicting results, however, in yet another study it was discovered that the crystal protein gene/s is located at a number of sites, including multiple plasmid locations and in one case on the chromosome itself (Kronstad et al., 1983).

These plasmid investigations reveal that B. thuringiensis contains a complex array of plasmids and the plasmid pattern varies from serotype to serotype, and even within isolates of the same variety. Furthermore, depending on the isolate, one or more plasmids may carry crystal protein coding genes and the loss of a variety of plasmids has been correlated with the loss in crystal producing capacity.

1.4.4.2 Genetic transfer systems and crystal protein gene cloning.

Transduction and transformation systems for the transfer of DNA into recipient B. thuringiensis cells have been developed in recent years (Fischer et al., 1981; Martin et al., 1981; Thorne 1978). However, the ability to transform B. thuringiensis was variety-dependent when plasmid PC 194 from Staphylococcus aureus was used (Fischer et al.,

1984; Martin et al., 1981).

Following on from the development of DNA transferring systems the cloning of the crystal protein gene from B. thuringiensis var kurstaki was reported (Schnepf and Whiteley, 1981). Further cloning studies have indicated that the crystal protein coding gene is located on a 45 kilo base pair plasmid and also of chromosomal origin in a given isolate of B. thuringiensis var kurstaki (Held et al., 1982). Klier et al. (1982) found that the location of the crystal gene was variety-dependent and that when the gene was cloned in Escherichia coli and B. subtilis inclusion bodies were formed which were toxic to insect larve. Of interest was this groups findings that crystal protein antigens were only detectable in transformed B. subtilis during and after sporulation. This suggested that the mechanisms for control of crystal protein expression were similar in B. thuringiensis and in B. subtilis.

Due to the close relationship between the different varieties of B. thuringiensis, B. cereus and B. subtilis it has been found that crystal-coding plasmids can be transferred between these species by mating (Gonzalez et al., 1982; Klein et al. 1983). Thus, B. subtilis, which had been transformed using a crystal-coding plasmid from B. thuringiensis was able to transfer crystal producing capacity to an acrystalliferous B. thuriensis var kurstaki mutant; furthermore, using the same transformed B. subtilis strain by cell mating, a recipient B. thuringiensis var israelensis strain was conferred with the ability to produce both types of parasporal crystal, toxic to lepidopteran and dipteran larvae (Klier et al., 1982).

Using crystalliferious strains of B. thuringiensis, mating with acrystalliferous strains of the same species allowed expression of

crystal-producing capacity in the latter. The size of the transmissable crystal-coding plasmid varied with the donor strain between 44 and 75 megadaltons (Gonzalez et al., 1982). With similar methods the same group also achieved cyrstal-coding plasmid transfer between crystalliferous B. thuringiensis and acrystalliferous B. cereus; the recipient B. cereus strain not only accepted the plasmids, but also produced crystals of the same antigenicity as the donor strain.

From this short account of some of the most recent and exciting investigations into the genetics of B. thuringiensis it can be seen that considerable advances have been made in this field during the last few years. What has emerged during these studies is that the crystal protein gene of B. thuringiensis varies in its location depending on the strain. In some it is chromosomal, and in other it is plasmid borne, with a number of these strains having more than one crystal-coding plasmid. The size of the crystal-coding plasmids is variable, but most of the available evidence suggests that high molecular weight plasmids are involved. The crystal protein gene has been cloned into a number of recipient bacteria, and, in some cases, these transcipients have been used to confer crystal-producing status on acrystalliferous B. thuringiensis mutants.

It is unlikely that this series of studies into B. thuringiensis will produce an organism capable of producing vastly increased amounts of parasporal crystal, because, as it is, the crystal may comprise up to 30% of the dry weight of the sporangium of wild-tyupe strains (Rogoff and Yousten, 1969). However, the study of this organism and the genetic control of the expression of its sporulation-specific parasporal crystal is being used as a convenient system for investigations into the control of bacterial sporulation.

1.5 THE PARASPORAL CRYSTAL OF BACILLUS THURINGIENSIS

As was mentioned previously, B. thuringiensis produces a proteinaceous, crystalline inclusion at the same time as spore formation takes place, 1.4.1., (Bulla et al., 1980; Norris, 1971). This particle has been previously called the crystalline inclusion body, delta-endotoxin, protein crystal, or the parasporal crystal or body. However, it will be referred to here as the parasporal crystal, the protein crystal, or simply as the crystal.

The parasporal crystal, and to a varying extent the spore wall, is the location of the toxicity associated with the particulate fraction of B. thuringiensis cultures, and this is the reason a large body of research has been devoted to its formation, molecular biology and mode of action which are summarized in the following sections.

It is important to note that toxicity to insects of intact parasporal crystals is exhibited only when the insect larva eats the material. Parasporal crystal of B. thuringiensis do not have contact toxicity, nor are they active when injected into the haemocoel of the insect.

1.5.1 Toxicity Spectra of the Parasporal Crystals of the Varieties of B. thuringiensis.

The parasporal inclusions of B. thuringiensis are almost exclusively toxic to insects from the orders Lepidoptera and Diptera with a small number of reports describing toxicity to insects from other orders (Van der Geest and de Barjac, 1982; Krieg and Langenbruch, 1981). Thus, the varieties of B. thuringiensis can be divided into two host spectrum categories, those which are toxic to lepidopteran insects (leptox varieties), and those which are toxic to dipteran insects

(diptox). It should be mentioned that leptox varieties are not toxic to all lepidopteran insects in the same way that all dipteran species are not affected by the diptox varieties of B. thuringiensis, indeed the diptox strains are usually found only to be toxic to members of the mosquito and blackfly families, Culicidae and Simulidae respectively,Figure 1.2.2. Table 1.5.1.shows the host preferences for the B. thuringiensis varieties. From this table it can be seen that the majority of varieties are toxic to lepidopteran insects while only one, B. thuringiensis var israelensis, is exclusively toxic to dipteran species.

1.5.1.1 Variation in preferred insect host between varieties of B. thuringiensis.

The majority of B. thuringiensis varities are toxic to lepidopteran insects, Table 1.5.1. However, research over the years has shown that tnere is considerable variation is the response of a given insect host to many of the varieties with which it was challenged (Dulmage and Co operators, 1981). This has led to the notion that, when preparing an insecticide against a particular pest it is important to ensure that the most virulent variety of B. thuringiensis against the insect is used.

The basis for this variation in host spectrum of isolates of B. thuringiensis is the slightly different toxins which make up the parasporal crysal produced by different varieties (Burgerjon and Martouret, 1971; Calabrese et al., 1980). In addition, it is also possible that the spores contribute some of the insectical activity, the exact amount depending on the bacterial variety and insect host (Burgerjon and Martouret 1971; Schesser and Bulla, 1970; Mohd-Salleh and Lewis, 1982). It has also been found that isolates within the

TABLE 1.5.1

Host toxicity of the varieties of *B. thuringiensis*

VARIETAL NAME	H-SEROTYPE NUMBER	LEPTOX	DIPTOX	REFERENCE
thuringiensis	1	+	+	Lacey and Mulla, 1977;
finitimus	2	-	-	Hall et al., 1977
alesti	3a	+	-	
kurstaki	3a,3b	+	+	Hall et al., 1977;
sotto	4a,4b	+	-	Sasamanti et al., 1982
dendrolimus	4a,4b	+	-	
kenyae	4a,4c	+	-	
galleriae	5a,5b	+	-	
canadensis	5a,5c	+	-	
entomodicus	6	+	-	
subtoxicus	6	+	-	
aizawai	7	+	-	
morrisoni	8a,8b	+	-	
ostriniae	8a,8c	+	-	
tolworthi	9	+	+	Hall et al., 1977
darmstadiensis	10	+	+	Padua et al., 1980;
toumanoffi	11a,11b	+	-	Finney and Harding, 1982
kyushensis	11a,11c	+	-	
thompsoni	12	+	-	
pakistani	13	+	-	
israelensis	14	-	+	
dakota	15	ND	ND	
indiana	16	ND	ND	
tohokuensis	17	ND	ND	
kumatotoensis	18	ND	ND	
tochigiensis	19	ND	ND	
yunnanensis	20	ND	ND	
pondicheriensis	20	ND	ND	
colmeri	21	ND	ND	De Luca et al., 1984
shandongiensis	22	ND	ND	
wuhaniensis	-[b]	+	-[a]	
fowleri	-[b]	-[a]	-[a]	

Unless otherwise stated, data are from Krieg and Langenbruch (1981).

[a] parasporal crystals not found to be toxic.
[b] no flagella
\+ some isolates toxic at high doses
ND: not determined

same varietal group have been found to synthesize antigenically different crystals which has been correlated with preferred toxicity against different insects. However, this situation is extremely complex and a major international programme is being conducted into the problem which is reviewed by Dulmage and Co operators (1981).

With regard to diptox varieties of B. thuringiensis only one has been shown to be exclusively pathogenic to dipteran insects, Table 1.5.1. Thus the concept of variable host spectra in B. thuringiensis var israelensis does not apply. In addition, of the different isolates of this variety which have been discovered since the first reported isolation by Goldberg and Margalait (1977), no evidence for increased toxicity of one or more isolates has been presented.

1.5.1.2 Contribution of spores to the insect toxicity of B. thuringiensis

The relative contribution of spores and parasporal cyrstals to the insect toxicity of B. thuringiensis varieties has been the topic of a number of studies, and has also been reviewed by Lecadet (1970).

What has emerged during these studies is that insects susceptible to B. thuringiensis can be grouped according to whether they are killed by parasporal crystals and/or spores of a single variety, or a number of varieties of B. thuringiensis. However, in the main, most affected insects are killed exclusively by parasporal crystals from a number of varieties with a smaller number more effectively, or exclusively, killed by crystals supplemented with spores of a range of varieties (Krieg and Langenbruch, 1981; Mohd-Salleh and Lewis, 1982; Schesser and Bulla, 1978; Sommerville et al., 1970; Tyrell et al., 1981).

The reason why spores enhance, or are required for toxicity of some varities of B. thuringiensis against some insects is not fully understood, but may be due to a number of factors. (1) The presence of parasporal crystal toxin in the spore wall of some varieties has been established (see Section 1.5.6) and this could account for the activity of the spores, especially if the spore wall toxin was slightly different, but complementary, to that in the parasporal crystal. (2) The spore may contain another, as yet undescribed, toxin. (3) Infection of the insect tissues by germination and multiplication in tne host may stress the insect to the point of causing death, or by weakening the larva sufficiently such that tne toxic action of the crystal may kill it.

The implication of the variable effect of spores and crystals of different B. thuringiensis strains on different insects further emphasises the necessity to use the most appropriate variety when controlling a given pest.

1.5.2 Biogenesis of the Parasporal Crystal and Spore in B. thuringiensis

The sporeforming events in bacilli have been extensively studied and reviewed (Aronson and Fitzjames, 1976; Holt and Leadbetter, 1969; Walker, 1970). In fact few aspects of the spore forming bacteria have received as much attention due to the importance of the sporulation event as a model for cellular differentiation.

1.5.2.1 Sporeformation in B. thuringiensis and other bacilli

The stages of sporeformation in B. thuringiensis have been studied and found to conform to the classical pattern of sporulation in bacilli. A full description of the results of these investigations is beyond

the scope of this thesis but have been adequately dealt with elsewhere (Bechtel and Bulla, 1976; Bulla et al., 1980; Fast, 1981; Norris, 1971). The steps involved in sporulation of bacilli can be summarized as follows:-

Stage 1	Axial filament formation
Stage 2	Forespore septum formation
Stage 3	Engulfment
Stage 4-7	Spore wall development
Stage 8	Spore maturation, and sporangial lysis

1.5.2.2 Crystal formation in B. thuringiensis

The exact stage at which crystal formation begins is uncertain. Bechtel and Bulla (1976) reported that assembly of the crystal began at stage 3 when studying B. thuringiensis var kurstaki. But, Mikkola et al. (1982) found that significant crystal formation had occurred by stage 2 in B. thuringiensis var israelensis. Both these studies used electron microsciope data to support their claims. Using a biochemical approach with B. thuringiensis var thuringiensis, it was found that crystal synthesis began between stages 2 and 3, and was substantially complete by Stage 4 (Lecadet and Dedonder, 1971). These results were largely confirmed in the study of Sommerville (1971) who used B. thuringiensis var alesti and B. thuringiensis var tolworthi. Thus it appears that crystal formation begins early during sporulation with some evidence to suggest that this event starts sooner in some varieties than in others.

Regardless of the variety studied, crystal formation always followed the end of log phase growth and took place by the assembly of protein

synthesized de novo at the beginning of sporulation (Herbert and Gould, 1973; Lecadet and Dedonder, 1971; Monro, 1961; Sommerville, 1971).

The locus and mechanism of assembly of the parasporal crystal in B. thuringiensis has been the topic of conflicting reports. Sommerville (1971, 1978), Sommerville and James (1970), Scherrer and Sommerville (1977) found evidence to suggest that assembly of the crystal took place on the exosporium and began at stage 2-3. However, this was not confirmed in the comprehensive electron microscope study of Bechtel and Bulla (1976) who found nascent crystals present at least one hour before the synthesis of the exosporium, which has been shown to begin during stage 4 (Bulla et al., 1980). Despite the assertion that the exosporium may serve as a template for crystal assembly (Sommerville, 1978) the fact that exosporium synthesis occurs after crystals are first seen, and also because the crystal protein subunit tends to aggregate so strongly such a template may not even be needed (Fast, 1981), some authors have tended to reject the notion of the template role of the exorposium during parasporal crystal synthesis (Bulla et al., 1980). Indeed, it has been suggested that crystal assembly is largely complete by the time exosporium synthesis even begins (Bulla et al., 1980). Furthermore, the same authors have reported evidence showing association between the developing crystal and the forespore membrane, which has been supported in another study (Mikkola et al., 1982).

Despite some disagreement regarding the function of the exosporium during parasporal crystal assembly, electron microscope studies have always shown that this crystal is formed outside the exosporium (extraexosporial), except for B. thuringiensis var finitimus where the crystal is deposited inside the exosporium (intra-exosporial), along with the spore itself (Aronson and Fitzjames, 1976; Bulla et al.,

1980; Mikkola et al., 1982). This intra-exosporial deposition of the parasporal crystal has also been reported for B. popillae (Aronson and Fitzjames, 1976; Bulla et al., 1978), and for B. sphaericus 2297 (Dr. J. F. Charles, personal communication). Because most strains of B. thuringiensis deposit their parasporal crystals extraexosporially, following lysis, the spore and crystal are released into the culture broths as independent entities. This has proved to be of considerable importance as it has allowed the purification of crystals from spores by a variety of techniques (see Section 1.5.7.1).

1.5.2.3 Ovoid inclusion synthesis

In addition to the synthesis of the parasporal crystal during the early stages of sporulation, another particle described as a"non-crystalline ovoid inclusion" has been reported (Bechtel and Bulla, 1976; Bulla et al, 1980). Only one such inclusion was formed per cell and it was not established if it possessed insecticidal properties.

1.5.3 Lattice Arrangements in the Crystal

A number of studies have provided evidence to show that the arrangement of protein molecules in the parasporal crystal does have long-range order, and that the particle is not simply an amorphous body (Bulla et al., 1980; Holmes and Munro, 1965; Norris, 1969 a, b). These studies, using evidence from electron microscopy and X-Ray diffraction techniques, have suggested that the protein molecules are ellipsoid or rod-shaped, and are arranged in pairs with each monomer having a molecular weight of about 130,000. These results were all obtained using leptox strains of B. thuringiensis.

Mikkola et al. (1982), using crystals purified from both leptox and diptox strains, found the regular lattice arrangements in the leptox crystals. In the diptox strains the inclusions were found to be made up of a number of subinclusions of varying electron densities. Similar results were found by other workers when studying diptox strains (Charles and de Barjac, 1982). Of the various subinclusions making up the insecticidal particle in B. thuringinesis var israelensis, some were found to have a crystalline arrangement, while others were not (Mikkola et al., 1982). The significance of the multiple inclusion types, and their relative insect potencies are not yet known. Suffice to say at this stage that this is one of the many factors to be described during this introduction which differentiates these two types of B. thuringiensis strains.

1.5.4 Relationship between Parasporal Crystal Shape and Insect Toxicity Type

Parasporal crystals of leptox strains are rhomboidal (Bechtel and Bulla, 1976;Bulla et al., 1980; Norris, 1969 a, b.) However, recent studies on B. thuringiensis var israelensis and on diptox isolates of normally leptox varieties, have revealed that crystals demonstrating exclusive toxicity to mosquitoes are round and irregularly shaped; further more, these parasporal crystals are usually made up of a number of "subinclusions" of varying shape and electron density and are held together in an unordered manner (Charles and de Barjac, 1982; Mikkola et al., 1982; Padua et al., 1982).

Mikkola et. al. (1982) have suggested that B. thuringiensis may therefore be classed into two groups based on the shape and structure of the crystal formed, which can clearly be correlated to the type of insect toxicity demonstrated by the particles. This observation is

unlikely to be of major taxonomic importance for B. thuringiensis, but might possibly be of use when indentifying diptox isolates of varieties normally classified as leptox.

1.5.5 Parasporal Crystal Nets

Usingthe electron microscope, parasporal crystal complexes were seen to be bounded by a granular crystalline net in diptox crystals (Bulla et al., 1980; Charles and de Barjac, 1982; Mikkola et al., 1982). This net has also been observed in association with diptox crystals and, in these varieties at least, appears to be proteinaceous (Bulla et al., 1980; Fitzjames et al., 1984).

In the leptox strains this net has been found to be toxic to insects (Bulla et al., 1980; Fitzjames et al., 1984), but its toxicity has not yet been examined in the case of the diptox crystals.

A "meshlike-envelope" has also been described surrounding the parasporal inclusions of certain strains of B. sphaericus (Yousten, 1984). As yet, the exact role of these envelopes in either species has not been elucidated, but Bulla et al. (1980) drew attention to the fact that these structures bore some ultrastructural resemblance to the exosporia of a number of Bacillus and Clostridium species, including B. thuringiensis itself. They suggested that it might be exosporial material deposited around the crystals after they had been synthesized. This would agree with the findings of Fitzjames et al. (1984) who found that the formation of the net seemed to be associated with a late sporulation stage (6), when crystal synthesis has been shown to be largely complete (Mikkola et al., 1982).

1.5.6 Location of Parasporal Crystal Protein in the Spore Coat of *B. thuringiensis*

Numerous reports have provided evidence to suggest that some parasporal crystal protein is located in the spore coats of *B. thuringiensis*. These studies have mainly been done by preparing antisera against solubilized crystals and reacting these antisera with solubilized spore extracts (Aronson et al., 1982; Delafield et al, 1968; Lecadet and Dedonder, 1971; Lecadet et al., 1972; Scherrer and Sommerville, 1977; Sommerville et al., 1968, 1970; Tyrell et al., 1980). However, in only one of these studies were spore extracts actually shown to be insecticidal (Scherrer and Sommerville, 1977).

Additional evidence has been revealed using electron microscopy to locate crystal antigens in the spores, and in one case on the exosporium, of *B. thuringiensis* by treating ultra-thin sections with ferritin-labelled crystal antiserum (Short et al., 1974; Walker et al., 1975).

Some studies have even found a relationship between the parasporal crystal protein of *B. thuringiensis* and the spore coats of *B. cereus* (Aronson et al., 1982; Sommerville and Pocket, 1975; Sommerville et al., 1970), the spore coats of two other bacilli, *B. megaterium* and *B. subtilis* (Sommerville et al., 1970) as well as of *Clostridium roseum* (Sommerville et al., 1970). However, in only one of these studies which compared *B. cereus* spore extracts to solubilized parasporal crystals of *B. thuringiensis* was the extract shown to have insect toxicity (Sommerville and Pockett, 1975).

The considerable body of biochemical and immunological evidence described above has been used to suggest why spores of some varieties

of B. thuringiensis demonstrate insect toxicity. A number of authors have also suggested that because of this relationship, parasporal crystal production by B. thuringiensis may represent an overproduction of a component of the spore wall (Fast, 1981; Sommerville, 1978).

However, in a recent report Meenakshi and Jayaraman (1979) were able to separate the processes of crystal and spore formation by the addition of chloramphenicol during the first 3 hours of sporulation. This treatment had a pronounced inhibitory effect on spore formation, but not on the synthesis of the crystal. Thus the results of these authors would seem to suggest tnat crystal synthesis, although normally temporally linked to that of spore formation, was fundamentally independent of spore formation and therefore might not represent an overproduction of a spore wall component.

1.5.7 The Molecular Biology of the Crystal

Fundamental to our understanding of the toxicity of B. thuringiensis is the discovery of the biochemical nature of the parasporal crystal. The achievement of this goal has been attained only within the last few years, and looking back in retrospect it can be seen where errors were made due to a lack of fundamental knowledge of the chemistry of the crystal.

1.5.7.1 Purification of the parasporal crystal

The parasporal crystal is produced at tne same time as the spore and both have similar size and density which makes purification using conventional methods of filtration and simple centrifugation impractical.

The floatation technique described by Sharpe et al. (1979) is a simple method which can be used as a preliminary step for removing a large percentage of the spores present. This procedure requires no sophisticated equipment and a significant removal of spores can be effected in an hour.

Using an aqueous two-phase system based on Dextran Sulphate and Polyethylene Glycol 6000 (PEG) Goodman et al. (1967) and Delafield et al. (1968) obtained crystal preparations of 99% purity, again without using sophisticated equipment or large amounts of expensivematerials. Because of these advantages this was the type of system used during this work for the purification of crystals from B. thuringiensis var israelensis.

Several other techniques using density gradient centrifugation have been described, (Ang and Nickerson, 1978; Milne et al., 1977; Sharpe et al., 1975) which require the use of specialised equipment, and sometimes large amounts of costly chemicals; it was for tnese reasons that these techniques were not used during this work.

1.5.7.2 Proteases associated with the parasporal crystals of B. thuringiensis

Evidence to support the idea of there being proteases associated with parasporalcrystals in strong. Chestukhina et al. (1978,1980) using crystals purified from a number of varieties of B. thuringiensis found detectable serine protease and leucine aminopeptidase activity in the purified crystal preparations. They also found that when purified crystals were prepared and solubilized under conditions which did not attempt to prevent the action of proteases, that the gel electrophoresis patterns of such solutions were markedly different

from when protease activity was prevented. In their protease-inhibited solutions electrophoresis revealed only the presence of a small number(1 or 2) of high molecular weight proteins, whereas when solutions with uninhibited protease activity were electrophoresed, bands of high molecular weight as well as of lower molecular weight appeared, with the number of the latter increasing with the time of incubation of the solutions before electrophoresis. After prolonged incubation (48h at 37°C) only bands of the lower molecular weight variety were seen in solutions where proteases were not inhibited.

Other workers have also detected protease activity in association with purified crystal preparations, (Chilcott et al., 1981, 1983; Nickerson and Swanson, 1981). The latter authors have also suggested that inhibition of protease activity during purification and processing of crystal preparations in desirable for the reliable determination of native crystal biochemistry.

1.5.7.3 Solubilization of the parasporal crystals

Parasporal crystals of B. thuringiensis are insoluble in water at neutral pH. This fact has contributed largely to the slow elucidation of their biochemistry (Luthy, 1980).

Since the mid 1960's a large number of systems has been described for the dissolution of the crystals. It would not be possible to discuss all of these methods here, but they have been the subject of a number of reviews and the reader is referred to these for more detailed information (Bulla et al., 1980; Cooksey, 1971; Fast, 1981; Huber and Luthy, 1981; Luthy 1980; Luthy et al., 1982; Nickerson, 1980).

Basically, strong reducing agents such as dithiothreitol (DTT) at pH

9-10 are required to dissolve leptox crystals and maintain their native state (Luthy, 1980). Native leptox crystal protein was also obtained by titrating a crystal suspension with NaOH at pH 12 (Bulla et al., 1977, 1979, 1981). Crystals dissolved under these conditions retained insect toxicity. However, crystals may also be completely dissolved using 0.1M NaOH which gives a non-insecticidal solution (Bulla et al., 1976; Huber and Luthy, 1981; Luthy, 1980). By contrast to the leptox crystals, diptox crystals may be dissolved in the absence of reducing agents using low NaOH concentrations. However, diptox crystals dissolved under these conditions are not toxic to mosquito larvae, but are lethal to cultured cells and erythrocytes (Armstrong et al., 1985; Thomas and Ellar, 1983a).

Solubilization of the crystals seems to follow a set pattern. Firstly, swelling of the crystals occurs at high pH, or in the presence of denaturing agents, followed by dissolution after addition of disulphide cleaving reagents. No effect on the crystals is caused by the use of reducing agents alone (Huber et al., 1981). Using protease-containing gut juice from the silkworm, Bombyx mori, Tojo and Aizawa (1983) also found that dissolution of the crystal followed swelling at alkaline pH, after which the protease was able to act on the crystal. Dissolution and swelling did not occur at neutral pH, regardless of the presence of protease.

Once solubilized, crystal protein solutions can tend to reaggregate, but this behaviour can be prevented by the use of alkylating agents such as iodoacetimide, (Bulla et al., 1977, 1981) which block sulphydryl groups.

The reason cleavage of S-S bonds is required for dissolution of the leptox crystals is apparently due to the fact that these bonds are

responsible for the intermolecular attractions between the protein subunits in the crystal (Dastidar and Nickerson, 1978). Swelling caused by alkaline, or denaturing conditions allows the interchain disulphide linkages to be broken by the use of reducing agents (Huber et al., 1981). These conditions allow crystal dissolution with the retention of toxicity to take place (Huber et al., 1981). It is apparent from the severe treatments required to achieve crystal solubilization that a complex series of forces including disulphide links and non-covalent bonding is operative in maintaining the conformation of the native crystal.

1.5.7.4 Composition of the parasporal crystal.

Analyses of solubilized crystals from a number of leptox varieties have not shown significant differences in amino and composition (Huber and Luthy, 1981). As with crystal solubilizing systems, a large number of authors have published data for amino acid compositions of B. thuringiensis parasporal crystals and their results have been reviewed. (Bulla et al, 1980; Cooksey, 1971; Fast, 1981).

Typically, amino acid composition data suggest relatively high frequency of glutamate and aspartate residues of 10-15% each, which accounts for the low isoelectric points observed for solubilized crystals, (Huber and Luthy, 1981). Half-cystine, methionine, tryptophan, and histidine occur far less regularly, about once each for every hundred residues (Cooksey, 1971; Fast 1981).

Amino acid analysis of crystals from the diptox B. thuringiensis var israelensis have been reported to be significantly different from those of leptox crystals (Huber and Luthy, 1981; Tyrell et al., 1981) where lysine, threonine, proline, alanine, methionine, valine and

isoleucine levels were higher and arginine, serine, glutamate and glycine levels lower for the diptox crystals. This difference underlines the difference in the parasporal crystals produced by the leptox and diptox varieties of B. thuringiensis which has been described before, 1.5.4.

The data concerning the carbohydrate content of the crystals are conflicting. Bulla et al. (1977, 1981) reported that crystals of B. thuringiensis var kurstaki contained about 5% carbohydrate made up of 3.8% glucose and 1.8% mannose. In a study by Nickerson (1980) in which 16 strains of B. thuringiensis were studied, carbohydrate contents of 1-5% were found, however the possibility that these sugars were not covalently bound was not excluded. Bateson and Stainsby (1970) reported finding 12% carbohydrate which was mainly glucose, but lesser amounts of mannose, xylose, arabinose were also present in the B. thuringiensis var thuringiensis crystals. Holmes and Munro (1965) reported 0.5% carbohydrate, but suggested this may have come from contaminating spores. Huber etal. (1981) found no significant quantities of carbohydrate in crystals from B. thuringiensis var thuringiensis, but only after extensive washing and suggested that non-covalent bonding of sugars to the crystal surface would occur. A similar lack of carbohydrate in crystals from B. thuringiensis var dendrolimus was found by Nagamatsu et al. (1984).

Tyrell et al. (1981) using a range of varieties found significantly higher levels of carbohydrate in crystals from B. thuringiensis var israelensis than in crystals from leptox strains. The diptox crystals were found to contain glucose, mannose, fructose, rhamnose, xylose and galactosamine, whereas in keeping with the results of Bulla et al. (1977) the leptox crystals were found only to contain glucose and mannose.

The question of carbohydrate content of the parasporal crystals therefore remains open due to the lack of agreement between the various published reports. However, even in the cases where workers did find evidence for carbohydrate in the crystals this material was not found to bear a relationship with the crystal toxicity (Nickerson, 1980). Thus, it is accepted that even if it was to be confirmed that the parasporal crystals do contain carbohydrate, the the sugar would be unlikely to contribute to the insect toxicity (Huber et al., 1981).

1.5.7.5 Parasporal crystal protein subunits

Numerous molecular weight determinations have been made on the proucts of crystal solubilization resulting in reported molecular weights of solubilized proteins ranging from less than 5,000 to greater than 200,000.

The variation in the results reported seems to be caused by a number of factors:-

(1) The method of crystal solubilization

(2) The method of molecular weight determination

(3) Possible actual variation in the molecular weight of subunits from different varieties.

Of these, it is most likely that the method used for crystal solubilization caused the variation. As has been previously mentioned (1.5.7.2) proteases adsorbed to the crystal surface, if left in an active form, can cause hydrolysis of the crystal protein leading to the detection of fragments of lower molecular weight than the original subunits. Also, if unusually harsh solubilization conditions are used this may lead to the denaturation and partial breakdown of the subunits.

In the most recent studies it has been proposed that the parasporal crystal is comprised of one or two protein subunits of similar molecular weight of around 130,000 daltons (Bulla et al., 1980; Fast 1981; Huber and Luthy, 1981).

This conclusion has been arrived at on the basis of a number of recent reports, Table 1.5.2. Molecular weight determinations done under non-denaturing conditions suggest that the subunit of $1.3\text{-}1.5 \times 10^5$ daltons may actually exist as a dimer in the native crystal (Huber

TABLE 1.5.2

Results of recent reports indicating molecular weights of the subunits of the parasporal crystal of B. thuringiensis

VARIETY	MOL. WTS. ($X10^{-3}$) OF SUBUNITS UNDER DIFFERENT CONDITIONS OF DETERMINATION		REFERENCE
	Non denaturing	Denaturing	
kurstaki	ND	134	Bulla et al., 1977, 1979, 1981
range of leptox varieties.	230±3	130	Huber et al., 1981
israelensis	24	24	
kurstaki	ND	135	Yamomoto and Iizuba, 1983
kurstaki	ND	135	Yamomoto and McLaughlin, 1981
kurstaki	ND	120	Tojo and Aizawa, 1983
kurstaki	ND	134	Tyrell et al., 1981
israelensis	ND	26	
tolworthi	ND	120	Herbert et al., 1971
dendrolimus	230	ND	Nagamatsu et al., 1978
	ND	145	Nagamatsu et al., 1984
israelensis	ND	26	Aronson et al., 1982
range	ND	130-145	Chestukhina et al., 1980, 1982
range	ND	135-165	Calabrese et al., 1980

ND: Not determined

et al., 1981; Nagamatsu et al., 1978) who both found a protein of 2.3 x 10^5 daltons under their conditions. This idea is supported by X-Ray diffraction study of Holmes and Munro (1965) whose data suggested the presence of a dimer of the same molecular weight in the crystals of B. thuringiensis var thuringiensis (see Section 1.5.3).

Investigations into the B. thuringiensis var israelensis crystal consistently demonstrated the presence of a major protein of about 25,000 daltons (Aronson et al., 1982; Huber et al., 1981; Tyrell et al, 1981). However, two of these groups also detected material of molecular weight 1.3 x 10^5 which suggests that this variety's subunit may be similar in size to the leptox crystals, but that under conditions which do not denature the leptox subunit, the diptox subunit is significantly degraded. Alternatively, it may actually be that the subunit of the B. thuringiensis var israelensis crystal has a completely different molecular weight to that of its leptox counterparts. Either case further emphasises the fundamental differences in the molecular nature of these two types of parasporal crystal, which has already been suggested by other lines of evidence, 1.5.4.

The results of a number of groups who have studied the crystal subunits of a range of B. thuringiensis varieties with regard to molecular weight determinations by polyacrylamide gel electrophoresis (PAGE) are summarized in Table 1.5.3.

Huber et al. (1981) found that all the leptox varieties studied produced a crystal subunit under their conditions of molecular weight 1.3 x 10^5. Chestukhina et al. (1980) reported that each variety, except B. thuringiensis var galleriae, produced only a single subunit, but that the molecular weight of the subunit varied from 1.30 - 1.45 x 10^5 daltons depending on the variety. Using B. thuringiensis var

TABLE 1.5.3

Molecularweightsof crystal subunits of a range of B. thuringiensis varieties

VARIETY	MOLECULAR WEIGHT ($X10^{-3}$) OF SUBUNIT/S		
	Calabrese et al., 1980	Chestukhina et al., 1980	Huber et al., 1981
thuringiensis	160;143	145	130
finitimus	ND	130	130
alesti	153	135	ND
kurstaki	155;143	130	130
sotto	164;153	ND	130
dendrolimus	162;148	135	ND
kenyae	148;63	ND	ND
galleriae	157;147;59	130;135;135;130;65*	130
canadiensis	155;139	ND	ND
entomodicus	47;40	ND	130
subtoxicus	155;137	ND	ND
aizawai	153	ND	130
morrisoni	155;148;142	135	130
tolworthi	148;63	130	130
darmstadiensis	148	135	ND
toumanoffi	157;150;143	ND	ND
thompsoni	47;40	130	ND
israelensis	ND	ND	25

ND: not determined

* results varied with isolate

galleriae they found that the number of subunits and their molecular weight depended on the isolate used, Table 1.5.4. Calabrese et al. (1980) obtained rather different results in their study with most varieties forming at least one high molecular weight species and some forming as many as three. Lower molecular weight bands of 50,000 - 70,000 daltons were also detected in some instances which were probably the result of limited proteolysis, as higher molecular weight bands were also seen in the gel tracks. The high molecular weight materials observed as the major components of most crystal varieties all fell in the 1.35 - 1.65 x 10^5 dalton range which was similar to the results of the other two groups, Table 1.5.3.

Considering the evidence for the model of the parasporal crystal subunit as a high molecular weight material it is unusual that a number of reports have claimed to have isolated low molecular weight fragments of less than 5000 daltons, some of which have been claimed to be toxic (Aronson and Tillinghast, 1976; Fast and Angus, 1970; Fast and Martin, 1980; Fast and Milne, 1979; Sayles et al., 1970). In an attempt to assess the validity of these results Andrews et al. (1981) repeated some of the work of the above authors and failed to find toxicity in any fragment of less than 68,000 daltons. These were also the findings of two other reports, (Bulla et al., 1979; Lilley et al., 1980).

Thus, it seems from the considerable body of results published that the most likely model for the composition of the parasporal cyrstals of *B. thuringiensis* is that two subunits of approximately 1.3 - 1.5 x 10^5 daltons form a dimer which is the basic crystal forming unit. The leptox crystals may be dissolved in aqueous solution, but require the presence of reducing agents and high pH or denaturing materials to effect the dissolution. The question of the presence and toxicity of

very low molecular weight fragments in the crystal, as yet, remains unresolved with only the evidence of a small number of reports to support the idea.

1.5.7.6 The protein toxin from the parasporal crystal

Leptox crystals dissolved under relatively mild conditions have been reported to retain their toxicity (Huber et al., 1981; Yamamoto and McLaughlin, 1981) when the toxin is administered *per os*, but are non-toxic when injected into the haemocael. This suggested that the high molecular weight subunit is a protoxin which is activated, presumably by proteases, to form a lower molecular weight toxin in the insect gut.

A number of publications suggested that major products of the solubilization of the parasporal crystals had molecular weights of about 1.3×10^5 and $6\text{-}7 \times 10^4$ (Bulla et al., 1977, 1979, 1980,; Lilley et al., 1980; Yamomoto and McLaughlin, 1981).

Using leptox strains of *B. thuringiensis* Bulla et al., (1979) found that the parasporal crystal could be dissolved under mild conditions to yield a protein of 130,000 daltons which was toxic only *per os*. When this material was left for a number of days under alkaline conditions a protein of molecular weight 68,000 was produced which was toxic *per os* and by injection into the haemocoel. Similar results were obtained by Lilley et al. (1980), Yamomoto and McLaughlin (1981), Tyrell et al. (1981), Yamomoto (1983), Yamomoto and Iizuka (1983) and by Tojo and Aizawa (1983). These results have allowed the proposal to be put forward by Bulla et al. (1980) that the toxic moiety is a component of the parasporal crystal which is produced following alkaline solubilization to release what is called the "protoxin".

This protoxin is then activated to produce the "toxin" by proteases found in the alkaline insect gut (Lilley et al., 1980; Tojo and Aizawa, 1983). The toxic moiety of leptox crystals has been reported to have a molecular weight of 65,000 ± 5,000, and is apparently relatively resistant to further proteolytic degradation. However, if further proteolysis does occur, a decrease in toxicity results (Lilley et al., 1980; Tojo and Aizawa, 1983).

The situation with crystals of B. thuringiensis var israelensis is less clearcut. Dissolution of the crystals under alkaline conditions similar to those used to dissolve the leptox crystals produces proteins with a range of molecular weight from 20,000 - 140,000 (Armstrong et al., 1985; Davidson and Yamomoto, 1984; Huber et al., 1981; Pfannenstiel et al., 1984; Tyrell et al., 1981; Yamomoto et al., 1983).

In most cases, despite the range of proteins observed, the major component was a protein, or a number of proteins, of 25,000 - 28,000 molecular weight (Armstrong et al., 1985; Thomas and Ellar, 1983a; Tyrell et al., 1981). Studies on the 25,000 - 28,000 daltons proteins have provided evidence to suggest that alkali solubilization produces a 28 kilodalton (kd) protein which is proteolytically cleaved to produce a 25 kd moiety. This latter species is toxic to cultured lepidopteran and dipteran cells, neonatal and adult mice (on injection), and is cytolytic to human, rabbit and sheep erythrocytes (Armstrong et al., 1985; Pfannenstiel et al., 1984; Thomas and Ellar, 1983; Yamomoto et al., 1983).

Thus, it seems likely that the parasporal cyrstal of B. thuringiensis var israelensis is composed of at least one high molecular weight species or protoxin. And, that on alkali solubilization (which would

occur in the guts of mosquito larvae which have a pH of greater than 9; Dadd, 1975) this protoxin yields a 28 kd species which is proteolytically converted to a relatively protease-resistant toxin (Armstrong et al., 1985).

Even though evidence has been described to suggest that the 25kd protein is proteolytically derived from the 28kd species and that the former is cytotoxic to a number of cell types (Armstrong et al., 1985), it is not yet established if the 28kd precursor possesses toxicity, per se. Neither has it been shown conclusively that the 25/28 kd cytolytic factor/s is responsible for the in vivo insect toxicity of the native crystals. This difference in the toxic moiety and degradation pattern of leptox and diptox crystals further underlines the divergence between these two types of B. thuringiensis strain which is also revealed by their different insect toxicity spectra and parasporal crystal morphologies, (See Sections 1.5.1. and 1.5.4).

1.5.8 Mode of action of the Parasporal Crystal

In both leptox and diptox strains of B. thuringiensis the primary site of action is the midgut epithelium of the target insect (Luthy and Ebersold, 1981). The toxic effect follows ingestion of native crystals by the larva and crystal solubilization and activation of the protoxin to release the toxin by the action of the proteases in the alkaline insect gut. Death usually occurs in 30 - 60 minutes depending on the dose ingested and is due to the toxic nature of the parasporal crystals and not to an invasion of the insect tissues, although this may follow the decease of the insect (Luthy and Ebersold, 1981).

1.5.8.1 Histopathology of the effects due to the crystal toxin

The general pattern of ultrastructural cellular disruption of susceptible target tissues is similar for both leptox and diptox insects using both intact larvae and cultured cell lines (Luthy and Ebersold, 1981).

The affected cells swell and develop balloon-like protrusions on their surface, which arise from microvilli, within minutes of toxin ingestion. Later, the epithelial cells separate from each other and often burst releasing their cytoplasmic contents into the gut lumen (Luthy and Ebersold, 1981). At the subcellular level the endoplasmic reticulum disintegrates; following this the mitochondria first of all condense and then swell with the loss of internal cristae. These changes were noted for cultured as well as native, midgut epithelial cells (Charles, 1983; Charles and de Barjac, 1982, 1983; Luthy and Ebersold, 1981). These alterations are accompanied by a dramatic increase in the permeability of the brush border membrane which is exposed to the gut lumen (Luthy and Ebersold, 1981). Thus, the effects of these toxins lead to gross histological alterations of the target cells. The effects of the crysals on cultured insect cells can only be seen following exposure to solubililzed crystals (Ebersold and Luthy, 1981; Thomas and Ellar, 1983 a,b). For solubilized leptox crystals, toxic effects are only observed with certain insect cell types which indicates that this toxin is highly specific (Luthy and Ebersold, 1981).

However, this is not the case for solubilized crysals from B. thuringiensis var israelensis which are toxic to mammalian, lepidopteran and dipteran cell lines (Armstrong et al., 1985; Thomas and Ellar, 1983a).

1.5.8.2 Gross physiological effects of the crystal toxin

Using a range of insects, a number of gross physiological effects have been noted which are consistent with membrane disruptions in target cells, both *in vivo* and *in vitro*.

Interference of glucose and ion transport in epithelial cells has been frequently reported (Fast and Donaghue, 1971; Fast and Morrison, 1972; Griego et al., 1978; Harvey and Wolfersberger, 1979; Pendleton, 1970).

Uncoupling of mitochondrial respiration was reported by an uncharacterized toxin isolated from a spore/crystal mixture (Faust et al., 1974; Travers et al., 1976).

Following reports that the leptox crystal caused gut paralysis in susceptible insects, one group used "purified toxin" to show a nerve-blocking effect on tissue from adult cockroaches, *Periplaneta americana*, an insect not known for its susceptibility to *B. thuringiensis* (Cooksey et al., 1969). Solubilized crystal protein from the parasporal crystals of *B. thuringiensis* var *israelensis* has also been found to inhibit nerve transmission in isolated ganglia from this insect (Chilcott et al., 1984).

Thus these studies reported distortions on a cellular scale and with the exception of the oxidative phosporylation uncoupling theory, none attmpted to suggest how tne crystal protein toxin exerted its affect on a molecular scale. Even at this the uncoupling model was only suggested on the basis of work done with mithochondrial preparations without explaining which molecular species was performing the alleged uncoupling nor how this species was capable of such an activity.

1.5.8.3 Molecular basis of the mode of action of the crystal protein toxin.

In a recent series of publications the mode of action of the parasporal crystal of B. thuringiensis was investigated using the purified, 65kd leptox toxin, and a purified preparation of the 28/25 kd diptox cytolytic factor (Knowles et al., 1984; Thomas and Ellar, 1983, a,b).

Using cultured insect cells, Knowles et al. (1984) tested the effect of preincubation of a number of typical cell surface components with the purified leptox protein toxin. They found that N-acetylgalactosamine, N-acetylmuramic acid, soybean agglutinin and wheat germ agglutin (which both bond N-acetylglucosamine) all inhibited the action of the toxin. This toxin was atoxic to suckling mice when injecting subcutaneously, and was haemolytically ineffective against human erythrocytes.

Preincubation with a variety of lipid preparations did not attenuate the effect of the toxin against cultured insect cells, suggesting the material did not act by interfering with membrane phospholipids as was shown for the toxin of B. thuringiensis var israelensis (Thomas and Ellar, 1983).

Thus, because two monosaccharides prevented the action of the toxin on cultured insect cells and because lectins which bound these sugars (and would thus have bound them on the cell membrane) inhibited the toxin also, it was proposed that the parasporal cyrstals of B. thuringiensis var kurstaki acted by binding to specific glycoprotein or glycolipid receptors on the cell membrane, and thereby caused cell leakage (Knowles et al., 1984).

A similar line of investigation led the same group to conclude that

the toxin from the B. thuringiensis var israelensis parasporal crystal worked by means of a detergent-like action on the phospholipid membranes of a range of cell types (Thomas and Ellar, 1983a) . These authors suggested that the mode of action of this toxin was similar to other cytolytic toxins such as Streptolysin S and Staphylococcus alpha-toxin acting on mammalian cells by a protein surfactant mechanism which destroys membrane integrity.

Thus these two studies, in addition to demonstrating a possible mode of action of the two toxins, provided further evidence to suggest the fundamental differences between the parasporal crystals of the leptox and diptox strains of B. thuringiensis which has been alluded to on previous occasions (1.5.1; 1.5.4; 1.5.7.6)

This difference was very clearly demonstrated in another study by Thomas and Ellar (1983b) using purified toxin materials. During this study it was found that alkali-solubilized B. thuringiensis var israelensis crystal exhibited a general cytolytic effect against cultured lepidopteran, dipteran and mammalian cells as well as against rat, mouse, sheep, horse and human erythrocytes. In addition, this soluble preparation was toxic when injected intraveneously to suckling and adult mice at doses of 15 - 20 ug of protein per gram body weight, but was atoxic when administered per os.

In contrast, solubilized B. thuringiensis var kurstaki toxin was lethal only to cultured lepidopteran cells and showed no other in vitro or in vivo toxicity and no haemolytic activity.

Native, intact parasporal crystals of both species showed no toxic activity against any of the cell lines, nor against any of the erythrocytes tested. Nor were the native crystals toxic when injected into mice (Thomas and Ellar, 1983b).

This study, as well as clearly demonstrating the difference between the two toxins also showed the very generalised lethal action of the alkali-solubilized B. thuringiensis var israelensis toxin which had not been previously reported.

In summary, it would appear that the toxins of leptox and diptox strains of B. thuringiensis, although they show similar histopathological effects, act primarily on the cell membranes of their target tissues by different molecular mechanisms. Effects on subcellular membrane systems have been reported (Charles, 1983; Charles and de Barjac, 1983; Luthy and Ebersold, 1981), but it has not yet been shown if these effects are due to the action of the toxins directly, or to the disruption of the cell membrane which has been shown to be caused by the bacterial toxins. Neither has it been ruled out that these B. thuringiensis crystal toxins have some other effect on another part of the target cell.

1.5.9 Function of the Parasporal Crystal

One of the major questions regarding the parasporal crystal, which, as yet, remains unsolved, is why this organism should devote a considerable part of its biosynthetic capacity to producing this particle which comprises up to 30% of the sporangial dry weight (Rogoff and Yousten, 1969). One immediate suggestion would be that the toxin is produced so that it may parasitize and colonise insect populations. However, B. thuringiensis is rarely, if even, found to be the causative agent of epizootic infections in nature (Dulmage and Aizawa, 1982). For this reason it is considered unlikely that this is the reason for the production of the crystal by this organism.

The parasporal crystal is a secondary metabolite and, as such, its

function might be similar to other postulated roles for other secondary metabolites produced by other bacilli. The most notable of these are the peptide antibiotics whose chemistry, biosynthesis and possible functions have been reviewed (katz and Demain, 1977). However, even according to the authors of this review, there is a lack of substantiating evidence to allow any of these functions to be accepted by a majority of workers in this field.

In their review on B. thuringiensis Bulla et al. (1980) suggested a number of possibilities regarding tne function of the crystal. These authors drew attention to the fact that for leptox, and to a lesser extent, for diptox strains of B. thuringiensis, spore coats have been found to contain the polypeptide comprising the parasporal crystal (see Section 1.5.6). They examined germination rates, and spore coat profiles of wild type and acrystalliferous mutants of B. thuringiensis var kurstaki and compared the same parameters in the closely related B. cereus. It was found that wild type B. thuringiensis var kurstaki spores contained the crystal protein as the major spore coat protein, and that the low molecular weight proteins typical of B. cereus spore coats were present in reduced amounts when compared to acrystalliferous mutants, or to spore coats of B. thuringiensis var israelensis. (Aronson et al., 1982). In addition, the B. thuringiensis var kurstaki spores were found to germinate slower than those of B. cereus. It was therefore suggested that the function of the crystal protein, both in the crystal and in the spore coats, was to enable germination to occur in the insect gut by the toxic action of the crystal on the insect and by providing nutrients for the germinating spore. Because of these two factors it was claimed that germination need not take place as rapidly as was observed for B. cereus spores in vitro (Bulla et al., 1980; Stahly et al., 1978).

Thus, the production of the parasporal crystal was stated to give B. thuringiensis an advantage when invading host insects.

These proposals put forward by Bulla et al. (1980) have not been substantiated by any other work and, in my opinion, represent mere conjecture on the basis of a small body of evidence. Their fundamental idea that the crystal gives the organism the ability to colonise insect hosts by providing nutrients seems misguided for two reasons; firstly, the insect gut is likely to contain a high nutrient level anyway, given that the function of this organ is to extract nutrients from the insect food so that they may be absorbed into the larval haemolymph; secondly, as has been previously stated, B. thuringiensis rarely, if ever, causes epizootics in nature, and therefore the organism would appear to be an inefficient parasite, despite the high toxicity of its parasporal crystals, thus the crystal does not seem to enable the organisms to establish itself as a major insect parasite under natural conditions.

However, given that the spore coats of most strains of B. thuringiensis studied do contain the crystal protein toxin, it is likely that this protein is important in the physiology of the spore. In this context, the suggestion that the crystal itself represents an overproduction of a spore coat component may have some validity. Despite this, it can only be concluded that the exact role of the parasporal crystal of B. thuringiensis remains elusive.

NOTE ADDED IN PROOF

A number of articles on the parasporal inclusions of B. thuringiensis var israelensis have recently been published (Lee et al., 1985; Hurley et al., 1985).

The first of these studies purified a number of types of subinclusion produced by this variety and found that each type was insecticidal (Lee et al., 1985). This group proposed that the subinclusions were of two basic types, dots (electron-dense), and refractile bodies (electron-transluscent). They claimed that the dots were synthesized first and then increased in size and eventually formed the larger refractile bodies, and that the change in electron density was accompanied by the synthesis of new gene products. However, the results of the pulse-labelling study which were used to support this idea may have been affected by intra-cellular protease activity which has been reported to be present during sporulation of bacilli, including B. thuringiensis (Lecadet et al., 1977; Maurizi and Switzer, 1980). In my opinion, therefore, the case for the crystals of B. thuringiensis var israelensis being comprised of 7 gene products, as was claimed by this group, is not supported by definitive evidence.

In the second study by the same group, whole parasporal inclusions of B. thuringiensis var israelensis were alkali-solubilized and two bioactive proteins detected (Hurley et al., 1985). One was a 65 kd protein which was mosquitocidal, but not cytolytic, while the other possessed cytolytic but no larvicidal activity and had a molecular weight of 28,000. This latter protein was almost certaintly the species isolated by previous

workers whose characteristics and mode of action have been studied (Armstrong et al., 1985; Davidson and Yamomoto, 1983; Pfannenstiel et al., 1984; Thomas and Ellar, 1983 a,b; Yamomoto et al., 1983).

Thus, it would appear that the mosquito toxicity of the native crystals and the cytolytic activity of the solubilized crystals are caused by different polypeptides, or different fragments of a single protein found in the inclusions of B. thuringiensis var israelensis. The origin of these two proteins in relation to the different subinclusion types was not established (Hurley et al., 1985).

1.6 MICROBIAL PROTEASES AND THE PROTEASES OF BACILLUS THURINGIENSIS

Production of protein degrading enzymes by many types of microorganism has been studied and commercially exploited for a considerable length of time. Among the most prolific producers of proteases are bacteria of the genus Bacillus which are known to produce a range of proteases during and after logarithmic growth. These proteinases are produced both extracellularly and intracellularly and are thought to be involved in a range of cellular processes (Doi, 1972; Maurizi and Switzer, 1980). There is considerable interest in these enzymes from a commercial point of view, as bacterial proteinases have been used in a number of commercial products, notably washing detergents (Ward, 1983). In addition, proteinases may cause degradation of protein fermentation products and therefore, a fundamental knowledge of these enzymes and methods for their inhibition are of genuine industrial importance, as well as of considerable academic interest. Other reasons for the study of the diverse range of enzymes arises from the observations that a number of cellular functions are thought to be at least partially controlled by proteolytic activity, with bacterial sporulation possibly being the most intensively studied (Doi, 1972; Holzer et al., 1975, Maurizi and Switzer, 1980).

In this section, therefore, a general overview of microbial proteases will be given with respect to protease classification and function. The section will then conclude with a description of the work done to date by other workers on the proteases of B. thuringiensis.

1.6.1. Classification of Microbial Proteases

The classification of microbial proteinases has proved difficult due to the wide range of specificities and mechanisms of action observed

for these enzymes. Thus location of attack, catalytic mechanism, substrate specificity, pH optimum and enzyme source are properties which have been used to distinguish various microbial proteinases, or peptide hydrolases as they are described by the Enzyme Commission (1978).

As described by Ward (1983) the Enzyme Commission recommended that these enzymes be primarily distinguished on the basis of whether the hydrolase degrades proteins or peptides at their termini (peptidases) or elsewhere (proteinases), Table 1.6.1. The peptidases were then furtner divided on the basis of which peptide terminus was hydrolysed or whether the enzyme hydrolysed dipeptides. Because the peptidases only hydrolysed tneir substrates at the terminal ends these enzymes are called exopeptidases.

Subsequent to tne site of substrate attack, proteinases and carboxypeptidases are classified according to their catalytic mechanism. Here the subdivision of Morihara (1974) have been adopted for the discussion of the proteinases subgroups, which puts these enzymes into four major classes, the serine, thiol, acid and metalloproteinases, Table 1.6.1.

1.6.1.1 Serine proteinases

These enzymes have serine and histidine at their active sites and are most active at neutral to alkaline pH. They are inhibited by diisopropyl flourophosphate (DFP) and phenylmethylsulphonyl flouride (PMSF) which covalently bind the active site serine. Morihara (1974) proposed that these enzymes be sub-divided into four groups:

1) trypsin - like proteinases which are produced by a number of Streptomyces species and exhibit specificity for basic amino acid residues.

TABLE 1.6.1

Basis of peptide hydrolase classification by the Enzyme Commission (Enzyme Nomenclature, 1978)

PEPTIDE HYDROLASE TYPE	PEPTIDE HYDROLASE SUBTYPE, BASED ON SUBSTRATE TERMINUS ATTACKED	CATALYTIC MECHANISM	AMINO ACID SUBSTRATE SPECIFICITY
	Dipeptidases		
Exopeptidases (Peptidases)	Aminopeptidases (Cleavage at N-terminus)		Used below level of catalytic mechanism to classify individual enzymes within each group
	Carboxypeptidases (Cleavage at C-terminus)	Serine Carboxypeptidases	
		Metallo-Carboxypeptidases	
Endo-peptidases (Proteinases, Proteases, Proteolytic enzymes. Peptidyl-peptide hydrolases)		Serine proteinases	
		Thiol proteinases	
		Acid proteinases	
		Metallo-proteinases	

2) alkaline proteinases produced by a wide range of bacterial, fungal and yeast species and preferentially hydrolyse peptide bonds involving aromatic or hydrophobic residues. The best known of this sub-group and indeed of microbial serine proteinases in general, are the alkaline, extracellular subtilisins produced by the bacilli. It is these enzymes which are used as the protease component of household detergents and which are the most commonly produced of all microbial enzymes (Markland and Smith, 1971; Ward, 1983).
3) Myxobacter alpha-Lytic protease is produced by Sorangium sp and is specific for small, aliphatic residues, such as alanine, at the carboxyl side of the splitting point.
4) Staphylococcal proteinase hydrolyses acidic residues at the carboxyl side of the splitting point and is produced by S. aureus.

Other serine proteineases are produced by Gram-negative bacteria and some yeasts, but are poorly characterized (Morihara, 1974).

1.6.1.2 Thiol proteinases

Thiol proteinases have cysteine at their active site and are optimally active at neutral pH. They are activated by reducing agents such as cysteine or mercoptoethanol and inhibited by sulphydryl reagents such as p-chloromercuribenzoate (pCMB) which covalently bind to the sulphydyl group of the residue at the enzyme's active site. Two subgroups of this microbial enzyme type have been described based on pH optima, and specificity to synthetic substrates (Morihara 1974). Clostripain is produced by Clostridium histolyticum and exhibits a stringent specificity against basic amino acid residues on the carboxyl side of the point of hydrolysis. Streptococcal proteinase is

produced by group A streptococci as a zymogen whose active form exhibits a broad specificity to synthetic and protein substrates.

1.6.1.3 Acid proteinases

Acid proteinases are widely distributed in yeasts and fungi but are rarely found in bacteria; they are optimally active at pH 3-4 and have aspartic acid residues as their active sites (Morihara, 1974). These enzymes are inhibited by diazoketone compounds and are divided into pepsin-like and renin-like acid proteinases by Morihara. Both forms require aromatic or hydrophobic residues at both sides of the splitting point.

1.6.1.4 Metalloproteinases

Metalloproteinases, or metal chelator sensitive proteinases, require a divalent metal ion for activity and are inhibited by metal chelators such as EDTA and o-phenanthroline. Morihara (1974) has divided these enzymes into four major subgroups based on pH optima and preferred substrates.

The neutral metalloproteases are widely distributed in microorganisms and show specificity for hydrophobic or bulky amino acid residues and have a pH optimum near 7.0. Unlike the serine and thiol proteinases they do not show appreciable esterolytic or amidase activity on N-acylated amino acid derivatives. These are zinc - dependent enzymes with molecular weights of 35,000 - 40,000 and are typically represented by thermolysin from Bacillus thermoproteolyticus (Matsubara and Feder, 1970; Pangburn et al., 1973; Ward, 1983) and neutral protease from B. subtilis (Matsubara and Feder, 1970). Other bacilli reported to produce a neutral metalloprotease are B. cereus,

B. megatenium, B. thuringiensis, B. polymyxa, B. stearothermophilus and B. amyloliquefaciens, with the enzymes of the latter two being used for industrial purposes (Ward, 1983). Other organisms known to pronduce a metalloproteinase are Pseudomonas aeruginosa, Streptomyces griseus, Aspergillus oryzae, S. naraensis, and Clostridium histolyticum (Matsubara and Feder, 1970; Morihara, 1974; Ward, 1983).

Alkaline metalloproteinases are produced by Gram-negative bacteria such as P. aeruginosa and Serratia sp, are most active at pH 7-9 and exhibit very broad specificity (Morihara, 1974) The molecular weights of the enzymes produced by these two species are 48,400 and 60,000 respectively. These enzymes require about ten times as much EDTA as the neutral metalloproteinases to cause inhibition. The Serratia sp enzyme contains zinc as its active metal but the active metal in the P. aeruginosa enzyme is unknown.

Myxobacter proteinases I and II are produced by Myxobacter strain AL-1 which are inhibited by EDTA and have alkaline pH optima of 8-9. Protease I is cell wall lytic whereas number II is not. Both proteinase types are poorly characterized.

1.6.2. Functions of Microbial Proteinases

The best understood role of extracellular microbial proteinases and their mammalian counterparts is nutritional; that is, to hydrolyse large peptides into smaller molecules which the cell can accumulate and utilize. Microbial and mammalian proteases also play a more complicated function in the regulation of metabolic processes, but this aspect of their role in nature is much less well understood.

The topic of the role of proteinases in nature has been extensively

reviewed and the reader is referred to the following works which explore the subject in more detail than will be done here: Doi (1972, 1977), Holzer et al., (1975), Goldberg and Dice (1974), Goldberg and St. John (1976), Maurizi and Switzer (1980), Ward (1983). This section will briefly summarize the role of proteinases in microbial cell turnover, cellular differentiation and enzyme/protein modification and modulation.

1.6.2.1 Protein turnover

In growing and sporulating bacterial cells proteins are continually being degraded to amino acids at rates of 1-18% per hour (Doi, 1972; Pine, 1972; Ward, 1983). However, all proteins are not degraded at the same rate with some proteins, amounting to less than 10% of the total, being turned over every hour and a much larger fraction, 70% under starvation conditions, is relatively stable to degradation in E. coli (Nath and Koch, 1970, 1971). In contrast, virtually all intracellular proteins in mammalian cells are continually being degraded and resynthesized, (Goldberg and Dice, 1974).

Generally speaking, protein turnover rates in growing cells are lower than in resting or starved bacteria (Holzer et al., 1975). This is because in non-growing cells greater protein turnover is necessary to allow the organism to induce the synthesis of newly required enzymes and thus adapt to the conditions which caused the interruption in growth. This type of adaptation is especially necessary for cells in environments lacking amino acids as it provides an amino acid pool for the synthesis of these newly required proteins (Ward 1983).

During bacterial sporulation, mass degradation of the cellular protein compliment takes place in order to provide amino acids for the

synthesis of proteins required during the complex process. In B. subtilis this protein degradation has been correlated with an increase in intracellular protease activity (Doi, 1972). The protein turnover rate during sporulation is continually higher than the rate during vegetative growth with approximately 90% of the protein present at the end of the log phase being degraded during sporulation. The rate of turnover during spore formation has been found to vary in different Bacillus species from 1.6%/hour for B. brevis to 20-24%/hour for B. licheniformis (Maurizi and Switzer 1980).

As with the case of vegetative cells, rates of degradation of individual proteins are thought to vary from protein to protein, although direct evidence for this is limited (Maurizi and Switzer, 1980).

Despite the evidence supporting a correlation between turnover rates and intracellular protease levels during sporulation a number of instances have been found where protein turnover has not been found to correspond closely to the amounts of intracellular protease in some bacilli. This has led Maurizi and Switzer (1980) to suggest that intracellular protease may not totally control intracellular proteolysis and that prior denaturation and/or modification of the target proteins may also be an important control factor.

1.6.2.2 Proteinases during Sporulation

In addition to the role of protein turnover in sporulation discussed in 1.6.1.1 where proteases were seen to fulfill a largely nutritional role, protease activity during sporeformation has also been found to have other functions during this process. These include proteolytic modulation of gene expression and enzyme activity and proteolytic

modification of enzymatic and structural proteins (Maurizi and Switzer, 1980). These activities of proteases are not necessarily sporulation - specific and will thus be discussed in the sections dealing with these subjects on a more general basis.

1.6.2.3 Proteinases during Germination

Proteolysis during germination is required for the provision of an amino acid pool to the metabolically inert spore (Maurizi and Switzer, 1980). It appears that there are two proteolytic systems in the spores of B. megaterium; the first of these is active within 3 minutes of initiation of germination and accounts for the degradation of 15-20% of the total spore protein in up to 40 minutes following the initiation. This system has been termed System 1 by Peter Setlow who has conducted an extensive study into the germination of B. megaterium spores. Proteolytic System 1 is characterized by its sensitivity to Hg^{++} ions and its lack of a requirement for metabolic energy (Maurizi and Switzer, 1980). Setlow's System II is responsible for the proteolytic degradation of proteins synthesized de novo by the germinating spore. This system is clearly differentiated from System I by its inhibition by inhibitors of energy metabolism. In addition, System I and System II differ in the proteins which they will degrade. While System II degrades proteins newly synthesized during germination which have a range of molecular weights and are not soluble in dilute acid, System I is restricted in its activity to a small number of acid-soluble low molecular weight (6,000-12,000) proteins which are present before de novo protein syntnesis occurs in the germinating spore (Maurizi and Switzer, 1980).

In addition to Setlow and co workers studies with B. megaterium results obtained using other species have indicated that a similar

role is played by a proteinases in germinating spores of many sporulating bacteria (Maurizi and Switzer, 1980; Ward. 1983).

Thus, the proteolytic system of germinating spores appears to be instrumental in providing the spore with a pool of amino acids for the synthesis of enzymes essential to its metabolism and also possibly in providing a reserve of energy-yielding metabolites and of metabolic intermediates for the developing cell.

1.6.2.4 Modulation of gene expression

Proteolytic modulation of gene expression has been indicated in a number of works by a range of mechanisms (Doi 1972, 1977; Holzer et al., 1975; Maurizi and Switzer, 1980). Such effects may be caused by degradation of repressor or activator proteins, by post-translational modification of nucleic acid processing enzymes, or by the modification of the RNA translation aparatus. Some of these activities have been suggested to occur during sporulation although the importance of others is, as yet, questionable.

Maurizi and Switzer (1980) described a study where the proteolytic degradation of a gene repressor was necessary for the induction of bacteriophage lambda but stated that there was no experimental evidence for such a control system operating in bacterial sporulation. Therefore, the question of destruction of sporulation-specific repressors by proteinases remains unresolved.

A more clearcut example for the involvement of proteases in the modulation of gene expression is the conversion of the RNA polymerase from B. thuringiensis from a vegetative to a number of sporulating cell forms, (Klier et al., 1973; Klier and Lecadet, 1974; Lecadet et

al., 1977). These workers found that the transcription products of the various RNA polymerases of B. thuringiensis were detectably different (Klier and Lecadet, 1976; Klier et al., 1978; Rain-Guion et al., 1976). In addition, they found that a transcript preferentially synthesized by one of the forms was a relatively stable mRNA coding for the parasporal crystal protein. Thus, their evidence suggests the possibility of a general mechanism for the proteolytic modification of RNA polymerases for the selected synthesis of specific transcription products. More extensive evidence in other organisms and the examination of the expression in vitro of a number of sporulation-specific genes would be required to determine fully the role of this complex enzyme modification system in the regulation of sporulation.

The possibility that gene expression during sporulation may be controlled at the translational level either by proteolytic modification of vegetative translational initiation factors, or by proteolytic alteration of the ribosomal proteins themselves, according to Maurizi and Switzer (1980) is no more than simply a possibility. These authors stated that evidence for either mode of control is inconclusive and that evidence that specific proteolysis is involved is virtually non-existant.

1.6.2.5 Modulation of enzyme activity

Proteolytic modification using intracellular proteinases by post-translational partial or complete degradation of microbial proteins is known to occur (Holzer et al., 1975; Ward, 1983).

Chitin synthase from yeast is converted from an inactive zymogen to an active form by a protease from the vacuolar fraction prepared after metabolic lysis (Holzer et al., 1975).

The number of examples of enzyme inactivation by intracellular proteases is much larger than the number of enzyme activations. B. subtilis IMP - dehydrogenase and aspartate transcarbamylase disappear rapidly during the transition from stationary phase to sporulation (Holzer et al., 1975). These authors also described a wide range of other enzymes from a number of micro-organisms whose inactivation in vivo is assumed to be caused by intracellular proteinases.

Enzyme modification is defined as the alteration of protease properties such as substrate specificity, affinity for substrates and/or effectors, or heat stability without being a simple on/off conversion of the enzyme. Examples of this type of transformation are well known for some microbial enzymes.

Aldolase from B. cereus has been found to have different molecular weights when isolated from vegetative cells or spores; appropriate proteinases have shown that the vegetative cell form can give rise to the spore form in vitro but the significance of this conversion in vivo is questionable according to Holzer et al., (1975) and the same was said to be true for a range of other yeast enzyme modifications mentioned by these reviewers.

However, the conversion of RNA polymerase is B. thuringiensis from a vegetative to a sporulating cell form described by Klier et al. (1973, 1977), Klier and Lecadet (1974) and referred to in 1.6.3.4. does not seem to be artifactual, although the biological significance of this modification is not completely understood.

1.6.2.6 Protein maturation

Protein maturation or processing describes the removal of N-formylmethionine or methionine from newly synthesized peptides and the cleavage of precursor proteins during virus assembly.

Protein and peptide synthesis in prokaryotes is initiated by N-formylmethionine (f-Met) and maturation requires the deformylation and to a large extent the removal of the N-terminal methionine (Holzer et al., 1975). This is thought to occur firstly by a deformylation catalysed by a highly unstable enzyme followed by an aminopeptidase cleavage of the methionine residue, however this aminopeptidase has not yet been identified (Holzer et al., 1975).

It is well established that assembly of a number of types of virus particle arises from the linking of proteins derived from precursor molecules (Holzer et al., 1975). However, despite the abundance of evidence that such modification does occur, the precise proteolytic systems have not been well described (Holzer et al., 1975).

In addition to the two examples above, a number of studies have suggested that fully intact spore coats of B. cereus and B. subtilis can only be formed by the processing of precursor molecules of high molecular weight to proteins of about 12,000-13,000 daltons (Aronson and Pandey 1978; Maurizi and Switzer 1980). Aronson and Pandey (1978) suggested that the high molecular weight precursor in both species was about 60,000 daltons.

Thus, the involvement of protease in the maturation of proteins in specific instances has been established but evidence for this type of activity on a wider scale is lacking.

1.6.2.7 Degradation of nonsense proteins

Nonsense, or nonfunctional, proteins may arise from gene mutations, or mistakes made in the expression of wild-type genes. They are degraded by proteolytic systems so that the amino acids comprising these useless proteins may be released for use in the synthesis of functional gene products.

E. coli is known to possess a protein degradation system which can selectively destroy nonsense polypeptides (Holzer et al., 1975). This "scavenger system" is thought to be different from the system involved in the turnover of proteins in resting or starving cells. However, precise biochemical data on the nature of the enzymes comprising this system are not available.

1.6.3. Studies on the Proteases of B. thuringiensis

A limited number of studies has been done on proteolytic activities in cultures of B. thuringiensis using a range of sources of enzymatic activity. These studies will be described here with respect to the factors which affect protease production and to the characterization of the activities isolated from B. thuringiensis cultures.

1.6.3.1 Factors affecting protease production by B. thuringiensis

Synthesis of extracellular protease during logarithmic growth of B. thuringiensis var finitimus was found to be regulated by both catabolite and nitrogen metabolite repression (Egorov et al., 1983, 1984). These authors found that extracellular protease production occurred during exponential growth and sporulation, but that only the

earlier protease production was inhibited by nitrogen compounds. In addition they found that the addition of albumin, gelation or casein to a synthetic growth medium did not enhance protease production when protease activity was related to cell counts.

Studies by another Soviet group showed that in glucose transport mutants of B. thuringiensis var galleriae the catabolite repression by glucose on extracellular protease production was weakened as was the effect of this sugar on the process of sporeformation (Shevstov et al., 1982). Li and Yousten (1975) found that when B. thuringiensis var kurstaki was cultured in nutrient broth the addition of Mn^{++} or Ca^{++} was necessary for protease production. These authors reported that protease elaboration began at the beginning of the stationary phase in an extracellular form with protease levels steadily increasing till sporangial lysis began.

These results show that the regulation of protease synthesis in B. thuringiensis is under metabolite repression during logarithmic growth and that both extracellular protease production and spore formation at the end of exponential growth are repressed by glucose which is similar to the case of other bacilli (Schaeffer, 1969). The role of metals in enhancing extracellular protease activities described by Li and Yousten (1975) cannot easily be understood in the context of nutrient repression and derepression and may be very possibly due to enzyme stabilization in a medium where pH rose to over 8.0 by the time significant levels of activity were detected.

1.6.3.2 Characterization of proteases from B. thuringiensis

Reports describing proteases from B. thuringiensis tend not to give a coherent picture of the proteolytic enzymes produced by this organism

due to the range of protease sources and to the range of results obtained. Table 1.6.2. summarizes these studies.

As can be seen from this table the amount of information available on B. thuringiensis proteases is scant in that none of these reports were found to give agreeing data on the organism's proteases.

The production of extracellular neutral metalloproteases and alkaline proteases at sporeformation by bacilli is well known (Maurizi and Switzer, 1980; Schaeffer, 1969) and the enzymes described in Table 1.6.2. would seem to fall into one or other category. Nonetheless it seems surprising that these different groups should produce such divergent results using the same bacterial species. One explanation of the lack of agreement might be due to the use of different varieties of B. thuringiensis and were this the case then the different types of proteases detected may be used as a taxonomic tool for identifying the subspecies of this bacillus.

The report of Li and Yousten (1975) is the only one which describes the partial purification of a metalloproteinase isolated from the culture supernatant of sporulating B. thuringiensis var kurstaki. These authors found no evidence for the production of a serine extracellular protease as was described by Epremyan et al. (1981). A possible explanation for this apparent discrepancy is that B. thuringiensis may indeed produce both a metallo- and a serine proteinase, but that the former is produced early during sporulation and the second later on. This was stated by Fargette and Grelet (1976) but unsubstantiated by experimental evidence. Furthermore the relative amounts of metallo- and serine proteinases may vary from strain to strain and from medium to medium and therefore it could be quite likely that two groups studying extracellular proteases would actually purify two different enzymes.

TABLE 1.6.2

Summary of literature reports describing proteases from *B. thuringiensis*

Bacillus thuringiensis variety	Stage of growth	Location of protease	Protease pH optimum	Molecular weight	Effective inhibitors	Reference
thuringiensis	early sporulation	intracellular	ND	23,000	PMSF, 0.5mM EDTA, 5mM	Lecadet et al., 1977
kurstaki	stationary	extracellular	7.0	37,500	orthophenanthroline, 1.0mM	Li and Yousten 1975
galleriae	ND	extracellular	8.5	29,000	PMSF, 0.1mM pCMB, 0.1mM EDTA*	Epremyan et al., 1981 Stepanov et al., 1981
israelensis	postlysis	purified parasporal crystals	8.5, 10.0	ND	ND	Chilcott et al., 1983

ND: not determined *Inhibitor concentration not stated

Epremyan et al. (1981) found that the extracellular protease they purified bore significant resemblance to subtilisin BPN in terms of pH optimum, molecular weight and response to PMSF, a known inhibitor of serine proteases. However, their enzyme was partially inhibited by EDTA and had a different amino acid composition to subtilisin BPN and therefore these authors suggested that this enzyme occupied an intermediate position between the subtilisins and the intracellular proteinases of other bacilli. In a subsequent study by the same group Stepanov et al. (1981) found that this extracellular protease was also inhibited by pCMB, an inhibitor of thiol proteinases (1.6.2.2.), thus bearing a significant resemblance to a similar enzyme isolated from Thermoactinomyces vulgaris by Mizusawa and Yoshida (1976). This has led the Soviet workers to propose a new subfamily of microbial serine proteinases where the extracellular enzyme also contains a sulphydryl group at its active site.

The intracellular protease isolated from sporulating cells of B. thuringiensis var thuringiensis by Lecadet et al. (1977) had a lower molecular weight than the enzyme purified by Epremyan et al. (1981) was sensitive to both EDTA and PMSF and required Ca^{++} for maximum expression of activity. These properties are similar to the intracellular proteases from other bacilli except for the molecular weight where in other species the typical size of the intracellular proteinease is a dimer in excess of 50,000 daltons, (Maurizi and Switzer, 1981). However, Lecadet et al. (1977) did detect another intracellular protease which was not characterized which may have been the counterpart of the typical bacillus intracellular protease isolated at sporulation.

In a study examining protease activities associated with purified crystals from B. thuringiensis var galleriae and B. thuringiensis var

insectus evidence was found for adsorbed serine proteinase, neutral metalloproteinase and leucine aminopeptidase activities (Chestukhina et al., 1980). However, these enzymes were not characterized beyond establishing the inhibition patterns of the adsorbed activities, Table 1.6.3. These results showed that the serine proteinese activity was strongly inhibited by PMSF and DFP, but only weakly so by pCMB. In addition to the complete inhibition by EDTA of the neutral metalloproteinese activity, the serine protease inhibitors DFP and PMSF also inhibited this activity. Of PMSF and EDTA, only the latter inhibited the leucine aminopeptidase activity. These results suggest the presence of a mixture of proteinases adsorbed to the crystal surface which would be expected considering the range of proteases likely to be present in cultures of sporulating bacilli.

In a similar study using parasporal crystals purified from *B. thuringiensis* var *israelensis* cultures, proteolytic activity was also detected. However, the activity was characterized only to the extent that two pH optima of 8.5 and 10.0 were observed (Chilcott et al., 1983).

From these studies it appeared that the actual number and types of proteases produced by *B. thuringiensis* remained to be clarified, and thus it was the aim of the protease investigations during this work to determine the characteristics of the proteases produced by this organism.

TABLE 1.6.3

Effect of inhibitors on protease activities associated with purified parasporal crystals of B. thuringiensis var galleriae (Chestukhina et al., 1980).

INHIBITOR	pH	RESIDUAL ACTIVITY (%)		
		SERINE PROTEASE ACTIVITY	METALLOPROTEASE ACTIVITY	LEUCINE AMINOPEPTIDASE ACTIVITY
PMSF	8.5	0	54	100
DFP	8.5	0	67	ND
EDTA	7.0	ND	0	20
	8.5	ND	ND	ND
pCMB	9.4	15	ND	ND
pCMBS	7.0	10	ND	ND

All inhibitor concentrations 10mM
ND: not determined

1.7 MOSQUITO AND BLACKFLY BIOLOGY AND MOSQUITO BIOASSAY

Mosquitoes and blackflies are probably the most widely distrubuted transmitters of human and animal diseases as well as being the worst annoyance pests in the insect world. They range from the tropics and sub-tropics to the arctic regions where they are an annoyance pest of a seriously disturbing nature (Chandler and Read, 1961).

Mosquitoes exclusively transmit five important human diseases - malaria, yellow fever, dengue, filariasis and encephalomyelitis, while blackflies are responsible for the spread of onchocerciasis or river blindness in man and animals.

Both mosquitoes and blackflies are affected by *B. thuringiensis* var *israelensis* which is one of the reasons the WHO has established Malaria and Onchocerciasis programmes in the tropics using this bacterium as a means for the control of these diseases.

1.7.1 Mosquito Biology

All mosquitoes are found in the family Culicidae (1.2.1) with the most important genera being *Anopheles*, *Aedes*, *Culex* and *Psorophora* with respect to nuisance value and disease transmission. There are over 2000 species known, most of which occur in the tropics with a low number of species (but often in huge numbers) being found outside this region (Chandler and Read, 1961).

The general life history is similar for all mosquitoes and follows from egg to larva, through pupa and finally to adults which continue the cycle by producing eggs. An adult female may lay from 40 to 50 to several hundred eggs during her lifetime. Whereas *Aedes* and

Psorophora lay single eggs out of water in environments such as soil, Anopheles lays them singly in loose clusters on water and Culex lay them also on water, but in small boat-shaped rafts called egg-boats. The Aedes and Psorophora eggs laid out of water are deposited in areas which are likely to become submerged such as in marshes or ponds which will be flooded after rains or high tides, or just above the water line in tree holes. This device ensures that eggs will only hatch when there is an adequate supply of water. Furthermore, only a partial hatch may be expected so that eggs will remain after the water level subsides, to be hatched again once the required water level is restored. The eggs of these mosquitoes may remain viable in a dried state for months, and in some cases years, enabling the mosquito to survive long periods of dry conditions. Following egg deposition, eggs may hatch in a few days, or after a much longer period, but regardless of the deposition - hatching interval, hatching will only occur in water.

Larvae, which are always aquatic, are microscopic when first hatched and grow to a length of up to 20 mm in about 7 days under optimal conditions of temperature and food availability. They feed by filtering particles 0.5 - 10 um from the water (Schnell et al., 1984) which is the reason native crystals of B. thuringinesis var israelensis are considerably more toxic than the solubilized toxin. Of importance here is the alkalinity of the mosquito larval gut (Dadd, 1975) which, in conjunction with the gut proteases, allows solubilization of the crystal and expression of its toxic activity. Anopheline mosquitoes feed at the surface of the water whereas the other species are bottom feeders. This has a significant bearing on the effectiveness of particulate larvicides because parasporal crystals tend to sink out of the feeding zone of Anopheles species thus requiring higher doses of B. thuringiensis var israelensis to

control these mosquitoes (Dame et al., 1981; Nugud and White, 1982; Tyrell et al., 1979). Larvae go through four instar stages by moulting and losing the skin of the previous stage before finally developing into a pupa at the end of the fourth instar. Towards this stage the older larvae cease feeding which makes them completely resistant to the B. thuringiensis var israelensis parasporal crystal which is toxic only by ingestion (1.5.8).

The pupal stage, like the larval, is completely aquatic. Pupae last for about 2-5 days during which time they do not feed and therefore they, too, are unaffected by B. thuringiensis var israelensis.

The adults emerge from the pupae, rest on the old skin for some time to dry their wings and then take flight to begin life as a normal adult, or imago.

Male adults survive for 2-4 weeks and females for longer. During their lifetime male adults survive by feeding on nectar and it is only the females which take blood in order to be able to lay fertile eggs, and therefore it is the female of the species which is responsible for disease transmission.

In warm climates mosquitoes may go through as many as 15-20 generations in a year, but in cooler areas much fewer occur, with only one annual generation in the arctic regions.

The variation in habitats, feeding habits, mode of over-wintering, choice of breeding grounds and other behavioural patterns among mosquitoes is remarkable. For this reason, when controlling a given species, a thorough knowledge of its biology is required in order to avoid wasteful effort and succeed in a given control programme.

1.7.2 Blackfly Biology

Unlike mosquitoes, which breed in a variety of water types, blackflies breed exclusively in running water. Eggs are laid by the female while hovering over the deposition site, and usually hatch within a few days.

The eggs hatch to produce larvae which cling to rocks and vegetation by means of hooks. They feed by means of a filtering device which gathers particulate matter deposited on it by the water current. The larvae go through six instars in 7 to 14 days, before spinning a cocoon around themselves inside which they pupate.

Blackfly pupae live inside their cocoons without feeding for 3 to 8 days after which they float to the surface to emerge as adults. As with mosquito larvae and pupae it is only the actively feeding stages which are susceptible to the parasporal crystal of B. thuringiensis var israelensis.

Once emerged, the adults are short lived and usually lay their eggs soon afterwards. Normally they inhabit the higher levels of trees and may migrate distances of up to 100 miles during their lifetime (Chandler and Read, 1961). As with mosquitoes it is only the females which take blood, but only in bright light. Most blackfly species do not attack man and therefore it is only a limited number which are responsible for disease transmission in humans.

Blackflies were once all included in the genus Simulium, but now are divided into about 6 genera with Simulium still the most important (Chandler and Read, 1961). In Africa S. damnosum and S. neavei are the only truly anthroplic species responsible for the transmission of onchocerciasis, with S. ochraceum the main vector in Guatemala.

Because blackflies require running water for breeding and are thus difficult to rear in the laboratory, and because they do not transmit as many serious diseases as mosquitoes, their control using B. thuringiensis var israelensis has not received as much attention as the latter. However, because onchocerciasis is such a serious and widespread disease in Africa the WHO has decided that the threat to the human population is sufficient to merit the establishment of an eradiction scheme for the disease, and results to date have shown that this pest can be successfully controlled using B. thuningiensis var israelensis (Davidson, 1982a; Molloy and Jamnback, 1981; Undeen and Nagel, 1978).

1.7.3 Mosquito Larval Bioassay

A fundamental aspect to the quantitation of any insecticidal material is that its potency must be assessed by its activity on insects, and not according to its chemical or biochemical content (Rishikesh and Quelennec, 1983). In the present context the assay of fermentation broths of B. thuringiensis var israelensis was done against early fourth instar larvae of Aedes aegypti, the stage at which most reproducible bioassay results may be obtained.

An insecticidal material can be tested using mosquito larvae by either exposing a population of larvae to a range of insecticide concentrations and determining the amount required to kill 50% of the population or, the larvae can be exposed to a fixed concentration and the time required to kill 50% determined. Either parameter is an indication of the amount of insecticidal material present, and in statistical terms is defined as the median (50%) lethal response level (Finney, 1971). In practice, the most commonly determined median

lethal response level in the assay of insecticidal preparations is the concentration of insecticide required to kill half the insects, LC_{50}, and is normally expressed in ug/ml or ng/ml when dealing with insecticides based on B. thuringiensis var israelensis.

Regardless of the particular assay system chosen it is desirable to get all the larvae at the same age, so that the response of a given individual should be as close as possible to the response of the population at large (Finney, 1971). In practice this is virtually impossible as it would require the rearing of a batch of larvae in which growth of all individuals was synchronized; this simply does not happen. What does happen is that a wide span of ages is observed so that after 5 days of growth under optimal laboratory conditions representatives of all instars may be present with perhaps only 40-50% of the population in the last instar. Of these, the size and susceptibility to the insecticide may vary significantly (especially for the older fourth instars) but the variation is less than would be the case between second and fourth instars.

Thus it can, and does, happen that a larval batch prepared for a given assay differs significantly from another batch prepared under identical conditions for another assay. This phenomenon represents a drawback to the bioassay technique and is encountered by all workers in the field (Dr. GW White, London School of Hygiene and Tropical Medicine; Personal Communication).

Two approaches are taken to avoid problems of reproducibility which may be caused by larval variation in the assay technique currently recommended for the evaluation of B. thuringiensis var israelensis (de Barjac and Larget, 1979; Rishikesh and Quelennec, 1983). They are, firstly, to subjectively choose larvae which appear to be of the same size, and certainly of the same larval instar. The quality of the

results obtained depends on the experience of the counter, with the narrower the range of larval ages and susceptibilities, the more statistically satisfactory the results will be. Secondly, the use of a standard material which is evaluated each time a bioassay is done is obligatory. The median lethal response level of the larvae to a standard material will vary from assay to assay in the same way that it would for a test material. The insecticidal potency of the standard material would be known (by arbitrarily assigning it a value) and therefore the potency of the test material is determined by comparing the median lethal response levels of the standard and test preparations according to the following calculations:-

$$\text{Potency of Test Material} = \frac{LC_{50}\ \text{Standard}}{LC_{50}\ \text{Test}} \times \text{Potency of Standard}$$

Thus, it is in this way that the inevitable variation in the target insect may be accounted for to obtain meaningful data for the insecticidal activity of a test material on a day to day basis.

The conventional bioassay system used during this work for the evaluation of broth potencies was that of Rishikesh and Quelennec (1983) which was based on the recommendations of a number of investigations sponsored by the WHO and associated bodies (de Barjac and Larget, 1979; Dempah and Coz, 1979; Sinegre, 1981; Sinegre et al., 1981 a, b).

This method is based on the principle that when a number of individuals of a target population is exposed to a range of concentrations of a lethal agent, the mortality caused is a function of concentration, within a certain range of mortality. Outside this range increases or decreases in the concentration of the lethal agent either do not cause a linear change in the percentage kill, or do not

cause any change at all. In practice the most suitable levels of mortality are usually 10-90% (Finney, 1971; Busvine, 1971; Rishikesh and Quelennec, 1983).

The standard materials used were a pure parasporal crystal suspension prepared during this work (2.5), and IPS.82, an international standard prepared at the Pasteur Institute, Paris and used worldwide for the direct comparison of results from different laboratories.

The use of a pure crystal suspension as a standard material for a bioassay has not, to my knowledge, been previously reported but it was felt that its use was justified for a number of reasons.

1) The results obtained using this material indicated the bioactivity of a sample in terms of the actual amount of protein crystal present, and not in terms of arbitrarily defined units.

2) This suspension was stable, easy to use, highly active and had little or no tendency to clump which meant that it fulfilled the major requirements of a standard material.

3) Using a pure crystal suspension as a standard, it enabled the bioactivity of fermentation broths to be determined without first having to estimate the dry weight of the broths which is necessary when using IPS.82.

4) The use of two standard materials is more desirable than the use of one because uncharacteristic behaviour of one preparation may be compensated for by the other if it shows typical dose-response behaviour.

Insecticidal activity results derived using IPS.82 as the standard are called potencies and are reported as International Toxic Units (ITU) per mg or ml of material, given that the potency of IPS.82 has an

arbitrarily assigned value of 15,000 ITU/mg (Pasteur Institute bioassay protocol leaflet accompanying preparation). Using the pure crystal suspension, the bioactivity of an insecticidal material was determined as mg of crystal protein per ml of culture broth.

However, despite the availability of suitable standard materials, and the development of an acceptable larval rearing regime, the conventional bioassay technique for the evaluation of the larvicidal activity of *B. thuringiensis* var *israelensis* was a cumbersome technique.

A considerable length of time (6-8 hours) was required to prepare and select the larvae for the assay, and then to make them up to the desired volume before adding the insecticide to the larval cups (see section 1.4.3). It was for this reason that a less labour-intensive and shorter bioassay technique was investigated, and these studies are reported in section 3.4.

1.8 FERMENTATION DEVELOPMENT OF BACILLUS THURINGIENSIS

Members of the genus Bacillus are commonly used in fermentation processes for the production of a number of commercially interesting products. Table 1.8.1 summarizes some of the organisms and their uses. Most current industrial fermentations using bacilli are carried out under submerged conditions (Ward, 1983), and this is also the case for the fermentation of B. thuringiensis.

The aim when developing a fermentation process is to devise a system in which production of the metabolite is optimized at the cheapest cost per unit of product, in as short a cycle time as possible. Other requirements which must also be satisfied include, the use of suitable raw materials, and the use of a process which allows convenient downstream processing of the fermenter product. With particular reference to B. thuringiensis these conditions apply, but in addition, efficient sporulation and lysis must be achieved, and the use of medium components compatible with the formulation requirements is essential (Dulmage, 1981; Luthy et al., 1982).

There follows a short discussion on the general principles behind the design of a fermentation process and its scale-up. This is then followed by a description of the requirements for the fermentation of B. thuringiensis and the formulation of insecticidal materials based on this organism.

1.8.1 Fermentation Development

The field of fermentation process development is a wide, interdisciplinary one which could not be adequately reviewed here. Instead, some of the most important factors relating to process design and scale-up will be discussed.

TABLE 1.8.1

Some of the industrial applications of the bacilli

ORGANISM	INDUSTRIAL APPLICATION	REFERENCE
B. amyloliquefaciens	alpha-Amylase	Fogarty 1983
	alpha-glucosidase	
	Isoamylase	
	Proteinases	Ward, 1983
B. licheniformis	alpna-amylases	Fogarty, 1983
	Glucose Isomerase	Bucke, 1983
	Penecillinase	Priest, 1983
	Proteinase	Ward, 1983
	Bacitracin	Kuenzi and Auden, 1983
B. polymyxa	beta-amylase	Fogarty, 1983
	Pectic enzymes	Fogarty and Kelly, 1983
	Pullulanases	Fogarty, 1983
B. stearothermophilus	alpha-amylase	Fogarty, 1983
	Pectic enzymes	Fogarty and Kelly, 1983
	Proteinases	Ward, 1983
B. thuringiensis	Insecticides	Bulla and Yousten, 1979
B. sphaericus	Insecticides	Singer, 1980

1.8.1.1 General aspects of process design

Due to the wide variation in products and microorganisms associated with industrial fermentations, rigid generalizations with regard to process design are difficult to make. However, some of the major considerations affecting fermentation process design as outlined by Kuenzi and Auden (1983) are discussed here.

An ideal fermentation process would be one in which the entire procedure, from inoculum build-up to maximal product synthesis in the production fermenter, is achieved in as short a time as possible. The minimization of cycle time is important in order to achieve the maximum productivity from the plant investment and to reduce labour and energy costs which increase with increasing process duration.

The process should use cheap, readily availabe raw materials which are stable during storage and do not vary significantly in quality from batch to batch. The raw materials should not interfere with the process by the production of toxic materials during sterilization or of off-colours which may affect the final quality of some products. It is also important that the raw materials be compatible with the downstream processing of the final fermenter broth.

The product itself should be stable to the fermentation conditions used, and should also have a sufficiently high value by comparison to the costs of production to justify the development costs and effort required to design the fermentation process.

With regard to the microorganism, it should, ideally, be easily grown under industrial conditions without causing problems associated with

shear sensitivity and susceptibility to other conditions likely to be encountered in the fermenter. In addition, the organism should not cause severe problems during the fermentation with respect to excessive heat production, and decreases in the mass and energy transfer coefficients which have been noted in some antibiotic fermentations (Aramy et al., 1977). Furthermore, the organism should be stable with respect to its ability to elaborate high product levels in the fermenter on a continued, run to run, basis.

The organism should also produce a minimized level of by-products in order to maximise the fermenter yield of the product of interest, and to reduce the number and complexity of downstream recovery steps. This consideration is especially important in the organic acid and antibiotic fermentations where a range of related products may be synthesized by the microbe, but where only one is of interest. This type of problem is avoided by culturing under conditions which selectively allow predominant synthesis of a single entity, or by the use of mutants which produce low amounts of unwanted by-products (Kuenzi and Auden, 1983).

The recovery system used should be as inexpensive, simple and as short as possible. Following fermentation of 10-100 m^3 volumes the processing of such a large bulk of liquid will, inevitably, add significantly to the overall economics of the process. Thus, the greater the number and complexity of the recovery steps, the more expensive will be the entire production process due to the cost of the recovery operations and the probable yield loss caused by complex downstream processing.

Despite the importance of all these considerations when optimizing a fermentation process it is unlikely that a system can be optimized with respect to all these parameters because of their interdependent

nature. Thus, the use of an expensive raw material may be justified if its use improves product yield, or simplifies the recovery process. Alternatively, in the production of an intracellular product, it may be desirable not to allow the fermentation to proceed till highest product levels are achieved, because cell disruption at this stage may require unduly harsh conditions leading to a lowering of the overall yield of recovered product.

From this discussion it should be apparent that fermentation design may not be conducted along set patterns due to the immense range of variables which exist between different process requirements. Thus, the eventual process used will depend on the product, the organism producing it, the availability of suitable raw materials, the particular characteristics of the plant that will be used to produce it, and on the system that will be used to recover it from the fermenter broth. While it is not possible to give rigid recommendations as to how each individual process may be designed, it is hoped that the aspects discussed here will give an idea as to how such a problem might be approached.

Once such problems as raw material and organism suitability to the procuction of the metabolite of interest have been considered, the aspects related to large-scale production must be assessed. These questions are discussed in the next section.

1.8.1.2 Process scale-up

The most frequently encountered problems related to scale-up of fermentation processes are those associated with medium sterilization. inoculum development, use of industrial grade raw materials, and the tank mixing/aeration requirements of the large-scale cultures (Banks.

1979; Lilly, 1983). These problems arise due to a number of factors and have variable effects on the process depending on the requirements of the individual fermentation.

Medium sterilization of large volumes of fermenter broth on an industrial scale is affected by factors of poor heat transfer due to medium viscosity and the sheer liquid volume which needs to be sterilized. Thus harsh heat treatments of broth are required to achieve sterility which means that broths may be heated for 3-6 hours at temperatures higher than those normally used to sterilize broths in the laboratory. This greater severity may cause adverse effects on the nutritive quality of the medium which may be excarbated by the use of industrial grade materials which may have been avoided during laboratory fermentations (Banks, 1979). Problems relating to medium sterilization causing nutritive quality decrease may be reduced by separate sterilization of the medium components, and/or high temperature short time (HTST) sterilization using very short heating and cooling times by continuous sterilization of the medium outside the fermenter. Of course, a medium whose nutrient value is unaffected by prolonged sterilization would avoid the necessity to develop elaborate sterilization process alternatives.

Inoculum development on the large-scale may be affected by medium sterilization and mixing requirements, but is most commonly affected by the number of steps required to produce an inoculum volume adequate for seeding the production fermenter.

The necessity for the use of multi-step protocols for the production of an inoculum may, in some cases, lead to culture degeneration or an altered metablic state of the organism when used to seed the production vessel. These problems are most prevalent in fermentation processes using highly mutated organisms which are frequently employed

in the pharmaceutical industries for the production of antibiotics.

One of the most severe problems in scale-up of fermentation processes is the alteration in growth and productivity patterns in large stirred fermenters from those observed in the simple shake-flask systems (Banks, 1979; Lilly, 1983). These problems arise due to the difference in oxygen transfer and heat produced between the two types of vessel. In the large-scale fermenter short mixing times attainable in flasks or in small fermenters are not possible due to the bulk of liquid and the relative dimensions of the impellers used to agitate the culture. Thus the amount of dissolved oxygen in the medium is reduced and the dissipation of the heat produced by the culture is less efficient. The larger the fermenter, the more serious will be these problems.

These problems are worsened by the growth of the organism in the broth which further increases its viscosity, and reduces its heat transfer ability (Arany et al., 1977). Problems due to growth of the organism in the fermenter are even more serious in the case of fungi due to the formation of mycelium which again increases the power required to agitate the vessel. However, excessive shear caused by strong stirring may destroy the mycelial growth and thereby prevent product formation. Thus, in these cases, organism growth and vessel agitation must be carefully balanced so that a successful fermentation may be achieved.

From this brief discussion it is hoped that some appreciation for the problems involved in process design and scale-up has been conveyed. With regard to these considerations a process for the fermentation of B. thuringiensis was developed, in addition to some requirements specific to the production of this particular organism.

1.8.2 Fermentation Studies with B. thuringiensis

Previous fermentation studies on B. thuringiensis have most frequently described media for its production on a flask scale. However, many of these media were not considered during this work on the development of a suitable medium for the fermentation of B. thuringiensis because they either used expensive raw materials, and/or medium components which were not readily available. A few studies mentioned culturing conditions required for efficient fermentations in flasks, but none dealt with the development of a fermentation process on a larger scale.

The fundamental requirements for the fermentation of B. thuringiensis are that the process allows the maximum expression of insect bioactivity with respect to medium costs and cycle time. In addition to these necessities, the use of medium components compatible with the formulation of the fermentation products is esssential (Dulmage, 1981; Section 1.8.3).

Here, the previous work done on the fermentation of B. thuringiensis, with respect to medium development, and optimization of fermentation conditions for laboratory cultures of the organism, will be described.

1.8.2.1 Media for the production of B. thuringiensis

The medium components used in the production of B. thuringiensis must be cheap, easily available, and must not interfere with the downstream processing of the fermentation products. In addition, a balance of the carbon and nitrogen levels must be achieved to prevent servere pH fluctuations during vegetative growth which may inhibit the progress of the fermentation (Dulmage, 1981).

Due to commercial interest in the fermentation of B. thuringiensis, relatively few studies describing industrially useful media have been published. In addition, it is not uncommon to find that when production of B. thuringiensis is reported, that final yields, and even the medium constituents are not revealed (Couch and Ross, 1980; Margalait et al., 1983). However, some interesting studies have been reported by a number of authors (Dulmage, 1970, 1971, 1981; Dulmage and Rhodes, 1971; Obeta and Okafor, 1984; Smith, 1982) and some of the best media described by these authors, along with the most frequently quoted patented media are shown in Table 1.8.2.

These media are most frequently based on soyabean and cottonseed preparations, casein, corn steep solids or yeast extract as the nitrogen sources. Glucose, starch and molasses were the most used carbon sources. Leguminous seeds and cottonseed materials have been reported as being very good nitrogen sources for the B. thuringiersis fermentation (Dulmage, 1981; Obeta and Okafor, 1984; Salama et al., 1983), however these were not readily available and so it was the soya preparations which were most frequently used instead during this study.

1.8.2.2 Optimal conditions for the fermentation of B. thuringiensis

Few studies on the best conditions for the fermentation of B. thuringiensis have been reported. However, a number of reports suggest that growth and sporulation of the organism are maximised by high levels of aeration at a temperature of 28^{o}C - 32^{o}C (Dulmage, 1981; Dulmage and Rhodes, 1971; Luthy et al., 1982).

TABLE 1.8.2

Some of the most useful literature media for the fermentation of B. thuringiensis

MATERIAL	CONCENTRATION, g/l								
	MEGNA, 1963	DRAKE & SMYTHE, 1963	CRC, 1978 MOLASSES	CRC, 1978 STARCH	DULMAGE, 1971 B-4	DULMAGE, 1971 B-5	SMITH, 1982 2	SMITH, 1982 5	OBETA AND OKAFOR, 1984
Casein	-	20.0	-	-	-	-	-	-	-
Corn Steep Solids	17.0	10.0	1.0	2.0	-	-	-	-	-
Cow Blood	-	-	-	-	-	-	-	-	10.0
$(NH_4)_2SO_4$	-	-	1.0	-	-	-	-	-	-
Peptone	-	-	-	-	2.0	-	-	2.0	-
Cottonseed Flour	14.0+	-	-	-	10.0	-	-	10.0	-
Soyflour	-	-	-	-	-	15.0	18.0	-	7.5*
Yeast Extract	-	5.4	3.0	10.0	2.0	-	-	2.0	-
Cane Molasses	18.6	-	-	-	-	-	-	-	-
Corn Starch	-	45.0	-	13.0	-	10.0	5.0	-	-
Glucose	-	-	-	-	15.0	5.0	10.0	15.0	-
Sucrose	-	9.0	-	-	-	-		-	-
$CaCO_3$	1.0	-	1.0	8.0	1.0	1.0	0.5	1.0	1.0
Phosphate	-	8.0	-	4.0	-	-	0.7	-	-

* A variety of leguminous seed preparations used, all at 7.5 g/l
\+ Other oil-free cereal flours such as soyflour claimed to be suitable.

In a study on some fermentation conditions Smith (1982) found that insect toxin production was not increased by the use of buffers to control the medium pH.

Thus, the amount of published information on the fermentation of B. thuringiensis was not extensive at the beginning of this study, and most of what was reported referred to the media used for the fermentation. Therefore, one of the minimum aims at the outset was to develop a suitable medium for the fermentation of B. thuringiensis and to characterize the conditions under which maximal bioactivity was attained using this medium.

1.8.3 Processing and Formulation of B. thuringiensis

Once B. thuringiensis has been produced in the fermenter it must be harvested and the cells formulated into a material which can be sold as a final product.

1.8.3.1 Harvesting

Because it is the particulate fraction of the fermentation culture which is used to prepare the insecticide, harvesting is most appropriately done by centrifugation of the broth (Dulmage, 1981; Luthy et al., 1982). However, filtration of the culture by the addition of filteraid has also been described (Megna, 1963). Spray drying may also be used as the insecticice is resistant to high temperatures for short periods of time, but spray drying is usually only practical after prior centrifugation (Luthy et al., 1982).

1.8.3.2 Formulation definition, significance and description

For the purposes of the discussion, a formulation is defined as the form in which the insecticide is distributed to the user; the tank-mix, therefore, is considered the final dilution of the commercial formulation which is applied by the end-user.

Formulation of the particulate material from the fermenter broth is necessary to produce the organism in a stable form with a shelf-life of at least 12 months, and which can be easily and uniformly applied to the insect habitat. Correct formulation of B. thuringiensis is as important as the proper fermentation of the organism. The failure of the industry to produce suitable formulations for field use has retarded the use of B. thuringiensis by commercial growers (Couch, 1978).

Because of its importance to the production of insecticides based on B. thuringiensis, relatively little published work has appeared on its formulation. The few articles and reviews which are available tend to be vague and reveal few indications as to how formulation of B. thuringiensis is actually done (Couch, 1978; Couch and Ignoffo, 1981; Luthy et al., 1982; Soper and Ward, 1981).

A number of types of B. thuringiensis formulation are currently available and these are the wettable-powder, liquid or flowable, briquette, and granular formulations. Different types are used depending on the target insect, and, more importantly, on the host's habitat. Thus, a tank-mix prepared from a dry powder or flowable formulation is suitable for application to field crops and open water, or anywhere the insect habitat is not protected by covering vegetation. Granular formulations are produced by the absorption of its organism onto the surface of coarse particulate materials, such as

sand or ground corn cobs, or by the spray drying of a slurry of the organism in a granular form. They are used for application to mosquito breeding sites which are obscured by vegetation such as marshland or swamps. These granular formulations are useful in such habitats because the granules penetrate the foliage due to their weight in a way that could not be done by an insecticide applied as a fine spray.

The most recent formulation innovation is the introduction of the briquette form. These doughnut shaped briquettes are used to control mosquitoes in small ponds where insecticides distribution by aerial application is impractical. The briquettes float on the surface of the water and slowly release the insecticide over about 30 days to control larval populations feeding on the briquettes's release-zone.

The types of forumalation and their uses are summarized in Table 1.8.3.

1.8.3.3 Formulation manufacture

As has been mentioned before, only scant information as to how formulation manufacture is actually done is available in the literature. What is presented here, therefore, is a combination of this small amount of information and the accumulation of knowledge acquired from personal contact with the manufacturers of these products.

The formulation of B. thuringiensis may be done by centrifuging the organism from the fermenter culture and, either mixing the centrifuged paste with a variety of additives then drying the paste if a dry formulation is required, or, drying the paste and then mixing the

TABLE 1.8.3

The types and uses of currently available formulations of *B. thuringiensis*

FORMULATION TYPE	APPLICATION	TRADE NAMES, PRODUCERS
Flowable	Open crop fields and aqueous mosquito breeding sites.	Teknar, Sandoz Inc., USA Skeetal, Microbial Resources Ltd., UK Vectobac-AS, Abbott Labs, USA Dipel-LC; Abbott Labs, USA Thuricide-HPC; Sandoz Inc., USA
Wettable Powder	Open crop fields and aqueous mosquito breeding sites	Bactimos, Biochem Products, Belgium Vectobac; Abbott Labs, USA Dipel-HG; Abbott Labs, USA
Granules	Vegetation-covered mosquito breeding sites	Vectobac-G; Abbott Labs., USA Teknar; Sandoz Inc., USA Thuricide; Sandoz Inc., USA
Briquettes	Mosquito breeding pools	Bactimos; Biochem Products, Belgium.

additives (Couch and Ignoffo, 1981). Additives used in the preparation of dry formulations are included to maintain the activity and homogeneity of the formulations when stored, distributed and used under field conditions. The major additive is the diluent which is usually a clay of some sort, such as bentonite. The diluent usually comprises over 90% of the wettable powder formulation and is used to increase flowability. Other additives include surface-active agents to enable homogeneous wetting of the powder to take place, and thus, good dispersion in the tank-mix, so that problems of spray nozzle blocking should be minimized (Soper and Ward, 1981). Many formulations also contain UV protecting agents to protect the spores when sprayed onto foliage. However, the use of these agents may not be necessary as the crystals of B. thuringiensis and their insect toxicity are not affected by natural doses of UV radiation in sunlight. (Burges et al., 1975;Griego and Spence, 1978; Couch and Ignoffo, 1981).

Liquid formulations are probably prepared by resuspending the culture paste and mixing the suspension with water or a water/or emulsion. Thus there are two types of liquid formulation - the aqueous flowable, and the oil-based flowable. These formulations are stabilized by the addition of emulsifiers such as detergents, and by stabilizers or suspending agents (Couch and Ignoffo, 1981). Preservatives are also necessary to prevent bacterial and fungal growth in the liquid formulations. As with the dry powders, the final concentration of the active ingredient (centrifuged paste) in these formulations is of the order of 1-5% by weight as indicated by the commercial literature and product labels.

In principle, granular formulations are relatively less complex to produce because they simply require spray drying of a centrifuge paste

suspension (after additive inclusion) in the granule form; alternatively, carrier particles (walnut shells, corn cobs, corn meal, wheat bran) can be mixed with a spore/crystal suspension prepared from the centrifuge poste and then dried. However, additives must be used to prevent sudden release of the parasporal crystals from the granule so that residual activity of the formulation should be prolonged.

Regardless of the formulation type, it is important that production medium components be compatible with the properties of the final formulation. Thus, the use of coarse meals is undesirable, because their use would necessitate an additional grinding step in the production of homogeneous flowable or wettable-powder formulations (Dulmage, 1981).

1.9 AIMS OF PRESENT INVESTIGATION

The aim of this investigation was to develop a fermentation process which could be used on a large-scale for the production of an insecticide based on *B. thuringiensis* var *israelensis*.

Fundamental to achieving this aim was the necessity of understanding at least some of the physiological requirements for optimal biomass/bioactivity production using this organism. It was also necessary to establish and maintain a bioassay system which could be used to quantitate the bioactivity of the fermentation cultures; it was in this context that an improved bioassay was developed.

The proteases of this organism were studied, initially, with a view to their capacity to decrease the product yields in the fermenters by the degradation of the proteinaceous parasporal crystal. Following some studies on whole culture supernatants, during which the time of protease production was determined, as was the effect of various inhibitors and metal ions on these crude preparations, a number of proteases were purified from the culture broths and partially characterized.

SECTION 2: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Bacterial Isolates

Bacillus thuringiensis var israelensis was obtained in a number of freeze dried forms from Dr. J.F. Charles, Pasteur Institute, Paris, France. These were:

1) International Standard Material IPS.80 (IPS.80), a formulation originally produced using the 1884 (World Health Organisation, WHO, catalogue number) isolate (WHO, 1982).

2) International Standard Material IPS.82 (IPS.82), original isolate 1884.

3) Isolate 1884 supplied as a freeze dried unformulated preparation derived from a bacterial culture.

In addition, two other varieties of B. thuringiensis were obtained from the same source, namely B. thuringiensis var thuringiensis and the HD-1 isolate of B. thuringiensis var kurstaki (Dulmage 1970 a, b).

The IPS.80 and 82 preparations were prepared by the formulation of solid material harvested from fermenter cultures of the organism and distributed in 100 mg lots in sealed vials. Unless otherwise stated, the isolate used for this work was from IPS.80.

Spontaneous mutants of B. thuringiensis were obtained as described in 2.2.4.

2.1.2 Mosquitoes

Eggs of Aedes aegypti were provided by Dr. Graham White of the London School of Hygiene and Tropical Medicine (LSHTM) on dried filter paper.

2.1.3 Sources and Costs of Industrial Medium Components

The cost and sources of industrial medium components used to produce B. thuringiensis are outlined in Table 2.1.1.

2.1.4 Sources of Other Materials

British Drug House: Folin - Ciocalteau Reagent, Azocasein, Hammarsten Casein, Triton X-100, Dextran Blue 2000.

Oxid: Nutrient Agar

Merck: Tryptone Water, Standard II Nutrient Broth, Plate Count Agar.

Bio Rad: Dye Reagent Concentrate for protein assay.

Pharmacia: Sephadex G-100, Dextran Sulphate, Sodium salt, molecular weight 500,000, Dextran T500.

Sigma Chemical Company: Protease inhibitors; phenylmethyl sulphonyl flouride (PMSF, P-7626), p-chloromercuribenzoic acid (pCMB, C-4378), p-hydroxymercuribenzoate, Sodium salt (pHMB,H0752); Trishydroxymethylaminomethane (Tris, T1378); Protein molecular weight markers; Horse heart cytochrome c (Cytc, C2506), bovine pancreatic chymotrypsinogen A (Chymo, C4879), hen egg albumin (Oval, A5503), bovine serum albumin (BSA, A7888), Leucine p-nitroanilide (L9125).

Riedel de Haen: Na_2HPO_4, Phenol Red, Na_2CO_3

Unless otherwise mentioned all other chemicals were of Analytical Grade.

TABLE 2.1.1

Cost and sources of materials used in industrial production media for culturing B. thuringiensis

CONSTITUENT	SOURCE	COST IR£/TONNE
Nitrogen Sources		
Soyabean meal, (44% protein)	Unigrain Ltd., Dublin	200
Rapeseed meal	Irish Oil and Cake Mills, Drogheda*	150
Casein (Rennet)	Chamco Ltd., Dublin	1900
(Acid)	" " "	1800
Defatted SF	Sheffield Proteins	600
Full Fat SF	" "	800
Soya Peptone	" "	1000
Yeast Extract, YEP-16	Biocon Ltd., Cork	2000
$(NH_4)_2SO_4$	" " "	50
Corn steep solids	" " "	650
Carbon Sources		
Glucose monohydrate	Lennox, Dublin	500
Starch	Wheat Industries, Cork	300
Sucrose	Irish Sugar Co.	500
Cane Molasses	United Molasses, UK	65
Buffers		
$CaCO_3$	Eglinton Stone Ltd., Northern Ireland	50
Na_2HPO_4	Albright and Wilson Ltd., Dublin	600

* Ceased trading, 1984. SF: Soya Flour

2.2 MAINTENANCE OF BACTERIA

2.2.1 Standard IPS.80 and IPS.82 Materials

These formulations were stored at 4°C. At various time intervals, small amounts were removed from the vials and suspended in 5 ml sterile counting diluent (2.3.6.2). Nutrient agar slopes were either inoculated immediately from such a suspension, or, the suspension was streaked onto nutrient agar (NA) plates and incubated for 24 hours at 30°C to obtain isolated colonies. An isolated colony was then suspended in 5 ml sterile counting diluent and the NA slopes inoculated from this suspension. Regardless of which method was used, the suspensions were rigorously checked by microscopic examination to ensure purity.

Inoculated NA slopes were incubated for 1 week at 30°C and then stored at 4°C for up to 3 months, after which they were sub-cultured, or discarded.

2.2.2 Other *B. thuringiensis* Materials

Other bacterial sources received in the usual culture-collection freeze-dried form were similarly grown and stored on NA slopes. These strains were also subcultured on a 3 monthly basis.

2.2.3 Subculturing from Nutrient Agar Slopes

5 ml of sterile counting diluent was applied to the slope and the spores aseptically disturbed from the agar surface with a sterile loop. The slope was then shaken to form a homogeneous spore

suspension, whose microbiological purity was checked using a Nikon phase contrast microscope at X400 magnification.

This spore suspension was then used to directly inoculate 10 NA slopes which were incubated at 30°C for 1 week and then stored for up to 3 months at 4°C, when they were either subcultured further, or discarded.

2.2.4 Isolation of Spontaneous Mutants

Spore suspensions prepared from slopes (2.2.3) or sporulated bacterial cultures were diluted $1:10^4$ in counting diluent and streaked onto NA plates to obtain single colonies, after incubation at 30°C for 48 hours.

Colonies were then examined by removing a small amount of biomass aseptically and examining microscopically to check for the presence of spores and crystals. Mutants which did not produce crystals, or which failed to produce crystals and spores were thus isolated and subcultured on NA as described above, 2.2.3.

2.3 FERMENTATION PROCEDURES

B. thuringiensis was grown in flasks and fermenters using standard culture techniques. Unless otherwise stated, 3 stages were used to grow the organism.

2.3.1 Standard Shake-Flask Culturing Conditions

All shake flask culturing was done at 30°C ± 1°C on an L-H Engineering MK III B orbital shaker set at 150 RPM. Flasks were run in duplicate, unless specified otherwise.

2.3.2 First Incoculum Stage

The medium used exclusively for this stage was the tryptone water (TW) medium, which contained 15.0 g/l Tryptone Water, 7.1 g/l Na_2HPO_4, 0.2 g/l $MgSO_4.7\ H_2O$,0.001 g/l $FeSO_4.7H_2O$, 0.005 g/l $Zn\ SO_4.7H_2O$, 0.005g/l $CuSO_4.5\ H_2O$ made up using distilled water and autoclaved for 15 minutes at 15 p.s.i. to give a final pH of 7.0 - 7.2.

Usually 50 ml of medium / 250 ml conical flask were used, but when larger volumes of culture were required 200 ml / 1 l flask were employed.

The tryptone water medium was inoculated from a spore suspension prepared as described in 2.2.3. When a loopful of spore suspension was used to inoculate 50 ml of medium, it was necessary to grow the organism for 24 hours to achieve satisfactory growth. The incubation period was sometimes shortened to 12 hours, or less, by inoculating with up to 1.0 ml of the spore suspension.

When larger volumes of first inoculum stage (Inoc. 1) culture were

required the spore suspension was prepared differently. In this case, a 250 ml flask with about 50 ml solid NA was inoculated with 1.0 ml of a spore suspension prepared as in 2.2.3. The flask was then incubated for 1 week at 30°C to allow complete spore formation and lysis. The spores were then harvested by gently swirling 20-30 ml of sterile counting diluent (2.3.6.2) in the flask to produce a large-volume spore suspension, of which 5 ml were used to inoculate the 200 ml TW medium flasks.

Following inoculation the flasks were then incubated for 10-24 hours under the standard flask-incubation conditions (2.3.1).

2.3.3. Second Inoculum Stage

Following growth in the TW medium this Inoc. 1 culture was used to provide a 5% inoculum for the second inoculum stage (Inoc. 2) media.

2.3.3.1 Flask cultures

Unless otherwise stated, the Cas inoculum medium (20g/l Casein, 5.0 g/l Corn Steep Solids, 2.7 g/l Yeast Extract, 5.0 g/l Cane Molasses, 5.0 g/l Na_2HPO_4, pH7.0) was used for this stage with 200 ml of medium in a 1l Erlenmeyer flask. Following 5% inoculation the flasks were incubated as in 2.3.1 for 16-24 hours, unless otherwise indicated.

2.3.3.2 Fermenter cultures

Inoc. 2 fermenter cultures were inoculated with a 5% volume of the Inoc. 1 culutre, and inbubated at 30°C, stirred at 400 RPM, and aerated at 1.0 l/l/min, regardless of whether a Laboferm or Microgen fermenter (2.3.5) was used. Foaming was controlled by the addition of

20-30 ml of a 1:10 emulsion of polypropyleneglycol (PPG) antifoam, before sterilization.

The same medium was used in fermenters as was used in flasks (2.3.3.1) and when Laboferm vessels were employed 5l cultures were run, while 8l cultures were grown in Microgen fermenters. Fermenter cultures were incubated for the same period as was used for flask Inoc. 2 growth.

2.3.4 Production Stage

2.3.4.1 Flask cultures

1l or 2l Erlenmeyer flasks, usually containing 10% of the flask's capacity of medium, were inoculated with a 5% (or some other specified amount) volume of the Inoc. 2 culture. The flasks were then incubated for 48-72 hours, or some other specified incubation period, as described in 2.3.1. A variety of production media was used, the contents of which are indicated in the relevant results section, however, some of the more frequently used production media are described in Table 2.3.1.

2.3.4.2 Fermenter cultures

7 litre Laboferm fermenters containing 5l of medium, or 16 litre Microgen fermenters containing 10-12l of medium, or a 75 litre pilot fermenter containing 40l of medium were used for fermenter culturing of the production stage. 5% inoculum volumes of Inoc. 2 broths were used to seed the production media which were then aerated at 1.0 l/l/min and stirred at 400 RPM, at $30^{o}C$, unless otherwise stated. Emulsions of PPG (1:10 dilution) were used to control foaming in the

TABLE 2.3.1

Some of the more commonly used production media

COMPONENT	CONCENTRATION, g/l		
	DRAKE AND SMYTHE	MEGNA	DULMAGE
Casein	20.0	-	-
Defatted Soyaflour	-	14.0	15.0
Yeast Extract	5.4	-	-
Corn Steep Solids	10.0	17.0	-
Starch	45.0	-	10.0
Molasses	-	18.6	-
Sucrose	9.0	-	-
Glucose	-	-	5.0
Na_2HPO_4	8.0	-	-
$CaCO_3$	-	1.0	1.0

Laboferm and Microgen fermenters, but this antifoam was inadequate for use on a pilot-scale fermentation where foaming was reduced using Silcolapse 5,000 (Imperial Chemical Industries Ltd).

2.3.5. Fermenter Design and Operation

The three types of fermenter used and described here were made by New Brunswick Scientific, U.S.A. and varied considerably with respect to their construction, capacity and mode of sterilization.

2.3.5.1 Laboferm fermenters

These were the least sophisticated of the three types and were made of glass. The Laboferm fermenters had a total capacity of 7l, with a maximum working capacity of 5l. They were sterilized in a large Astell Hearson autoclave for 60-80 minutes at 121^{o}C. They were removed from the autoclave while still hot, 80-90^{o}C, and then stirred and aerated as quickly as possible to prevent caking of the starch in the medium which would have resulted in the blocking of the air and sampling lines.

Because of the extended autoclaving period required for sterilization of these fermenters, pH changes in these vessels during the sterilizing tended to be more severe than in the Microgen fermenters. This behaviour is well known in processes where long sterilization times are used (Banks, 1979). However the pH was always aseptically adjusted to 6.8-7.2 before inoculation.

Because of their size and number (12) in the laboratory these fermenters were best suited to large runs where a number of conditions and media were examined.

2.3.5.2 Microgen fermenters

These vessels were of stainless steel and had a total capacity of 16l with a maximum working volume of 12l. They were sterilizable *in situ* by the use of pressurized steam in the heating jackets, supplemented by the sparging of live steam into the culture medium, again under pressure. Because of the efficient heat transfer of these vessels, sterilization took only 20-30 minutes, and consequently, these fermenters offered a considerable attraction over the Laboferms from the point of view of shorter sterilization times. This ensured a lower degree of medium component deterioration and pH change during the sterilizing cycle.

The laboratory was equipped with three Microgen fermenters. Consequently, the scope for testing a number of culturing conditions/media was more limited than for the Laboferms. But, this was balanced by the sterilization cycle advantage, as well as the fact that pH and dissolved oxygen could be continuously monitored in the Microgen vessels using the relevant probes and recording instrumentation, with which the laboratory was also equipped.

2.3.5.3 Pilot fermenter

The pilot fermenter was also of stainless steel and had a total volume of 75l, but a lower working capacity of a maximum of 50l. However, I found that extreme foaming occurred using 50l and that foaming was more easily controlled when 40l were cultured.

Like the Microgens the pilot fermenter was sterilized using live steam, but the important difference was that the steam only had access

to the heating/cooling jacket and was not directly exposed to the medium during sterilization. This meant that much higher steam pressures of approximately 100-150 p.s.i. in the heating jacket were required to effectively achieve sterilization of the medium.

The steam boiler in the laboratory had not got the capacity to provide this pressure of steam. Therefore, what happened during sterilization was the temperature of the broth reached 100^{o}C within 10 minutes of the commencement of the cycle due to the thorough stirring of the medium enabling efficient heat transfer to occur. Thereafter, temperature and pressure increased slowly till the sterilizing conditions of 115^{o}C, 10 p.s.i. were achieved which were maintained for 60 minutes. After each sterilization the vessel contents were streaked onto NA plates to check for contamination, but none was ever found.

2.3.6 Quantitation of Growth in Bacterial Cultures

2.3.6.1 Culture sampling and sampling labelling

Samples of 5-10 ml were taken into universal bottles and the pH determined within 30 minutes using a Phillips PW 9410 digital pH meter. A microscopic examination was also undertaken within 30 minutes using a phase contrast Nikon Optiphot microscope at X400 magnification. The samples were then used for viable count determinations or stored at 4^{o}C or - 20^{o}C to await further examination or centrifugation for the production of a sample for protease estimation.

Samples were coded to distinguish each on the basis of medium used, experiment or run number, replicate number and culture age. The

medium name was abbreviated using 2-4 letters and the rest of the code followed in order of fermentation run number, replicate number and time, in hours, since inoculation at which the sample was taken. Thus a sample of the tryptone water medium taken 18 hours after inoculation from the second replicate flask during the fourteenth fermentation run was labelled:-

TW.14.2.18

2.3.6.2 Viable count determinations

For the determination of the total viable count (TVC; number of vegetative cells + sporulated cells + free spores) 1.0 ml of the culture broth was appropriately, diluted in counting diluent (CD; 1.0 g/l peptone or nutrient broth, 1.0g/l Na_2HPO_4 pH 7.0 - 7.2) and 1.0 ml of one or more dilutions was pour plated, in triplicate, using molten (50^oC) plate count agar prepared within 3 days of use. The agar was allowed to solidify and the plates were then incubated upside-down at 30^oC for 20-30 hours before counting the number of colonies per plate. The mean of the three repliacte figures was taken as the TVC, provided the percentage variation of the 3 values was less than 15%, otherwise the count was deemed unreliable. The most accurate counts were obtained when there were 100-300 colonies per plate.

For the determination of the spore count, the original broth sample was heated to 80^oC for 10 minutes and then treated exactly the same as for the ordinary TVC.

2.3.6.3 Estimation of proportions of vegetative cells, sporulated cells and free spores.

A broth sample was diluted, usually 1 in 5, in counting diluent and

examined microscopically using a Nikon Optiphot phase contrast microscope at a magnification of x400. Three microscopic fields were examined and the number of vegetative cells (VC), sporulated cells (SC) and free spores (FS) determined. A mean percentage of total cells figure was calculated for each cell type and expressed as the VC: SC: FS ratio.

The same data allowed the sporulation and lysis efficiencies to be calculated, where the sporulation efficiency was defined as the number of sporulated cells and the number of free spores as a proportion of the total cell population. The lysis efficiency was the percentage of free spores present in comparison to the total cell number.

2.3.6.4 Optical density meausrements

Spore suspensions, parasporal crystal suspensions and bacterial cultures grown in soluble media were quantitated by their optical densities at 600nm. The suspensions were appropriately diluted in counting diluent and the optical density determined in 1.0 cm glass cuvettes using a Pye Unicam SP6-550 spectrophotometer with counting diluent as the blank.

2.3.6.5 Dry weight determination

Production medium samples of 35 ml were used except where indicated otherwise. The particulate material in the broths was removed and cleaned by subjecting the samples to three cycles of centrifugation at 15,000 xg, 30 min. 4^{0}C and resuspension in distilled water before drying the pellets on pre-weighed glass petri dishes at 110^{0} overnight.

2.4 MOSQUITO REARING AND BIOASSAY

The mosquito rearing and bioassay facility was establisned at the NIHE in Dublin following a period of training in the techniques at the London School of Hygeine and Tropical Medicine supervised by Dr. Graham White of the Entomology Department.

Bioassays, adult rearing, larval and pupal production and egg drying and storage were all done in a constant temperature room maintained at 26°C ± 1°C, 40-50% relative humidity, in which there were no windows and wnere the artificial light was supplied by tungsten filament bulbs on a 12 hours on, 12 hours off cycle.

2.4.1. Maintenance of Adult Mosquito Colonies

The methods for rearing and maintenance of the mosquito colonies implemented at the NIHE were essentially those used at the LSHTM. Adult Aedes aegypti colonies were reared and maintained as a source of eggs which were used to produce larvae for the bioassay.

The adult flies were kept in cages made of nylon curtain netting fitted over a cubic frame 30 x 30 x 30 cm made from metal rods. One of the netting panels had a hole cut in it with a netting "tube" approximately 50 cm long attached to the aperture, which was knotted to close off the cage from the external environment. This tube was used as the access to the cage by which sugar solutions, pupae and egg papers were put into and removed from the cage. The cages were kept at 80% relative humidity (RH) by moistening a 20 x 20 cm section of absorbent gauze and laying it on the top cage panel and then covering the cages completely with a plastic cover. The adults typically numbered 1000-3000 individuals per cage and were fed on a sterile 10%

glucose solution which was replaced every 3 days.

Once a week the females were given a blood meal so that they could lay viable eggs. The sugar solutions were removed 24 hours before a blood meal to increase the desire of the females to feed. An adult guinea pig was anaesthetized by the intra-peritoneal administration of 0.44ml Nembutal (pentobarbitone) per kg body weight. The animal's back was shaved in order to make it easier for the females to obtain a blood meal, and then it was laid on top of the cage (after removal of the plastic cover and moist gauge). The females were allowed to feed for approximately 60 minutes by which time they were seen to be engorged with blood. After feeding, the sugar solutions were replaced and the guinea pig was returned to its own cage to regain conciousness.

Three days after the blood meal,an egg laying site was put into the cage. This was a conically rolled sheet of filter paper (Whatman No 1, 12 cm) pushed to the bottom of a paper cup so that it was moistened by the 10-20 ml of water in the cup. In order to make this site more like a natural egg laying locus, a few millitres of a pupal skin suspension were added to the water at the apex of the filter paper. The females laid their eggs on the filter paper over the next three days at the end of which the egg paper was removed from the cage. The papers at this stage were seen to be covered to varying degrees, depending on the adult numbers, colony age and success of the feeding operation, with the tiny black eggs of the mosquitoes.

Once harvested, egg papers were dried for a week in a cage identical to that used for raising the adults and then they were stored in a dessicator containing moistened paper towelling to preserve a RH of at least 80%.

A colony of Ae. aegypti mosquitoes was kept for 2-3 months with continuous supplementation of adult numbers by fresh pupae. After

this time the remaining adults were killed by leaving the cage at 50°C overnight and then the cage was cleaned, to be used again. At any given time 2-5 colonies were maintained.

2.4.2 Production of Larvae and Pupae

Larval batches were produced to supply insects for the bioassay or to stock the adult cages described in 2.4.1. Usually, when required to maintain adult numbers, larvae not used for bioassay purposes were allowed to proceed to pupation. Pupae were then removed and counted by hand and then put in the cages to emerge into adults.

2.4.2.1 Hatching the eggs

To produce larvae for bioassay purposes, an egg paper which had been kept in the storage dessicator for less than three months was used. Either the entire egg paper, or a section thereof, depending on the number of eggs it held, was placed in 500 ml of boiled distilled water to which about 100 mg of freshly dissolved ascorbic acid was added. Hatching was allowed to proceed for 1-3 hours. Use of deoxygenated water is desirable for successful hatching of the eggs (American Mosquito Control Association, 1970; Rishikesh and Quelennec, 1983) and this is the reason the water was boiled and treated with ascorbic acid.

2.4.2.2 Rearing the larvae

Once hatched, the tiny hatchlings (1 mm long) in 500 ml distilled water were made up to 2 l with tap water which had been autoclaved to remove traces of chlorine which may have inhibited the growth of the

larvae.

The larvae were fed dried beef liver which had been prepared by grinding, drying at 70°C overnight and then regrinding. On day one, 1.5 g of the dried liver and about 250 mg dried yeast were added to the larval water. The water was bubbled with a fish tank aspirator in order to prevent the formation of a scum on its surface which would have asphyxiated the larvae who breath by protruding a breathing siphon through the water's surface (Chandler and Reed, 1961). On day two or three, an additional 1.0 - 1.5 g of dried liver in 0.5 g amounts was added to the water depending on how many larvae were present and how much food remained.

If too little food was added larval development was retarded, resulting in a batch of small larvae whose susceptibility to the insecticide was high. On the other hand, if the larval density was low and the amount of food available high, then overdevelopment of the larvae occurred. This resulted in a batch which was unsuitable for use in a bioassay due to a commitment to pupation of an unacceptable proportion of the fourth instar larvae and a low insecticide susceptibility of the larvae not committed to pupation.

The larvae were allowed to grow for a total of 5 days by which time a large proportion of them should have been in the early fourth instar.

2.4.2.3 Pupal production

If pupae were required, the unused larvae from a batch used for a bioassay, or from a batch designed for pupal rearing alone, were allowed to grow by supplying as much food as was required and incubating for as long as it needed for the larvae to begin pupating.

Pupation usually began after around 7 days under the conditions used, but sometimes if the ratio of food availability to larval numbers was high then pupation was observed as early as 5 days from the hatching of the larvae. Once pupae formed they remained in that state for about 2 days, and so, in a pupating culture, pupae were harvested every other day, put in paper cups with distilled water and put into the adult cages to emerge.

The pupae were removed and counted by hand using pasteur pipettes modified by cutting and flame-melting the sharp edges close to the main barrel of the pipette. The pupae were not sexed.

2.4.3 Operation of the Conventional, Mortality vs Concentration, Bioassay

The background principles of the insect bioassay have been discussed previously (1.7.3.) The bioassay procedure described here is based on a number of almost identical protocols recommended by the WHO and is similar to the method learned at the LSHTM.

2.4.3.1 Preparation of the Larvae

Larvae grown as outlined in 2.4.2.2. were removed from the culturing water by use of a plastic tea strainer and suspended in a jug containing distilled water. They were then washed at least three times by pouring off as much water as possible and adding fresh distilled water. This preparation step was designed to remove as much particulate matter as possible in order that there should be minimal carry-over of food from the culture medium. Food removal is desirable as the presence of particulate material may compromize the effect of the insecticide (Rishikesh and Quelennec, 1983; Ramoska et al., 1982;

Sinegre et al., 1981 b).

2.4.3.2 Larval selection and counting

Washed larvae were put in a plastic jug containing distilled water and larvae which were subjectively judged to be early fourth instar (L4) were removed using a modified pasteur pipette and placed in waxed paper cups in a minimum volume (less than 5 ml) of water. 25 early L4 larvae were counted into each cup and the volume was made up to 100 ml $\pm$ 2 ml using distilled water.

Completed cups were arranged strictly in the order in which they were counted and then selected at random for use in exposing the insects to a given concentration of a particular material. This random selection process was essential to ensure that larval cups counted at around the same time were not exclusively used to bioassay a single preparation. This was because during the larval counting (which took 6-8 hours) the subjective idea in the mind of the counter of the acceptable larval size could vary, unknown to the person, so that larvae selected at the beginning may have been, on average, larger or smaller than those used later in the count.

2.4.3.3 Preparation of insecticidal materials

All insecticidal suspensions were diluted in bioassay diluent (BD; 0.01% Triton X-100, 0.4 g/l NaN_3, 5 g/l Na_2HPO_4 pH 7.0). Culture broths were diluted 1:1000 with a fresh dilution prepared for each assay.

A single pure parasporal crystal suspension whose protein concentration was 1.0 mg/ml (2.7.2) was used throughout. The neat

suspension was diluted 1:100 to give a 10 ug/ml stock which was stored at $4^{o}C$. This stock was further diluted one in ten to give a 1000 ng/ml suspension which was used for the bioassays of a single week and then discarded.

The IPS.82 material was originally suspended to give a concentration of 500 ug/ml. This suspension was examined by phase contrast microscopy and showed no tendency to form clumps. This suspension was divided into 10 aliquots of 10 ml, 9 of which were immediately frozen for use in bioassays over the following months. The 500 ug/ml suspensions were further diluted to give 5000 ng/ml suspensions which were used for the bioassays. Fresh 5000 ng/ml suspensions were prepared each week.

2.4.3.4 Addition of diluted insecticide materials to the larval cups

Insecticide dilutions were added to larval cups using micropipettes, with thorough vortexing of the dilutions after each addition. Each insecticide concentration was assayed in duplicate, with a minimum of 8 concentrations tested for each material. The objective was to obtain at least three mortality rates on either side of the LC_{50} value witnin the 10-90% mortality range where the percentage kill data are most statistically useful.

50-150 ul of the 1000 ng/ml suspensions were added to the larvae giving pure crystal concentrations from 0.5 - 1.5 ng/ml in the bioassay cups.

Final concentrations of 5 - 25 ng/ml of IPS.82 were obtained in the larval cups by adding 100 ul - 500 ul of the 5000 ng/ml suspensions.

For the culture broths 100 ul - 1000 ul of the 1:1000 dilutions were

used giving final culture dilutions between 10^{-6} and 10^{-5}.

At least 4 cups of larvae were exposed to 1000 ul of bioassay diluent as controls.

2.4.3.5 Mortality determination and insecticidal potency calculation

Larval mortality in the bioassay cups was determined after 24 and 48 hours by counting the numbers of larvae left alive in each cup. It was necessary to count the survivors because mosquito larvae are canabalistic and therefore a count of the number of corpses is likely to underestimate mortality (Rishikesh and Quelennec, 1983).

The mortality of control larvae was determined and when control mortalities of 5 - 10% were observed analytical mortalities were corrected by the use of Abbotts formula (see below), but in practice control mortalities of greater than 5% were never observed.

Abbotts Formula

$$M_T = \frac{M_O - M_C}{100 - M_C} \times 100$$

M_T = corrected mortality

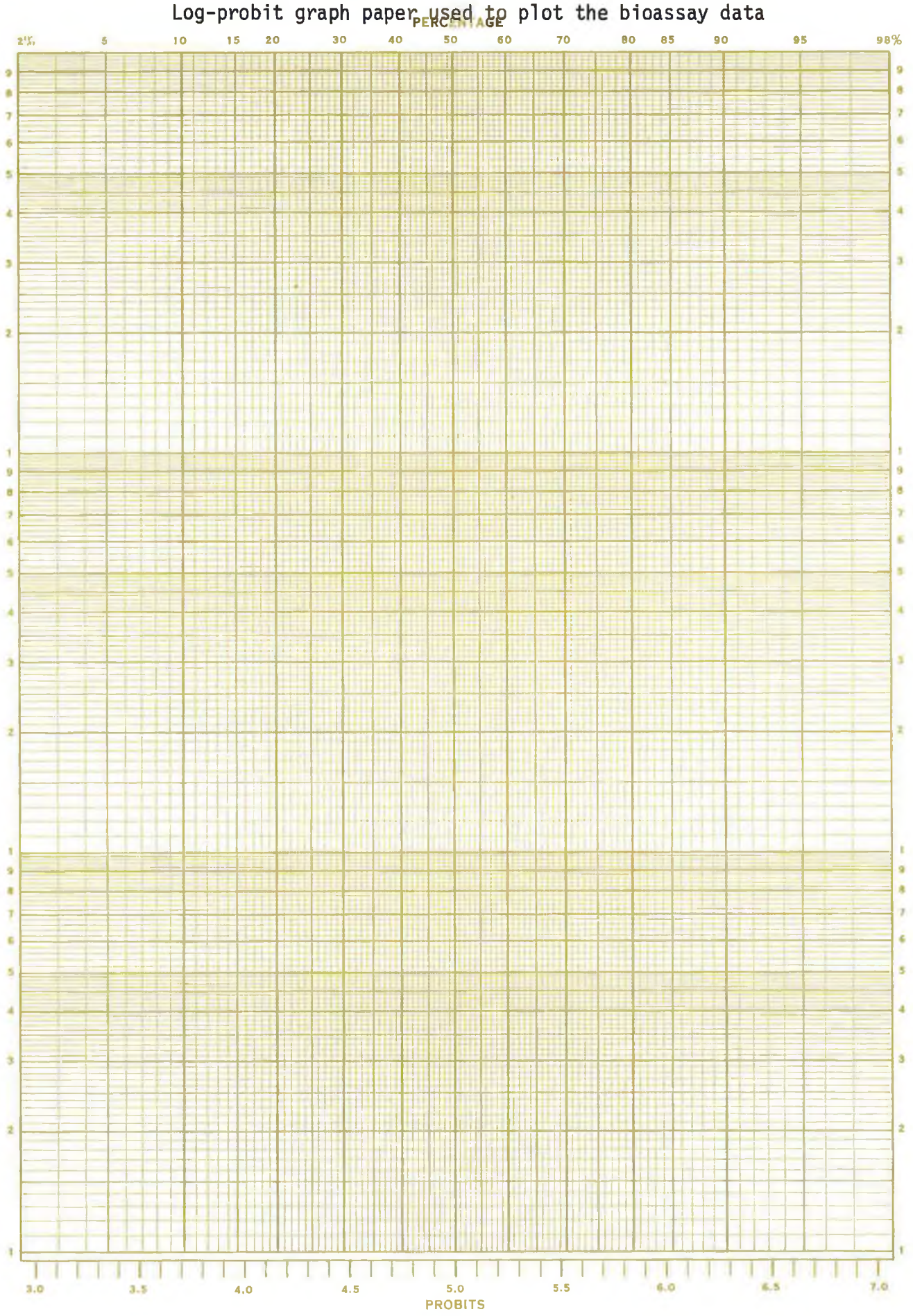
M_O = observed mortality

M_C = control mortality

Percentage mortalities were calculated and LC_{50} values for the standard materials and L Dil_{50} (dilution of culture broth required to kill 50% of larvae) values for fermentation samples were determined by plotting the data on log-probit graph paper, Figure 2.4.1.

FIGURE 2.4.1

Log-probit graph paper used to plot the bioassay data



The crystal protein concentrations and insecticidal potencies of the culture broths were calculated as described in Appendix 1.

In addition, the ratio of the median lethal response levels after 24 and 48 hours was determined for each material as was the ratio of the LC_{50} IPS.82/LC_{50} Crystal at both times.

2.4.4 Operation of the Novel, Mortality vs Time, Bioassay

The principle underlying this new Mortality vs Time (M/T) bioassay and the reasons for investigating this system are discussed elsewhere (1.7.3.).

2.4.4.1 The assay system

Larvae were grown, prepared and counted in exactly the same way as was done for the conventional bioassay, 2.4.3. The same pure crystal suspension as was used in 2.4.3. was also employed here to give final crystal protein concentrations of 4 - 10 ng/ml in the bioassay cups, with each concentration set up in triplicate.

Mortality was, again, determined by assessing the number of surviving larvae and counts were done every half hour from t1 - t3 and after that, every hour. Mortality counts were done more regularly in the first few hours because a proportionately greater level of kill occurred at this stage, Figure 3.4.2. Counting was not done after 8 hours exposure.

As with the conventional bioassay, mortality data was plotted on log-probit paper to determine the LT_{50} (see 1.7.3).

2.5 PURIFICATION OF PARASPORAL CRYSTALS

For the purification of the parasporal crystals, B. thuringiensis var israelensis was cultured in a soluble medium, the lysed culture centrifuged and a spore/crystal suspension prepared from the centrifuge paste which was used as the primary suspension from which the crystals were purified.

2.5.1. Production of a Spore/Crystal Suspension for the Purification of the Parasporal Crystals

A 5 l culture of B. thuringiensis var israelensis was produced in a Laboferm fermenter at 30°C, 400 RPM, 1l/l/min using the GNB medium, Table 2.5.1., following 5% inoculation by a 24 hour TW culture.

After 72 hours the culture was harvested by centrifugation at 15,000 xg, 30 min, 4°C using a Sorval RC 5B High Speed Centrifuge. Following centrifugation, the wet weight yield of the broth was determined and the paste was frozen. The dry weight of the broth was also determined as described in 2.3.6.5 using 100 ml broth samples, in triplicate.

TABLE 2.5.1

The GNB Medium used to produce the material for the Parasporal Crystal Purification Process.

Component	Concn, g/l
Glucose Monohydrate	10.0
Nutrient Broth (Merck, Std. II)	8.0
$MgSO_4.7H_2O$	0.3
$ZnSO_4.7H_2O$	0.02
$FeSO_4.7H_2O$	0.02
$MnSO_4.4H_2O$	0.05
$CaCl_2.2H_2O$	0.05
Na_2HPO_4	2.0

Nutrient Broth and phosphate autoclaved together in fermenter; rest of components autoclaved separately and added aseptically to sterile medium bulk.

The paste was thawed 3 1/2 months later and 5.0 g (the product of approximately 800 ml of GNB culture broth) was supended in 1.0 M Na Cl, 0.01% Triton X-100, 5.0 g/l Na_2HPO_4, pH 7.0, a supending medium with a high salt concentration to minimize protease activity (Delafield et al., 1968; Nickerson and Swanson, 1981). Following resuspension in this medium, the paste was badly clumped and was recentrifuged using the standard conditions of 15,000 xg, 30 min, 4°C. The pellet was then carefully suspended in bioassay diluent (BD, 2.4.3) without the formation of clumps. This centrifugation and resuspension procedure was repeated twice and the biomass finally suspended in 10.0 ml BD.

This was the Primary Suspension used for the crystal purification process and was quantified with respect to total viable count, dry weight and OD 600. Following the separation process its crystal protein concentration was determined by comparative bioassay with the pure parasporal crystal suspension.

2.5.2 The Parasporal Crystal Purification Process

The technique used here to purify the parasporal crystals from the Primary Spore/Crystal Suspension was essentially that of Goodman et al. (1967).

5.0 ml of primary suspension were added to 200 ml of the two-phase system to begin the separation process. The separation funnel was shaken vigourously by hand and allowed to stand at 4°C for 30 - 40 minutes. The upper polyethyleneglycol (PEG) layer was removed to complete the first pass. 100 ml of fresh upper phase was then added to the remaining phase and the process continued as was described for the first pass. In total, 7 passes were required to obtain crystals

of > 99% purity. Figure 2.5.2 diagrammatically describes the crystal purification system.

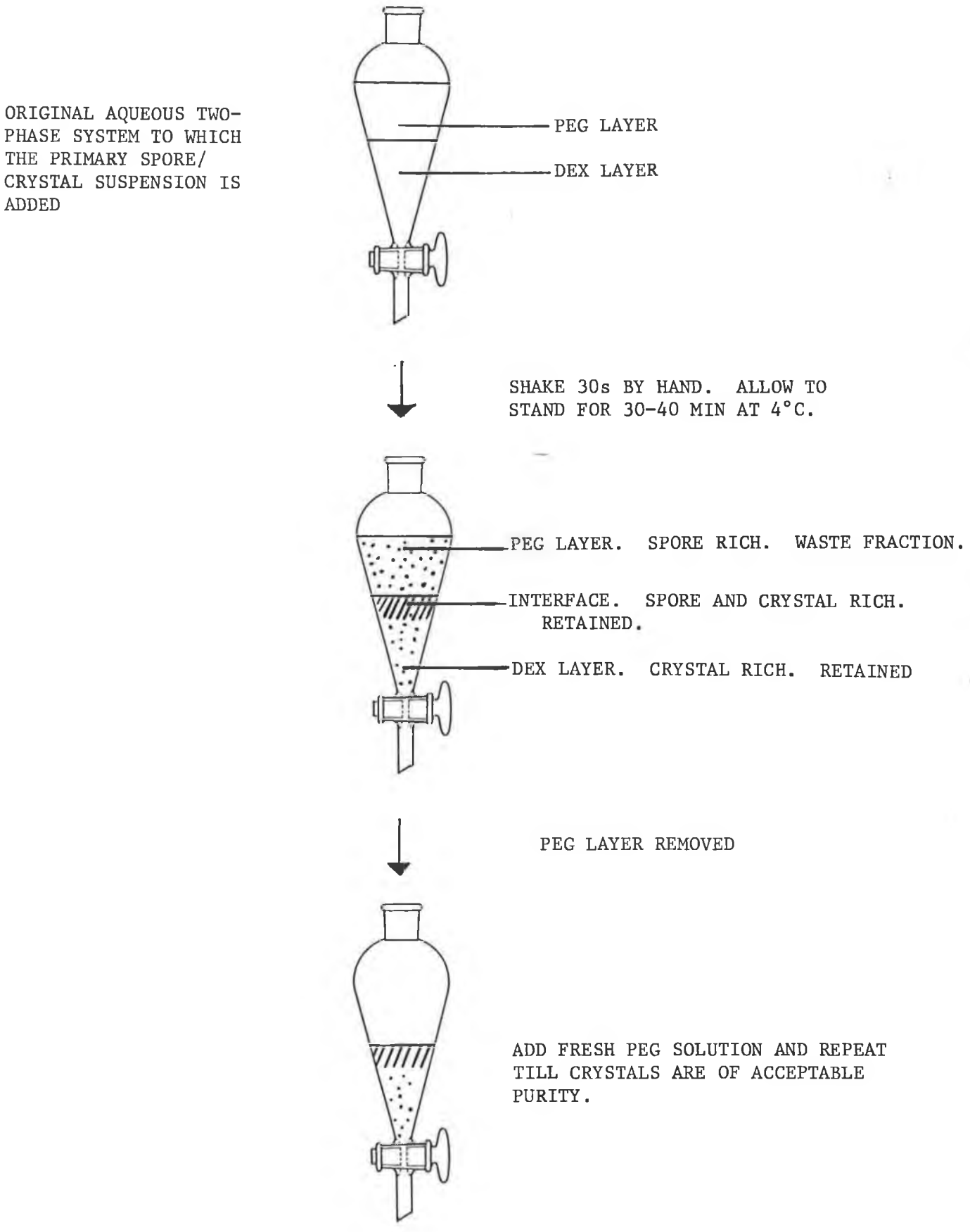
At each stage the volume, viable count, microscopic appearance and OD 600 of the PEG layers were determined. However, for the interface and dextran sulphate layers this type of analysis was done only after the final pass.

After the 7th pass the crystals were harvested from the interface/dextran sulphate layers by centrifugation and then washed three times by resuspension and centrifugation (same conditions used as for harvesting spores and crystals from the GNB medium, 2.5.1)

Finally the crystals were suspended in BD up to a volume of 10.0 ml. This crystal suspension was then analysed with respect to viable count, OD 600, dry weight, microscopic appearance and its protein concentration determined by the Coomassie-Blue Dye Binding technique (2.7.2).

FIGURE 2.5.2

The parasporal crystal purification process



2.6 MEASUREMENT OF PROTEASE ACTIVITIES

In each of the assay methods the standard temperatures used was 30°C and all absorbance measurements were made using a Pye-Unicam SP6-550 spectrophotometer with 1.0 cm glass or quartz cuvettes.

2.6.1 Caseinase Activity Determination

5.0 ml of a 6.0 g/l Hammarsten casein solution dissolved in 50 mM Tris, pH 8.0 prepared fresh on the day of assay was used as the substrate solution. After preincubation for 10 minutes at 30°C, 1.0ml of protease material diluted in protease diluting solution (PDS; 1mM $CaCl_2.2H_2O$, 10mM NaCl, 50 mM Tris, pH 8.0) was added to the substrate and thoroughly mixed. Casein hydrolysis was allowed to proceed for 30 minutes at 30°C at acid which time 5.0 ml trichloroacetic acid (TCA) reagent (0.11 M trichloroacetic acid, 0.22 M sodium acetate, 0.33 M acetic acid; Yasunobu and McConn, 1970) was added and the mixture thoroughly blended.

Precipitation of the remaining protein was allowed to proceed for a further 30 min at 30°C and then the tube contents were filtered through Whatman No. 1 filter paper. The absorbance at 280 nm (A 280) of the undiluted filtrates was measured using quartz cuvettes.

Enzyme blanks were prepared by adding the diluted enzyme to the TCA reagent, followed by 5.0 ml substrate solution and conducting the protein precipitation and filtration steps as described above.

The delta A280 (A280 analytical filtrate - A280 enzyme blank filtrate) values were calculated and those above 0.6 were not used due to the possibility that substrate limitation had occurred during the assay.

Acceptable delta A280 values were compared to a standard tyrosine curve prepared on the same day.

A unit of protease activity was defined as that amount which gave rise to the same absorbance as a 1 ug/ml solution of tyrosine per ml of enzyme solution, under the conditions used.

When activity vs pH determinations were done 10 mM Na_2HPO_4, 10 mM Tris was used as the buffer system for the substrate solution.

When the effect of inhibitors was being studied the inhibitors were prepared in tne protease diluting solution at a concentration six times as hign as would have been found in the final reaction volume (FRV) of 6.0 ml. Therefore, when the diluted inhibitor-treated enzyme (after a preincubation period of 30 minutes) was added to the substrate solution, tne resulting concentration was the one quoted.

A similar approach was adopted when preparing enzyme dilutions to estimate the effect of metals on the caseinase activity.

In testing the effect of PMSF on the caseinase activity the inhibitor was dissolved in PDS using dioxane while the effect of the relevant concentrations of dioxane were tested in a separate experiment.

2.6.2 Hydrolysis of Azocasein

1.0 ml of preincubated 5.0 g/l azocasein solution in relevant buffer (10 mM Na_2HPO_4 pH 6.0 - 7.5; 50 mM Tris pH 8.0 - 9.0; 100 mM $NaHCO_3/Na_2CO_3$ pH 9.5 - 11.0; all containing 10 mM NaCl, 0.5 mM Ca^{++}) was mixed with 1.0 ml enzyme appropriately diluted in PDS (2.6.1.). The reaction was allowed to proceed for 30 minutes and was quenched by the addition of 2.0 ml 50 g/l TCA.

The precipitate was allowed to develop for 15 minutes at 30°C whereupon the mixture was filtered through Whatman No. 1 filter paper. 1.0 ml of the filtrate was mixed with 1.0 ml 0.5 M NaOH and the absorbance at 440 nm (A440) determined using glass cuvettes. An enzyme blank was prepared in the same way as the test solution except the diluted enzyme was mixed with the TCA before addition of the substrate.

The delta A440 value of the test solution was determined by substracting the enzyme blank A440 from that of the test absorbance. Delta A440 values below 0.4 were assumed not to have suffered substrate limitation on the basis of some preliminary studies (data not presented).

The activity unit was defined as that amount of enzyme in the test material which gave rise to a delta A440 of 0.01 under the assay conditions.

2.6.2.1 Modified azocasein hydrolysis method

A modification of the azocasein digestion assay was developed to allow activity determination on gel filtration fractions which were low in volume and enzyme content.

For this modified method, 200 ul of the diluted or undiluted fraction was used as described in 2.6.2. except that the incubation period was increased from 30 to 60 minutes.

2.6.3. Determination of Leucine p-Nitroanilidase Activity

The method described here is based on guidelines from previous authors (Appel, 1974; Chestukhina et al., 1980; Pfleiderer, 1970).

The substrate was prepared fresh each day by dissolving 50 mg leucine p-nitroanilide (LNA) in 10 ml 10 mM HCl and then making up to 50 ml with distilled water to give a 4.0 mM substrate solution in 2 mM HCl. The typical A410 of this solution was 0.07. An aliquot of 2.5 ml of a protease solution suitably diluted in the appropriate buffer (10 mM Na_2HPO_4 pH 7.0 - 7.5; 50 mM Tris pH 8.0 - 9.0; 100 mM $NaHCO_3/Na_2CO_3$ pH 9.5 - 11.0) and preincubated for at least 10 minutes were mixed at t0 with 0.50 ml of a similarly incubated substrate solution. The reaction was allowed to proceed for 30 minutes at which stage the solutions were immediately read at 410 nm using glass cuvettes.

A substrate blank solution was prepared in parallel with the test enzyme solution by mixing 2.5 ml diluting buffer and 0.50 ml substrate solution and then determining the A410 after 30 minutes at $30^{o}C$.

An enzyme blank figure was calculated by measuring the A410 of the diluted enzyme preparation and taking 5/6 of this value as the enzyme blank.

The substrate/enzyme blank figure was thus the sum of the two individual control values and this was substracted from the A410 at t30 of the test solution to determine the delta A410 quantity.

The activity of the enzyme was then calculated given that the activity unit was defined as the number of nanomoles of product released per ml of neat enzyme per minute.

Molar Extinction Coefficient of p-nitroanilide

= 9620 M cm (Appel, 1974; Pfleiderer, 1970)

Thus A410 of 1 um/ml p-nitroanilide = 9.62.

If A410 = A, then the product concentration in the final reaction

volume (FRV) = A/9.62 um/ml.FRV = 3.0 ml, therefore the total amount of product released was $\frac{A}{9.62} \times 3.0$ um

Amount of product released per minute

$$= (A/9.62) \times 3.0 \times (1/30) \text{ um/min}$$

Therefore the activity in the neat broth was

$$\frac{A}{9.62} \times 3.0 \times \frac{1}{30} \times \text{Dilution Factor} \times 1000$$

nm NA/min/ml neat broth

$$= A \times 10.4 \times \text{Dilution Factor Units/ml}$$

For testing the effect of inhibitors and metals on leucine p-nitroanilidase (Leunase) activity, buffers containing the inhibitors/metals were prepared for diluting the enzyme so when the substrate was added the resulting dilution gave the required inhibitor/metal concentrations.

To test the effect of PMSF on leunase activity the inhibitor was dissolved in 1,4 dioxane with the effect of the given dioxane concentrations determined in a separate experiment.

During a preliminary study (data not shown) to determine optimum enzyme dilutions, delta A410 values of up to 1.2 were found to give linear release of nitroaniline under the assay conditions used. In addition, the substrate concentration in the FRV was 670 uM and the product concentration at t30 (based on molar extinction coefficient) was typically 50-100 uM. Therefore, the possibility that the hydrolysis was susbtrate limited was not considered likely due to the adequate substrate excess.

2.7 PROTEIN DETERMINATIONS

Two protein assay methods were used to accommodate two different types of material. For the determination of protein concentrations in culture broths and dialysed ammonium sulphate fractions the Folin method was used because of its simplicity and because it was assumed that no interfering substances were present in these materials. However, to determine the protein concentration in the pure crystal suspension, 2.5, solubilization of the material in NaOH was required and the pressure of other substances such as Triton X-100 and Na_2HPO_4 may have interfered with the Folin-Lowry method, thus the Coomassie Blue Dye - Binding technique was used.

2.7.1 Protein Determination Using the Folin-Lowry Method

Culture broth and ammonium sulphate samples diluted in distilled water were assayed according to the modification of the standard Folin-Lowry technique (Lowry et al., 1951), described by Plummer (1978). The Folin - Ciocalteau reagent was obtained from a commercial supplier, 2.1.

2.7.2 Protein Determination of Crystal Suspensions by the Coomassie Blue Dye-Binding Technique

The crystal material was dissolved by adding 0.5 ml of the suspension to 4.5 ml 0.1 M NaOH and incubating at 30°C for 2 h. Similar procedures for dissolving parasporal crystals had been described previously (Bulla et al., 1976; 1977, 1979; Dastidar and Nickerson, 1978).

The OD600 decrease was followed during the solubitization and reached

a constant value after 30 minutes. Following solubilization the solution was centrifuged at 15,000 x g, 30 min, 4°C, the supernatant was removed and its A280 determined in quartz cuvettes against 0.1N NaOH. For comparison the A280 of a 0.50 mg/ml BSA solution in 0.1N NaOH, 0.5 g/l Na_2HPO_4 was also determined.

To neutralize the alkali in the solubilized crystal solution, 2.0 ml was mixed with 0.5 ml of 0.5 M Na_2HPO_4 pH 7.0 and the final pH adjusted to 7.0 with about 25 ul of 9.3M H_2SO_4.

A standard protein curve using bovine serum albumin dissolved in the same solution to solubilize and then neutralize the crystal protein solution was prepared.

The protein content of the dissolved/neutralized crystal protein solution was low and in order for a significant A595 to be detected 200 ul of this solution was added to 5.0 ml of the diluted dye reagent. A similar adjustment was made for the standard protein solutions and apart from this alteration the technique used was identical to that recommended in the Bio Rad pamphlet accompanying the protein assay kit.

2.8 PROTEASE PURIFICATION TECHNIQUES

Crude culture-supernatant protease preparations were treated to purify the protease activities using Ammonium Sulphate Fractionation (ASF) and Gel Filtration (GF). The ASF was used to increase the specific activity of the protease preparations. This treatment was also used to eliminate an unidentified material which markedly increased the viscosity and volume of pellets using ammonium sulphate concentrations of greater than 30-40%. Only after ASF were samples considered suitable for application to a GF column.

2.8.1. Ammonium Sulphate Fractionation of Crude Culture Supernatants

This was the first step in the protease purification process. Final concentrations are expressed as % $(NH_4)_2$ SO_4 saturation as calculated by Green and Hughes (1955). All centrifugations were done at 15,000 xg, 30 min, 2°C. Ammonium sulphate was ground in a mortar and pestle to a fine powder and added slowly over 1-3 hours depending on the salt concentration and broth volume. During salt addition, the broths were continuallystirredwhile contained in vessels standing in ice-water baths to maintain a temperature of less than 4°C. When salt addition was complete the broths were stirred for a further 15-30 minutes before being left at 4°C overnight (O/N). Following O/N cold storage the precipitates were centrifuged, the pellets redissolved in 5-15 ml PDS (2.6.1) and pellets and supernatants dialysed O/N against the same buffer.

2.8.2 Dialysis of Culture Supernatants and Ammonium Sulphate - Treated Materials

All dialyses were carried out with constant stirring O/N at $4^{o}C$ in dialysis tubing that had been boiled for 30 minutes in 1 mM EDTA and then rinsed and soaked in distilled water. Sample volumes were measured before and after dialysis.

Ammonium sulphate fractions were dialysed against 30 volumes of protease diluting solution (PDS; 2.6.1).

To test the effect of dialysis on protease activities of crude culture supernatants, protease samples were dialysed against 100 volumes of PDS, or PDS without its Ca^{++} supplement, or against PDS without its Ca^{++} and with 1.0 mM EDTA.

2.8.3 Gel Filtration of Redissolved Ammonium Sulphate Pellets

2.8.3.1 Preparation of sample for gel filtration

200 ml of a supernatant from a fully sporulated and lysed culture broth (SM. 51. 1. 72) was brought to 45% ammonium sulphate saturation as described previously (2.8.1) and the pellet discarded. The supernatant was then adjusted to 60% saturation and the resulting pellet dissolved in 15.0 ml PDS at $4^{o}C$, dialysed and the dialysed preparation quantitated with respect to protease activity and protein concentration. 10.0 ml of the dialysed solution was applied to the column.

2.8.3.2 Equipment used

For the gel filtration of the protease sample a complete LKB column chromatography aparatus was used. The column itself was 2.6 x 75 cm, cooled by a cooling water bath. The buffer was pumped to the column by an LKB 2120 Variaperpex II peristaltic pump and the A280 of the eluent monitored by use of an LKB 2138 Uvicord S detector. Fractions were collected using an LKB 2070 Ultrorac II fraction collector while their A280 was plotted on an LKB 2120 potentiometric recorder.

The separating medium was Sephadex G-100 which was swollen by steeping in distilled water for 2 days followed by heating at 80°C for 2 hours, before being degassed under vacuum for 1 hour.

The gel was poured and packed with degassed distilled water before being equilibrated with GF buffer (100 mM NaCl, 1 mM Ca^{++}, 50 mM Tris, 0.4 g/l NaN_3, pH 8.0). The column volume was 370 ml. All GF operations after this were carried out at 4°C.

2.8.3.3 Standard sample application technique

The solution to be applied contained the protease or standard proteins, marker dyes and sucrose to increase sample density. This solution was applied directly to the column, after removal of the adaptor, from a syringe to which 1.2 mm internal diameter tubing was fitted. A piece of filter paper 2.3 cm in diameter had been allowed to settle onto the gel in order to flatten the gel surface and then the buffer above the gel bed was allowed to just barely run into the column to a depth of 1 mm, or less.

The sample solution was then applied to the column and allowed to run into the gel to the same extent as the buffer.

Then, 5-10 ml of fresh GF buffer was put on the column to prevent drying of the gel and the adaptor was carefully replaced so as to prevent trapping air bubbles beneath it. Having completed the operation the run was started by switching on the fraction collector.

2.8.3.4 Column running conditions

GF buffer was pumped through the column at a rate of 10.0 ml/hour. Fractions of 5.0 ml were collected by the pump calibration method (LKB instruction manual for the 2070 Ultrorac II Fraction Collector).

2.8.3.5 Column molecular weight calibration

Molecular weight calibration was done using cytochrome C, chymotrypsinogen A, ovalbumin and bovine serum albumin whose respective molecular weights are 12,400, 25,000, 45,000 and 68,000.

A solution was prepared containing 50 ul 1 mg/ml phenol red in 40 mM NaOH, 450 ul 5 mg/ml dextran blue, 500 ul each of the 4 protein standards (all 5.0 mg/ml in PDS) and 1000 ul 10% (W/V) sucrose. This solution was applied as described in 2.8.3.2 and the column run as outlined in 2.8.3.3.

The elution volume of the standard porteins was determined by measuring the A280 of the fractions and a standard plot of molecular weight vs elution volume drawn.

2.8.3.6 Gel filtration of protease sample

10.0 ml of a protease preparation described in 2.8.3.1 was mixed with

50 ul 1 mg/ml phenol red in 40 mM NaOH, 500 ul 5 mg/ml dextran blue and 2000 ul 10% (w/v) sucrose. The entire solution was then applied to the column and run as previously mentioned 2.8.3.2,3.

Collected fractions were stored at $4^{o}C$ for 1-2 days before their protease activity was determined by the modified azocasein digestion method, 2.6.2.1.

The A280 of the fractions was also determined by a spectrophotometer because the detector/recorder on line with the column only gave a continuous read out of the eluent, whereas, an individual A280 value for each fraction was required to be able to graphically represent the ultra-violet absorbance of the eluted fractions.

SECTION 3: RESULTS

3.1 FERMENTATION OF BACILLUS THURINGIENSIS

The aim of the investigation described in this section was to optimize the commercial fermentation of B. thuringiensis var israelensis with respect to insecticidal activity, cost and fermentation time.

There are a number of key stages/considerations in the development of an industrial fermentation process following the isolation of a suitable strain. These include the characterization of inoculum media, optimization of the production medium and the conditions required to produce maximum product activity, scale up, development of suitable culture storage procedures and techniques for ensuring strain productivity (see section 1.8.1.).

With respect to inoculum stage development it is important to obtain a protocol capable of producing an inoculum in one, or a series of steps. This protocol should give a high yield of biomass in a healthy condition which, when used to seed the production medium, will initiate growth and metabolite elaboration with a minimum log phase. In the B. thuringiensis fermentation this was achieved by growing the organism in a nutritionally rich medium where sporulation was prevented so that the vegetative cells were able to commence growing in the production medium after a short period of adaption to their new environment.

Production stage development should allow rapid elaboration of the product of interest at as low a cost as possible. In order to achieve this, medium components should be carefully selected to provide sufficient carbon and nitrogen to the organism without causing an imbalance of the two, if microbial biomass is to be optimized. In addition, medium components should not cause unacceptable changes in the pH of the broth and they should also be compatible with the

downstream processing of the culture.

Storage of the organism and development of techniques to monitor strain production ability are essential to the longterm implementation of a process on an industrial scale. Depending on the type of microorganism used the preferred storage conditions will vary, but freeze drying is generally accepted as the best method for longterm maintenance of microbes and this should be used where applicable/possible. Bacilli are usually easily stored by refrigeration on agar slants for many months due to their formation of heat-resistant endospores, and B. thuringiensis was conveniently maintained under these conditions.

With the fermentation of B. thuringiensis the most important parameter is the production of insect toxin which, in turn, is related to biomass production and sporulation and lysis efficiencies (Luthy et al., 1982).

Having regard to these considerations a process for the fermentation of B. thuringiensis was developed by first of all conducting some preliminary studies to obtain an idea about how best to approach optimization of biomass production. Following this, a small number of experiments was done varying culture conditions and media components, notably casein, to determine their effect on the growth of the bacterium.

An inoculum medium was then developed with the knowledge that a vegetative cell inoculum was required in order to minimize the log phase in the production medium.

The production stage was optimized by first assessing the potential of a number of literature - reported processes in terms of cost and

biomass production and sporulation/lysis efficiency. The best of these media were varied in their nutrient components to try to produce an even better medium. In addition, a number of media not bearing a direct relationship to those from the literature were also studied.

The most promising of these media were investigated to determine which produced the highest bioactivity and at which stage during the fermentation the maximal insect toxicity occurred.

Finally the process was scaled-up from flasks through laboratory fermenters to pilot scale.

With respect to strain maintenance, a study was done using slants which had been deliberately stored and subcultured under suboptimal conditions. The effects of these poor maintenance conditions on the fermentation were established and safeguards to prevent problems with strain deterioration recommended.

3.1.1 Preliminary Investigations

During this section a number of media and growth conditions were studied to determine their effect on the growth of the organism.

3.1.1.1 Effect of aeration and casein on sporulation of B. thuringiensis

During early experiments, when grown in the Drake and Smythe (D&S) medium (2.3.4) sporulation of B. thuringiensis was never observed. However, in one experiment when casein was omitted from the medium, high levels of growth and sporulation were observed (data not shown). It was therefore decided to investigate the reason underlying this phenomenon.

21 flasks containing 150 ml or 400 ml of both the original D & S

medium and the Drake and Smythe without casein medium (0 Cas) were inoculated with a 5% volume of a 24 hour Inoc. 2 culture (2.3.3) and incubated for 72 hours as recommended in 2.3.1. At various times during the fermentation viable counts, pH and VC:SC:FS ratios were determined and the results are presented in Table 3.1.1.

These results clearly indicated that a high level of growth and sporulation of B. thuringiensis was obtained using the D & S medium only when the casein was omitted from the medium and when a high level of aeration was used to culture the organism. It should be noted that a high level of aeration on its own was not sufficient to induce sporulation in the D & S medium. In addition, omission of the casein from the medium and the use of a low aeration rate did allow sporeformation to take place, but at a much slower rate than was observed using the high aeration conditions. Furthermore these results show that omission of casein from the D & S medium did not significantly alter the viable counts observed, Table 3.1.1.

Thus, it appeared that casein in the D & S medium acted as an inhibitor of sporulation and that in its absence, in conjunction with the use of a high level of aeration, high levels of growth and sporulation occurred.

The effect of aeration on growth and sporulation of B. thuringiensis was also studied using the Megna medium (2.3.4). One litre and two litre Erlenmeyer flasks containing 10% and 20% of their capacity were inoculated and incubated similarly to the Drake and Smythe flasks in the experiment described above; viable counts, pH and VC:SC:FS ratios were determined at various time intervals and the results obtained presented in Table 3.1.2.

TABLE 3.1.1

The effect of aeration and casein on the growth and sporulation of *B. thuringiensis* when grown in 2l flasks

MEDIUM	BROTH VOLUME (ml)	VIABLE COUNT $x10^9$/ml (t48)	pH at tx					VC:SC:FS at tx		
			0	24	36	48	72	24	48	72
D&S	400	1.1	7.0	5.4	5.4	5.5	5.5	100:0:0	100:0:0	100:0:0
	150	1.1	7.0	5.5	5.6	5.7	6.8	100:0:0	100:0:0	100:0:0
0 Cas	400	1.0	7.0	6.7	5.8	5.9	5.9	100:0:0	100:0:0	10:90:0
	150	1.5	7.0	7.0	7.1	7.1	7.1	60:30:10	15:5:80	10:10:80

B. thuringiensis was grown on the Drake and Smythe medium and on the Drake and Smythe without casein medium using different broth volumes in 2l flasks to give different levels of aeration, so that the effect of casein and aeration on the sporulation and growth of the organism could be determined.

Once again, it was found that the flasks with the lowest amount of medium grew and sporulated considerably quicker than the flasks containing the higher level of medium. In accordance with the Drake and Smythe results, lower rates of aeration were seen only to delay sporulation rather than inhibit it completely. In contrast to the Drake and Smythe medium, no evidence was found for a substance inhibiting sporulation in the Megna medium because similar levels of growth and sporulation occurred in most flasks by t120.

An important observation was made with the Megna medium where the pH profile of the culture followed the growth of the bacterium. Initially, in the early stages of growth the pH fell to 6.0 or less and thereafter when vegetative growth became less vigourous and sporulation began the pH gradually increased. Then as sporulation and lysis proceeded, a furtner pH increase was observed with the final pH reaching a value of close to 9.0 in most cases. This general pattern of pH/growth was observed for many media in later experiments and has also been reported by other workers (Tyrell et al., 1981).

It was also noticed that severe clumping of cells occurred during logarithmic growth of B. thuringiensis, a feature of this organism which has also been reported (Meenakshi and Jayaraman, 1979). This clumping continued through to sporulation and only began to break down late during sporeformation and lysis. The significance of this clumping phenomenon was that viable counts of cultures of clumped cells gave unreliable estimations of the bacterial numbers and that increases in the viable count seen late in the fermentation process, Table 3.1.2, were due to clump-breakdown and sporangial lysis and not to a change in the number of cells present. Therefore viable counts were usually only done on unclumped vegetative cell cultures or on fully lysed broths.

TABLE 3.1.2

Effect of aeration on growth and sporulation of B. thuringiensis when cultured in the Megna medium in 1l and 2l flasks.

Flask Volume (l)	Broth Volume (ml)	Viable Counts at tx X 10^9/ml				pH at tx					VC:SC:FS at tx (%)		
		8	24	48	120	8	24	32	48	120	24	48	120
2	400	0.5	0.6	0.6	1.7	5.85	6.00	5.95	7.25	8.35	100:0:0	100:0:0	40:5:60
	200	0.8	0.4	1.5	2.2	6.00	7.10	7.40	8.20	9.10	100:0:0	40:5:55	5:0:>95
1	200	0.6	0.6	0.6	2.0	5.95	6.00	7.00	7.45	8.90	100:0:0	20:80:0	10:0:90
	100	0.7	0.6	2.0	2.0	6.20	7.60	8.00	8.65	9.25	50:50:0	<5:40:60	5:0:95

* Initial pH was 7.0

3.1.1.2 Effect of the use of vegetative cells and free spores as inoculum sources

In an attempt to confirm that the use of vegetative cells was preferable to spores as the inoculating cell type and to determine the difference in the log phase observed using these two types of cell, two experiments were conducted where different media were inoculated with vegetative cells and with spores and the growth and sporulation observed following inoculation.

In the first experiment, a series of 11 flasks containing 200 ml of TW medium (2.3.2) were inoculated with either spores or vegetative cells such that the initial viable count in both types of inoculum was similar. The TW medium was used because growth could be readily quantified by optical density as well as viable count, in addition to the fact that maximal levels of growth occurred quickly without the formation of spores so that the effect of inoculum type on vegetative growth alone could be estimated.

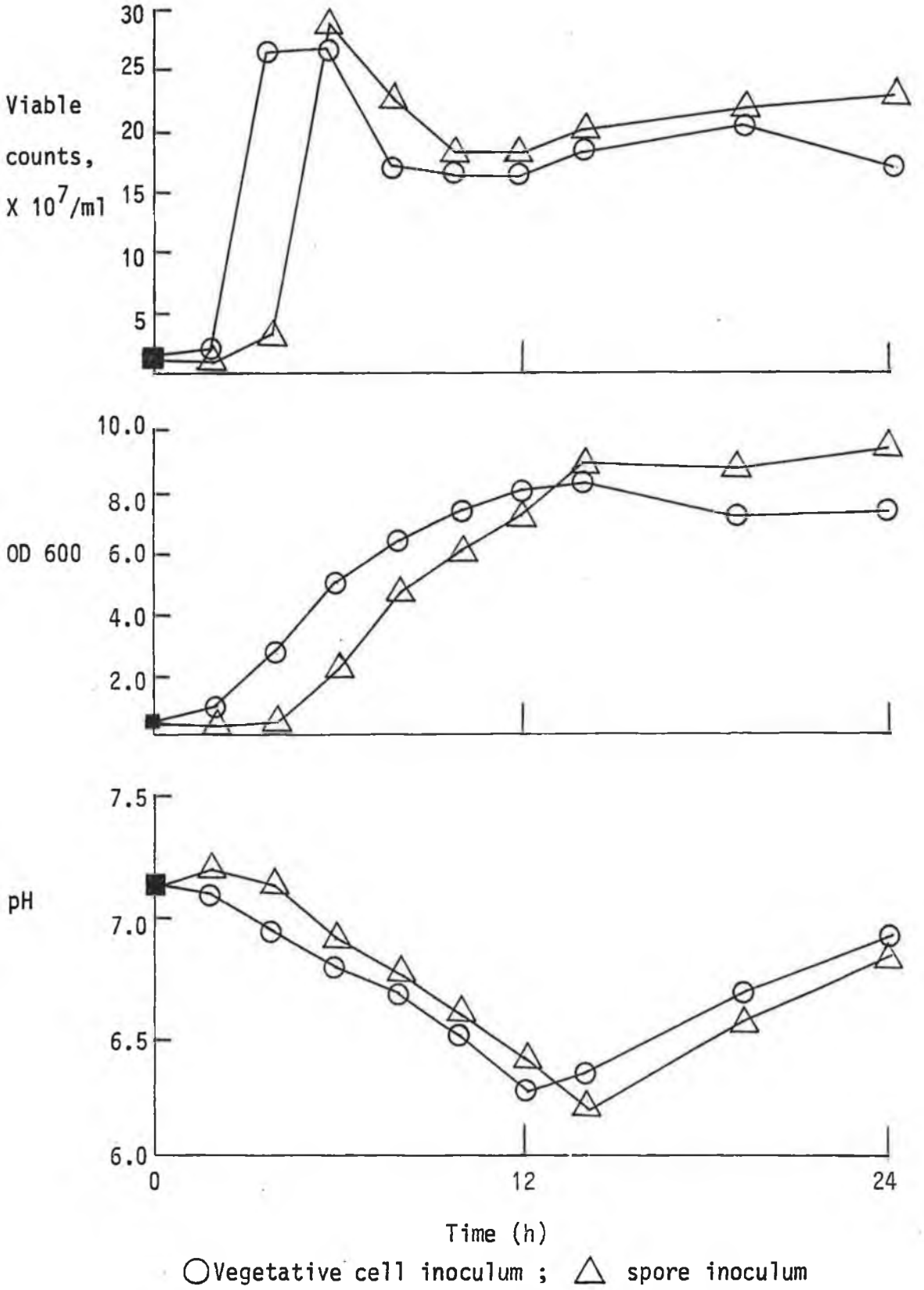
The vegetative cell inoculum for this experiment was prepared as described in 2.3.2 and the spore suspension used was prepared as outlined in the same section. Following inoculation the flasks were incubated as in 2.3.1 and growth of the cultures monitored for 24 hours by measuring pH, viable count and OD 600. The results obtained are presented in Figure 3.1.1.

In the second of these experiments a similar approach was used to see if the results obtained using the soluble TW medium could be observed using an industrial medium.

Vegetative cell and spore inocula were prepared as for the TW experiment above and 100 ml of the SM medium (Table 3.1.8) in 1 litre

FIGURE 3.1.1

Effect of using a vegetative cell and a free spore inoculum to seed the soluble TW medium when culturing B. thuringiensis in flasks.



flasks were inoculated and incubated as in 2.3.1. Viable counts, pH and sporulation efficiencies were followed during growth of the organism and the results obtained are presented in Figure 3.1.2.

The results of these two experiments clearly demonstrated the shorter log phase caused when vegetative cells were used to inoculate both types of medium. In addition, the use of vegetative cells gave higher sporulation efficiencies (% of cell population which formed spores) than did the spore inoculum. In the industrial medium there were higher levels of growth in the vegetative cell-inoculated cultures than in the spore-inoculated cultures during the early stages of the fermentation. However, the viable counts at the end of the production stage were similar in both types of flask, Figure 3.1.2.

These results mitigated for the use of vegetative cells as the inoculating cell type in order to produce a more sporulation - efficient fermentation, and, to a lesser extent, to reduce the fermentation time by a reduction of the lag phase.

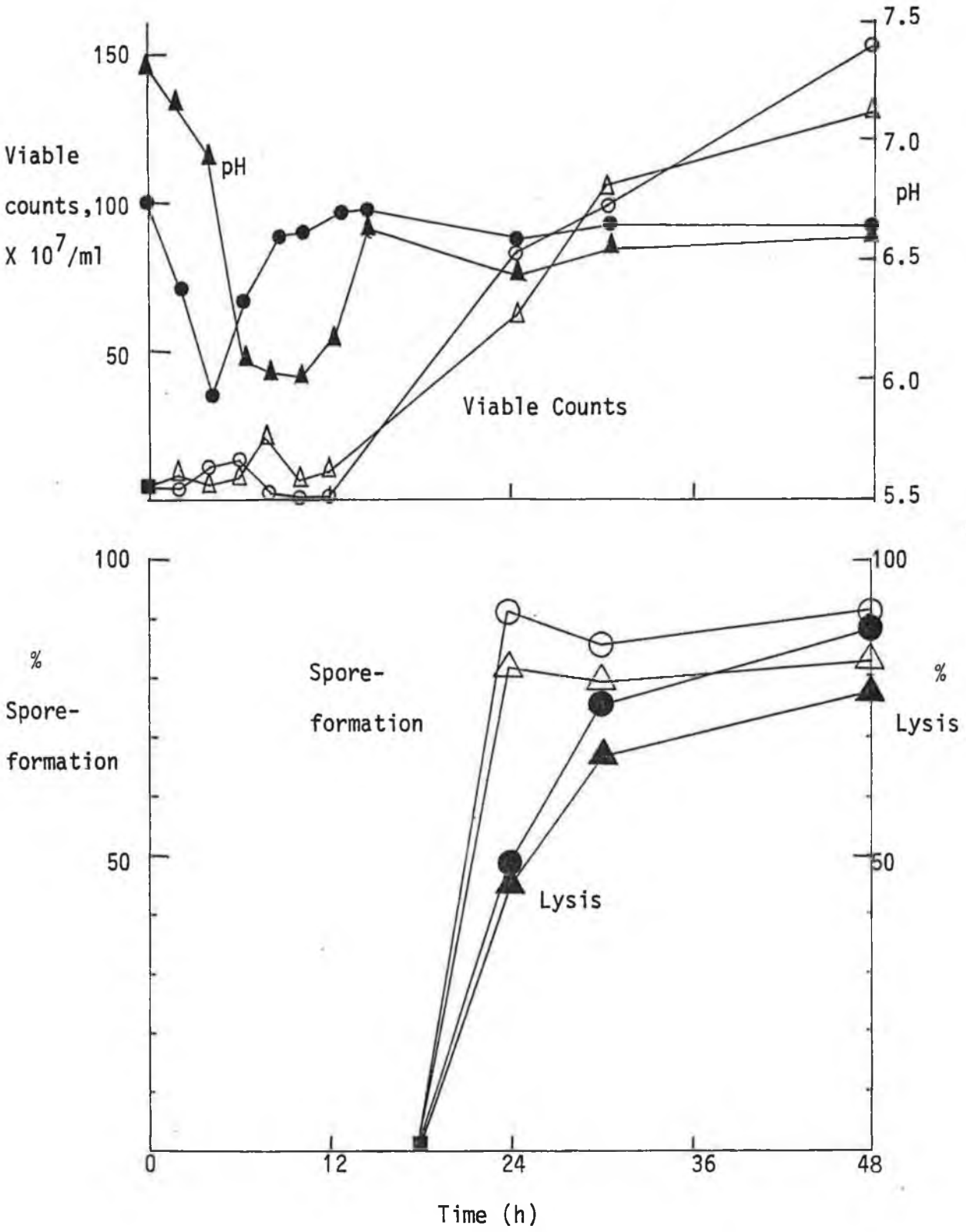
3.1.2 Development of Inoculum Production Process

Up to this point it had been demonstrated that an inoculum - producing process should produce a high level of biomass, most desirably consisting of vegetative cells. Also, it was shown that sporulation of B. thuringiensis could be prevented by the use of low level aeration and the inclusion of casein in the medium, 3.1.1.2.

Therefore, for the purposes of designing a protocol for the growth of an inoculum on an industrial basis, a three-stage process was to be used because it was likely that at least this many stages would be required on a commercial scale. Furthermore, it was decided that the TW medium (2.3.2) would be used as the Inoc. 1 medium because it was

FIGURE 3.1.2

Effect of using a vegetative cell inoculum and a spore inoculum to seed the industrial SM medium when used to culture B. thuringiensis in flasks.



○ , ● Vegetative cell inoculated flasks

Δ , ▲ Spore inoculated flasks

B. thuringiensis cultured in 1l flasks containing 100 ml SM medium under standard conditions.

easily prepared, gave extremely reproducible results and it prevented sporulation while maintaining viability of the cells for extended periods (data not shown). However, the cost of this medium meant that it could not be used after this stage and thus a second inoculum stage medium had to be developed.

For this reason, an investigation was carried out to discover a medium which would produce a high cell yield at a low cost in a maximum of 24 hours, in which the cells were in a vegetative state when used to inoculate the production medium.

3.1.2.1 Development of the medium

One litre flasks containing 100 ml of various media were inoculated with a 5% volume of a 24 hour TW culture (2.3.2) and incubated under standard conditions, 2.3.1. Table 3.1.4 describes the growth of B. thuringiensis with respect to viable count, pH and VC:SC:FS proportions when cultured in these media, whose contents and costs are described in Table 3.1.3.

The media which best prevented spore formation were numbers 1, 2, 5, 7, 8 but medium 8 gave high cell density by microscopic examination and was cheaper than media 1 and 2. The low viable counts were caused by the severe clumping which affected all cultures, especially at t20, t24. Because of the good growth observed microscopically (data not shown), its relatively cheap cost and its ability to prevent spore formation completely up to t20, medium 8 was selected as the second inoculum stage medium. After its discovery it was found that by using 200 ml medium 8 per 1l flask, sporulation could be prevented completely for at least 30 hours. Hereafter this medium was called the Casein Inoculum Medium.

TABLE 3.1.3

Contents and Costs of Media Investigated for Use as Inoc. 2 Media

COMPONENT/	CONCENTRATION, g/l							
PARAMETER	1	2	3	4	5	6	7	8
Casein	20.0	20.0	-	-	-	-	-	-
Corn Steep Solids	10.0	5.0	10.0	10.0	10.0	20.0	20.0	5.0
Yeast Extract	5.4	2.7	10.0	15.0	20.0	5.4	5.4	2.5
Molasses	-	-	-	-	-	-	-	5.0
Sucrose	30.0	15.0	30.0	30.0	30.0	30.0	15.0	-
Na_2HPO_4	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Cost, IR£/m^3	55.4	46.4	27.0	34.0	41.0	27.0	25.1	44.3

TABLE 3.1.4

Growth observed for B. thuringiensis when cultured in a variety of media tested for use as Inoc. 2 media

MEDIUM	pH AT Tx				VIABLE COUNTS AT Tx ($x10^7$/ml)			VC:SC:FS		
	0	20	24	30	20	24	30	20	24	30
1	7.00	7.53	7.63	7.74	24	18	24	100:0:0	95:5:0	60:40:0
2	7.13	7.60	7.73	7.81	23	35	23	100:0:0	85:15:0	10:90:0
3	7.04	7.70	8.00	8.27	30	39	81	30:70:0	10:80:20	10:30:70
4	7.00	7.49	7.73	8.09	7	21	60	30:70:0	10:60:30	5:50:45
5	7.04	7.46	7.73	8.00	14	31	50	100:0:0	25:75:0	10:90:0
6	7.09	7.44	7.70	7.96	33	29	33	30:70:0	10:90:0	5:85:15
7	7.05	7.48	7.68	8.06	19	35	46	10:90:0	10:85:0	10:80:10
8	7.26	7.56	7.70	7.89	31	28	25	100:0:0	50:50:0	10:90:0

3.1.2.2 Determination of optimum inoculum size

In order to determine the minimum inoculum volume which would allow quickest growth and sporulation of the organism in the production stage 1%, 5%, 10%, 15% and 20% inoculum volumes of a 24 hour casein inoculum flask culture were used to seed the production medium. One hundred millilitres of SM medium (Table 3.1.8) per 1 litre flask were used as the production stage and following inoculation, the flasks were incubated as in 2.3.1 while pH, viable counts and sporulation efficiencies were monitored over a 48 hour period. The results obtained are shown in Table 3.1.5.

The data produced during this experiment showed that there was no significant difference in the growth patterns for the various inoculum sizes. It was decided to use the 5% inoculum volume because this inoculum level gave more reproducible results than the 1% volume as well as allowing more rapid spore formation and lysis. In addition, this inoculum volume was approximately the same size as had been used by previous workers when developing commercial fermentations (Dulmage, 1970b; Margalait et al., 1983; Goldberg et al., 1980).

However, inoculum volumes as low as 0.5% have been recommended, not to increase yields, but to improve reproducibility (Dulmage, 1981).

Thus following the completion of this experiment, an inoculum medium which yielded a high level of biomass in a vegetative cell state, in 24 hours had been described and the optimum inoculum level for seeding the production stage medium determined. It therefore remained to elucidate the growth characteristics of B. thuringiensis in this medium to establish over which range of culture ages this medium could be used to inoculate the production medium.

TABLE 3.1.5

Growth of B. thuringiensis in the SM medium following inoculation by various volumes of a 24 hour casein inoculum flask culture

INOCULUM SIZE	pH AT Tx					VIABLE COUNTS AT Tx ($x10^7$/ml)			VC:SC:FS: AT tx		
	0	6	24	30	48	24	30	48	24	30	48
1%	6.7	6.0	7.0	7.1	7.3	25	100	100	15:85:0	10:40:50	10:5:85
5%	6.7	6.0	6.3	6.5	6.7	77	105	110	10:45:45	10:30:60	5:5:90
10%	6.7	6.1	6.3	6.5	6.7	75	100	100	10:50:35	10:50:40	5:10:85
15%	6.7	6.4	6.3	6.5	6.7	74	91	125	10:60:30	15:10:40	15:10:75
20%	6.7	6.6	6.5	6.5	6.6	91	95	117	10:60:30	10:50:40	5:10:85

Cultures grown in one litre flasks containing 100ml medium under standard conditions.

3.1.2.3 Patterns of growth of B.thuringiensis when cultured in the casein inoculum medium

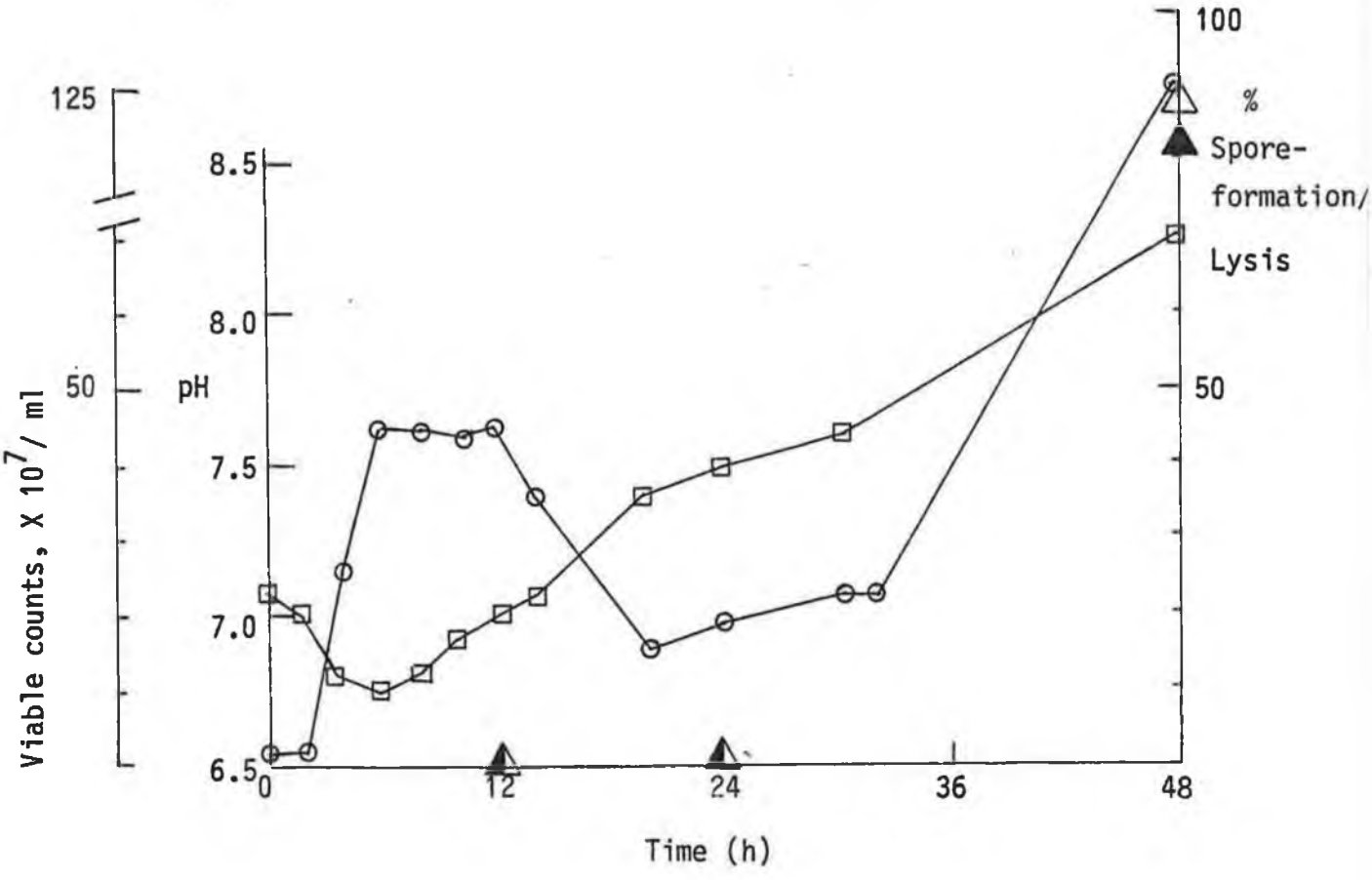
As was mentioned above, having determined the optimum medium for the production of a seed culture for inoculating the production stage, and also having established the optimum inoculum volume, it was necessary to investigate the optimum time of inoculation.

This was done by growing B. thuringiensis in the casein inoculum medium in flasks and monitoring viable counts, pH, and VC:SC:FS proportions for up to 48 hours.

A 5% inoculum volume of a 24 hour TW culture (2.3.2) was used to inoculate the casein inoculation medium. For this flask experiment 200 ml of casein medium/1 l flask were cultured as in 2.3.3.1. The results obtained are shown in Figure 3.1.3. Except for the sporulation profiles, the patterns of growth of B. thuringiensis in the medium were similar to those observed when the organism was grown in the Megna medium, Table 3.1.2. In both cases, the pH dropped during vigourous vegetative growth, and rose as vegetative growth slowed down and the cells reached the post-exponential stage. Following this, sporulation took place giving rise to a rather steep increase in the pH. Once again, clumping caused an apparent decrease in viable counts after about 6-8 hours of growth, but bacterial numbers increased again once a high degree of lysis was achieved. Most importantly, the patterns of growth of the organism in the casein inoculum medium showed that no spore formation occurred for at least 30 hours after inoculation and therefore it was accepted that casein inoculum cultures up to 24 hours old could be used as satisfactory inocula for the production stage media. Vegetative growth had ceased at around t10-t12 so it was accepted that the casein inoculum culture could be used to seed the production stage between 14 and 24 hours;

FIGURE 3.1.3

Growth of B. thuringiensis in the casein inoculum medium in flasks.



○ Viable counts ; □ pH ; △ % Spore-formation ; ▲ % Lysis

B. thuringiensis was 5% inoculated with a 24 hour TW culture and grown in a 1l flask with 200 ml casein inoculum medium under standard conditions.

although 24 hour cultures were most frequently used as inoculum sources, cultures as young as 14 hours were successfully used for the same purpose.

3.1.3 Characterization and Development of Production Media by Optimization of Biomass Production and Sporulation

In general, a production medium for a fermentation process is optimized by selecting for best product elaboration with respect to medium costs, fermentation cycle time, and process reproducibility. In addition, other factors, such as the compatibility of the medium with the downstream processing and the effect of the medium components on medium viscosity and mass/heat transfer may affect the suitability of a given medium.

With respect to the fermentation of B. thuringiensis the production medium should yield a high level of insect toxin in a cycle time of less than 72 hours. For this to occur, a high degree of sporulation and lysis is required because the crystalline protein toxin is produced only during sporulation and its toxicity is expressed by release from the mother cell sporangium (1.8.2).

Thus, in this study the production medium was optimized by, first of all, evaluating a number of literature-reported process with respect to biomass production and sporulation. Variations of these literature media, as well as other media, were then studied by evaluating the same growth parameters, medium costs and cycle times. Finally, the production media were quantified with regard to the levels of bioactivity present, enabling a decision to be made on the basis of this parameter, as to which of the media investigated was most suited as the production medium for the fermentation of B. thuringiensis.

3.1.3.1 Comparative evaluation of literature-reported production media

A number of production media have previously been described in the scientific literature (Dulmage, 1970b, 1971; Goldberg et al., 1980) as well as in patents (Drake and Smythe, 1963; Megna, 1963; Mechalas, 1963; CRC, 1978). Five of these processes were investigated, but the Mechalas (1963) one was ignored because it described a semi-solid fermentation which was considered unlikely to be as successful as the submerged fermentation procedures. Some of the other media were not assessed because they were too expensive (Goldberg et al., 1980), and/or some of the medium constituents (such as cottonseed flour) were unavailable (Dulmage, 1970).

The literature media studied are described in Table 3.1.6. To assess these media, 200 ml volumes in 2 l flasks were 5% inoculated with a 24 hour casein inoculum medium culture prepared as described in 2.3.3.1 and the production flasks incubated under the standard flask incubation conditions, 2.3.1. During growth in these media, pH, viable counts and VC:SC:FS proportions were monitored. Table 3.1.6 indicates the maximum levels of biomass and sporulation observed for these media, as well as the time at which these levels were recorded.

A considerable variation in the levels of biomass production and sporulation was observed for these media. In only one medium, the Drake and Smythe, was sporulation never observed (see also 3.1.1) and in the others high levels of sporulation were achieved in as little as 24 hours and as high as 72 hours. The viable counts were usually in the 1-2 x 10^9/ml range except for the CRC Molasses medium which typically gave lower viable counts. The levels of sporulation and biomass production were apparently functions of the quality and quantity of the media's nutrient composition. Thus, in the nutritionally rich Drake and Smythe medium, which had a nutrient

TABLE 3.1.6

LITERATURE PRODUCTION MEDIA ASSESSMENT

COMPONENT/ PARAMETER	CONCENTRATION, g/l				
	DRAKE & SMYTHE 1963 (D & S)	MEGNA 1963 (MEG)	CRC MEDIA, 1978 MOLASSES (MOL)	CRC MEDIA, 1978 STARCH (ST)	DULMAGE 1971 (DUL)
Casein	20.0	-	-	-	-
Defatted Soya Flour	-	14.0	-	-	15.0
Yeast Extract	5.4	-	3.0	10.0	-
Corn Steep Solids	10.0	17.0	1.0	2.0	-
$(NH_4)_2 SO_4$	-	-	1.0	-	-
Starch	45.0	-	-	13.0	10.0
Molasses	-	18.6	14.0	-	-
Sucrose	9.0	-	-	-	-
Glucose	-	-	-	-	5.0
Na_2HPO_4	8.0	-	-	4.0	-
$CaCO_3$	-	1.0	1.0	8.0	1.0
Total Solids (% W/V)	9.6	4.6	1.6	3.3	3.1
Cost (IR£) Per 1000 l	67.00	20.70	6.30	23.00	14.30
Max. TVC, $x10^9$/ml	2.0-2.5	2.0-2.5	0.3-1.1	0.7-1.5	1.2-2.5
Time of 90% Lysis (h)	>90*	72	24-30	48-72	48-72

* Sporulation of B. thuringiensis was never observed in the medium, even when the culturing period was extended up to 96 hours.

solids content of 9.6%, good growth was observed, but sporulation was inhibited due to the excessively high nutrient content and/or the presence of a sporulation inhibitor derived from the casein, 3.1.1.1. On the other hand, in the nutritionally poor CRC Molasses medium, high levels of sporulation and lysis occurred rapidly, but with the production of a low amount of biomass. The media with an intermediate nutrient content gave relatively high levels of biomass and sporulation, but in a longer time than was required by the CRC Molasses medium for full lysis. Interestingly the CRC Starch medium, which contained 10g/l of yeast extract, was inhibited in its sporulation by this nutrient, because when lower levels of yeast extract were used much more rapid sporulation and lysis was observed (data not shown).

This series of results demonstrated very clearly the necessity of using carefully balanced levels of individual nutrients so that the production medium should be sufficiently rich to allow growth and sporulation in an acceptable time period. These data, and those presented in 3.1.1.1, underlined that the production medium should not be excessively nutritious as this was likely to delay, or even inhibit completely, the progress of sporulation and lysis.

Thus, of the five media studied it was the Drake and Smythe, the Megna and the Dulmage media which produced the highest levels of biomass. The Drake and Smythe medium did not permit sporulation, although it did if the casein was omitted (3.1.1.1) and was the most expensive of the five media studied. The Dulmage medium was considerably cheaper and produced only slightly lower counts than the other two, while the Megna medium was more expensive than the Dulmage, but allowed better growth. These latter two media required 48-72 hours for lysis to reach acceptable levels. It was, therefore, on the

basis of biomass production capacity that these three media were used to optimize the production medium for the fermentation of B. thuringiensis.

3.1.3.2 Assessment of literature-reported media variations

Having carried out a preliminary evaluation of the production media described previously, the best of these media were selected (3.1.3.1) and varied by changing the concentration of some of the recommended components, omitting them altogether, or adding in nutrients not used in the original medium formulations. Again, as in 3.1.3.1 these variant media were optimized, at this stage, with regard to biomass production and sporulation. Thus, a medium which struck an acceptable balance between biomass yield, medium cost, and fermentation time was sought.

One litre or two litre Erlenmeyer flasks containing 100 ml or 200 ml of medium, respectively, were 5% inoculated with 24 hour casein inoculum medium cultures prepared as in 2.3.3.1 and the production flasks incubated as in 2.3.1. Total viable counts, pH and the proportions of vegetative cells, sporulated cells, and free spores were determined during the culturing period. The results obtained for the most important medium variants are presented in Tables 3.1.7, 8, 9. Data for many other medium variants which, when tested, gave low yields are not presented. It should also be noted that the data presented here were derived from a number of fermentation runs.

The most significant results obtained with the Drake and Smythe variants (Table 3.1.7) were as follows. Firstly, the use of sucrose as the carbon source, even at very high levels of 45g/l, allowed rapid sporulation and lysis, but only low viable counts were obtained.

TABLE 3.1.7

Useful variants of the Drake and Smythe (1963) medium, and results obtained using these media.

COMPONENT	CONCENTRATION, g/l								
	D&S	V1	V2	V3	V4	V5	V6	V7	V8
Casein	20.0	-	-	-	-	-	-	-	-
Corn steep solids	10.0	10.0	10.0	5.0	5.0	5.0	10.0	5.0	10.0
Yeast Extract	5.4	5.4	5.4	3.0	2.7	3.0	2.7	2.7	5.4
Starch	45.0	-	-	-	-	20.0	-	-	45.0
Molasses	-	-	-	-	20.0	-	50.0	50.0	-
Sucrose	9.0	45.0	30.0	15.0	-	-	-	-	9.0
Na_2HPO_4	8.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	8.0

PARAMETER	MEDIUM								
	D&S	V1	V2	V3	V4	V5	V6	V7	V8
Total Solids (% w/v)	10.0	6.0	5.0	2.8	2.8	3.3	5.5	5.0	7.5
Cost/m^3 (IRx)	67.0	22.4	20.5	17.0	11.3	18.8	17.0	14.2	30.5
Max. Viable Count ($x10^9$/ml)	2.0-2.5	1.0	1.0	1.0	1.5-2.0	1.5-2.0	1.2-1.5	1.0-1.5	2.0
Time of 90% lysis (h)	>96*	30-40	24-36	24	48	48	48	48	48

B. thuringiensis was grown in 1l or 2l flasks containing 10% of the flask capacity of medium and incubated under standard conditions. These are the results of a number of fermentation runs.

* Sporulation never observed in this medium.

This was possibly due to poor utilization of the sugar by the organism which would account for the poor growth and rapid sporulation. Media using this sugar were not inexpensive, and thus were not considered commercialy useful, despite their rapid rates of sporulation. Secondly, when molasses was used to provide carbohydrate levels similar to those obtained when 20g/l of starch were used similar levels and rates of sporulation were observed using both carbohydrate sources, but viable counts were higher using the starch (media V5, V6, V7). Lastly, the V8 or OCas medium which was nutritionally very rich gave no better results than media V4 and V5 which were considerably cheaper.

Thus, the most economically attractive variants of the Drake and Smythe medium were V4 and V5 which gave viable counts of 1.5-2.0 x 10^9/ml and high levels of lysis within 48 hours at a reasonable cost.

In general, the Dulmage medium variants were better than the Drake and Smythe variants from the cost, biomass production and sporulation points of view, Table 3.1.8. Most notable of these media was the SM medium which was the second cheapest of those examined, but which gave consistently high counts and high levels of sporulation and lysis within 48 hours. This medium was not improved by the addition of 1 g/l of yeast extract, SMY (V5) of Table 3.1.8. In addition, it was shown that soyabean meal was the best of the soya preparations used. However, the meal, as supplied, was not suited to insecticide formulation requirements (Dulmage, 1981) and thereafter soyabean meal ground in a domestic kitchen blender was used. Glucose was found to be a suitable carbon source (Medium SM.G, V7, Table 3.1.8), but in view of its cost, by comparison to that of starch and molasses, it was not frequently used in medium formulations.

The general trend exhibited by the Megna medium variants was one of

TABLE 3.1.8

Useful variants of the Dulmage (1971) B-5 medium, and results obtained using these media.

COMPONENT+	CONCENTRATION g/l							
	Original	V1 FFSF	V2 SBM	V3 SP	V4 SM	V5 SMY	V6 S.20M	V7 SM.G
Defatted SF*	15.0	-	-	-	-	-	-	-
Full Fat SF*	-	15.0	-	-	-	-	-	-
Soyabean meal	-	-	15.0	-	15.0	15.0	15.0	15.0
Soya peptone	-	-	-	15.0	-	-	-	-
Yeast extract	-	-	-	-	-	1.0	-	-
Glucose	5.0	5.0	5.0	5.0	-	-	-	10.0
Starch	10.0	10.0	10.0	10.0	10.0	10.0	-	-
Molasses	-	-	-	-	10.0	10.0	-	-
$CaCO_3$	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

*SF: Soyaflour

PARAMETER	MEDIUM							
	ORIGINAL	V1	V2	V3	V4	V5	V6	V7
Total Solids (% w/v)	3.0	3.1	3.1	3.1	3.3	3.6	3.0	2.5
Cost/m^3 IR£	14.3	8.5	13.0	20.5	6.6	8.6	4.3	8.0
Max. Viable counts ($x10^9$/ml)	1.2-2.5	1.9	2.3	1.2	2.0-3.0	2.0	1.6	2.0
Time of 90% lysis (h)	48-72	48-72	48	72	48	48	48	48

+ Each medium also contained 0.2 g/l $MgSO_4.7H_2O$, 0.01 g/l, $FeSO_4.7H_2O$, 0.01 g/l $ZnSO_4.7H_2O$.

B. thuringiensis was grown in 1l or 2l flasks containing 10% of the flask's capacity of medium and incubated under standard conditions. These are the results of more than one fermentation run.

high biomass production with a cycle time of 48 to 72 hours, at a cost 2-4 time higher than the SM variant of the Dulmage medium, Table 3.1.9. When starch, glucose and sucrose were compared as carbon sources (media V5, V6, V7 Table 3.1.9) starch and glucose gave similar biomass production and fermentation cycle times. However, viable counts using sucrose were lower than for the other two carbon sources, and, in addition, the time required for 90% lysis to be observed was 72 hours. This delay of sporulation and lysis using sucrose was in contrast to results obtained earlier using Drake and Smythe variants, Table 3.1.7. The reason for this dichotomy of responses to sucrose as the carbon source was probably due to the high level of nitrogen sources in the V7 variant of the Megna medium. Among the Megna medium variants examined V3, V5 and V6 were most useful from the point of view of biomass production, however all these media required 72 hours for full lysis to occur and were at least twice as expensive as the SM variant of the Dulmage medium.

Table 3.1.10 compiles the most useful variants of the three literature media which were considered to have the greatest potential as production media from considerations of cost, biomass production, and fermentation cycle time.

On the basis of low cost, high biomass production and a production stage time of 48 hours within which at least 90% lysis took place, the SM (V4) variant of the Dulmage medium was selected as the most likely medium to be useful for producing B. thuringiensis var israelensis on an industrial scale.

TABLE 3.1.9

Useful variants of the Megna (1963) medium, and results obtained using these media.

COMPONENT	CONCENTRATION, g/l							
	ORIGINAL	V1	V2	V3	V4	V5	V6	V7
Defatted SF*	14.0	14.0	14.0	25.0	14.0	14.0	14.0	14.0
Corn steep solids	17.0	5.0	-	5.0	5.0	5.0	5.0	5.0
Yeast extract	-	-	4.0	2.0	2.0	2.0	2.0	2.0
Molasses	18.6	18.6	18.6	18.6	20.0	-	-	-
Starch	-	-	-	-	-	10.0	-	-
Glucose	-	-	-	-	-	-	10.0	-
Sucrose	-	-	-	-	-	-	-	10.0
$CaCO_3$	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

* SF: Soyaflour

PARAMETER	MEDIUM							
	ORIGINAL	V1	V2	V3	V4	V5	V6	V7
Total solids (% w/v)	4.0	3.8	3.7	5.1	4.1	3.1	3.1	3.1
Cost/m^3 (IR£)	20.7	12.9	16.0	22.7	15.0	15.7	18.7	18.7
Max. viable counts ($x10^9$/ml)	2.9	2.1	2.0	3.3	2.1	2.5	2.6	1.9
Time of 90% lysis (h)	72	48-72	48-72	72	48-72	48	48	72

These are the results of a single fermentation, except in the case of the original Megna medium. B. thuringiensis was grown in 2l flasks containing 200ml medium; following inoculation according to standard procedures, the flasks were incubated under standard conditions.

TABLE 3.1.10

The most useful variants of the literature media for the fermentation of B. thuringiensis from the point of view of biomass production and sporulation efficiency.

COMPONENT	CONCENTRATION, g/l						
	DRAKE & SMYTHE VARIANTS		DULMAGE VARIANTS		MEGNA VARIANTS		
	V4	V5	V4	V7	V3	V5	V6
Soyabean meal	-	-	15.0	15.0	-	-	-
Corn steep solids	5.0	5.0	-	-	5.0	5.0	5.0
Defatted SF*	-	-	-	-	25.0	14.0	14.0
Yeast extract	2.7	3.0	-	-	2.0	2.0	2.0
Glucose	-	-	-	10.0	-	-	10.0
Molasses	20.0	-	10.0	-	18.6	-	-
Starch	-	20.0	10.0	-	-	10.0	-
$CaCO_3$	-	-	1.0	1.0	1.0	1.0	1.0
Na_2HPO_4	5.0	5.0	-	-	-	-	-

* SF: soyaflour

PARAMETER	MEDIUM VARIANTS						
	DRAKE & SMYTHE		DULMAGE		MEGNA		
	V4	V5	V4	V7	V3	V5	V6
Total solids (%)	2.8	3.3	3.3	2.5	5.1	3.1	3.1
Cost/m^3 (IR£)	11.3	18.8	6.6	8.0	22.7	15.7	18.7
Max. Viable Count ($x10^9$/ml)	1.5-2.0	1.5-2.0	2.0-3.0	2.0	3.3	2.5	2.6
Time of 90% lysis (h)	48	48	48	48	72	48	48

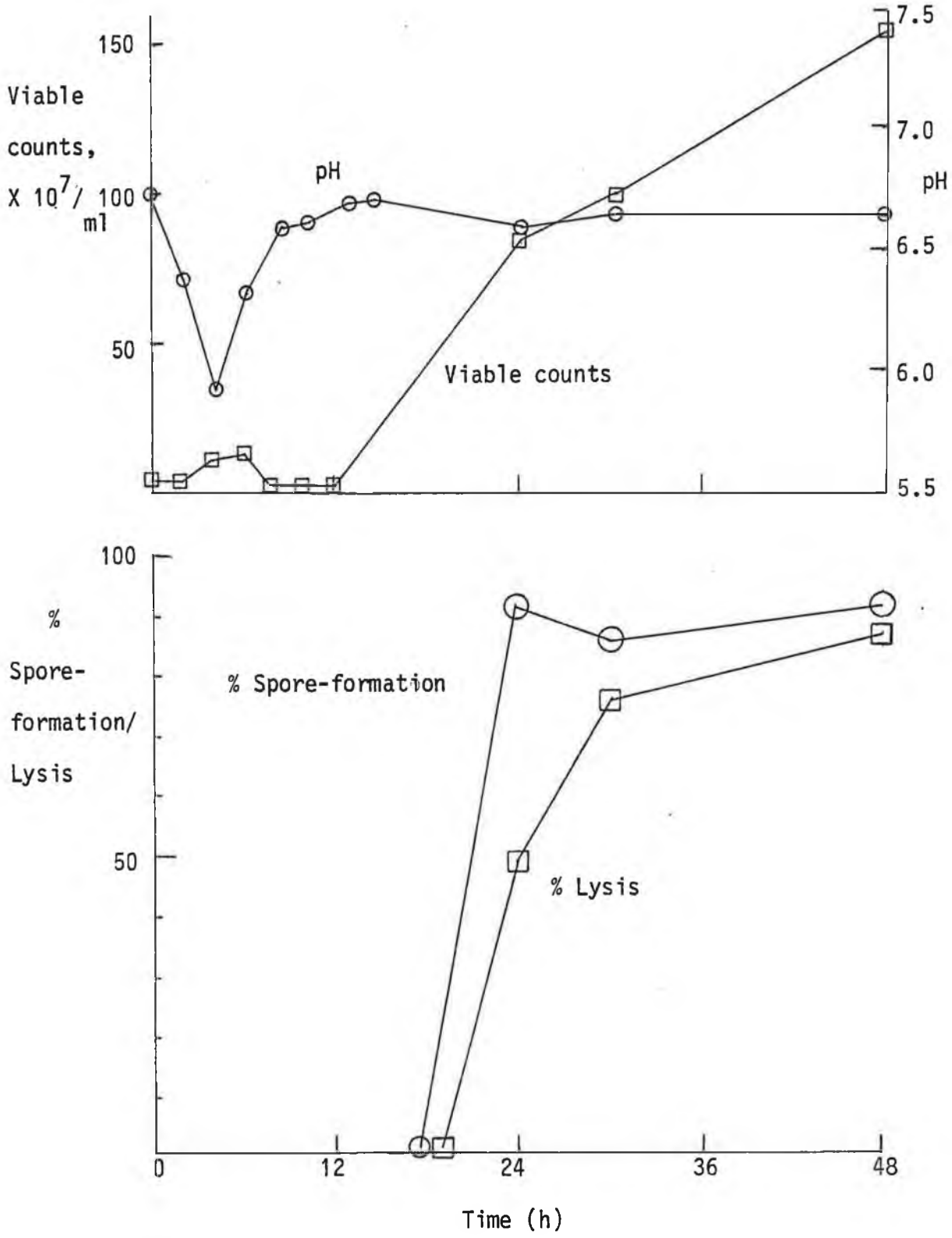
3.1.3.3 Characterization of growth of B. thuringiensis in the optimized production medium

In the previous section the selection of a medium optimized to support maximal growth and sporulation of B. thuringiensis was described. In order to quantify the growth of the organism in the medium, a number of flask fermentations were run. The flasks contained 100 ml or 200 ml of production medium per 1 litre or 2 litre flasks, respectively, and were inoculated with a 5% volume of a 24 hour casein inoculum medium prepared as described previously (2.3.3.1). Following inoculation, the production flasks were incubated as in 2.3.1. During the fermentation pH, viable counts, and sporulation patterns were monitored and Figure 3.1.4 shows a growth curve obtained from such a fermentation, while Table 3.1.11 shows a summary of data from a number of fermentation runs.

The growth pattern of B. thuringiensis when cultured in this medium was similar to that observed when other media were used to produce the organism, Fig. 3.1.1-3. Here, the pH began at or near neutrality and dropped to about 6.0 during the first 6 hours of growth. After this, the pH climbed steeply for a short while and then only slowly till about t24, at which stage the pH either remained at its t24 value or continued to rise till, in some cases, it reached a final pH at t48 of close to 8.0. At t24 it was usual to record almost complete sporeformation with lysis typically in the 10-50% range. Therefore, during the ensuing 24 hours sporangial lysis was completed giving about 90-95% free spores by t48. During flask fermentations, viable counts were almost always in the 2.0 - 2.5 x 10^9/ml range by t48 when lysis was complete and clumps of sporulated cells had broken down, Table 3.1.11.

FIGURE 3.1.4

Patterns of growth, pH, and sporulation during flask culture of B. thuringiensis when cultured in the SM medium.



B. thuringiensis was cultured in 2l flasks containing 200 ml of SM medium following inoculation with a 5% volume of a 24 hour inoculum culture. The production flasks were then incubated under standard conditions.

TABLE 3.1.11

Summary of results for flask cultures of B. thuringiensis in the SM production medium.

RUN	VIABLE COUNTS AT t48 $x10^9$/ml	VC:SC:FS t48
27	3.0	5:5:90
29	1.4	10:10:80
44	2.2	5:0:95
49	2.4	10:0:90
51	2.1	10:0:90

1l or 2l flasks with 10% of their capacity of culture medium inoculated and incubated under standard conditions.

One characteristic of the fermentation observed was that at t24 at least 95% sporeformation was typically observed, but that by t48 the proportion of vegetative cells was often of the order of 10-15% and in rare cases even nigher. This "regeneration" of the culture has been observed by other workers (Dr. Ray Quinlan; Dr. Peter Luthy, personal communications) and was probably caused either by germination of newly released spores, or by the resumption of growth of the vegetative cells which failed to sporulate by t24. This regeneration was most likely the result of the high degree of lysis which occurred late in the fermentation, releasing a high level of nutrients back into the culture broth, thus making it possible for a certain proportion of the cells to resume growth.

No further investigation into this phenomenon was conducted as no evidence was found to suggest that the regeneration had a significantly detrimental effect on the fermentation.

3.1.4. Characterization of Production Media with respect to Bioactivity Production

In the previous section, 3.1.3, the optimization and characterization of media with reference to biomass production and time of lysis was described. However, it was not possible to assume that the media which produced the highest amount of biomass were necessarily the ones which produced the greatest bioactivities. The relationship between viable counts and insect toxicity has been investigated previously and it has been reported that there is not always good correlation between viable counts and bioactivity (Burges and Thompson, 1971; Dulmage 1970b, 1971, 1981; Smith, 1982; Luthy et al., 1982). Therefore, it was necessary to determine, firstly, the time at which insect toxicity was maximal during the fermentation and secondly, the media which

produced the highest bioactivity levels.

Detailed descriptions of the method used for bioactivity measurements are outlined in section 2.4.3. The main features of the assay system used are the dilution of the fermentation broth 1:1,000 and dispensing various aliquots of 100 - 1000 ul of the diluted culture into cups containing 25 larvae in 100 ml distilled water. This gave a range of dilutions of the culture broth from 10^{-6} to 10^{-5}. The mortality of the insects in the various culture dilutions was recorded and compared with the insect mortality in cups treated with a standard material. The amount of bioactivity in the broth sample was then calculated by comparison of the median lethal response levels of the test and standard materials.

3.1.4.1 Determination of the time of maximum insect toxin production

For this series of experiments B. thuringiensis was cultured in flasks using media and procedures described in 3.1.3. The time at which the maximal level of insect toxin (maximum bioactivity) production occurred was determined using the pure crystal suspension described in 2.5.2 as the standard material. The bioassay procedure used was that mentioned in 2.4.3.

The maximum bioactivity could just as well have been determined using the international standard material, IPS. 82. However, it was felt that only one standard material was needed, and that, because of the fact that the crystal suspension was so easily employed in the bioassay system it was used for this series of determinations.

Because the crystal suspension was used as the standard material, bioactivitiy data were calculated as milligrams of crystal protein per millilitre of fermentation broth. If IPS.82 had been used, the

bioactivity would have been quoted as international toxic units per millilitre of bacterial culture.

During these experiments, in addition to the determination of broth bioactivities, the pH, viable counts and proportions of vegetative cells, sporulated cells and of free spores were also measured. The viable counts were determined only when lysis and clump breakdown were complete.

Results obtained for the literature media are presented in Figures 3.1.5 - 8 and for a selection of variant media in Figures 3.1.9 - 12. Data are not presented for the Drake and Smythe medium because significant insect toxicity (greater than 0.01 mg crystal protein/ml culture broth) was never detected in cultures of B. thuringiensis var israelensis using this medium.

The data presented in figures 3.1.5 - 12 clearly show that maximal crystal protein levels occurred late in the fermentation and almost invariably coincided with the time at which maximum cell lysis was observed.

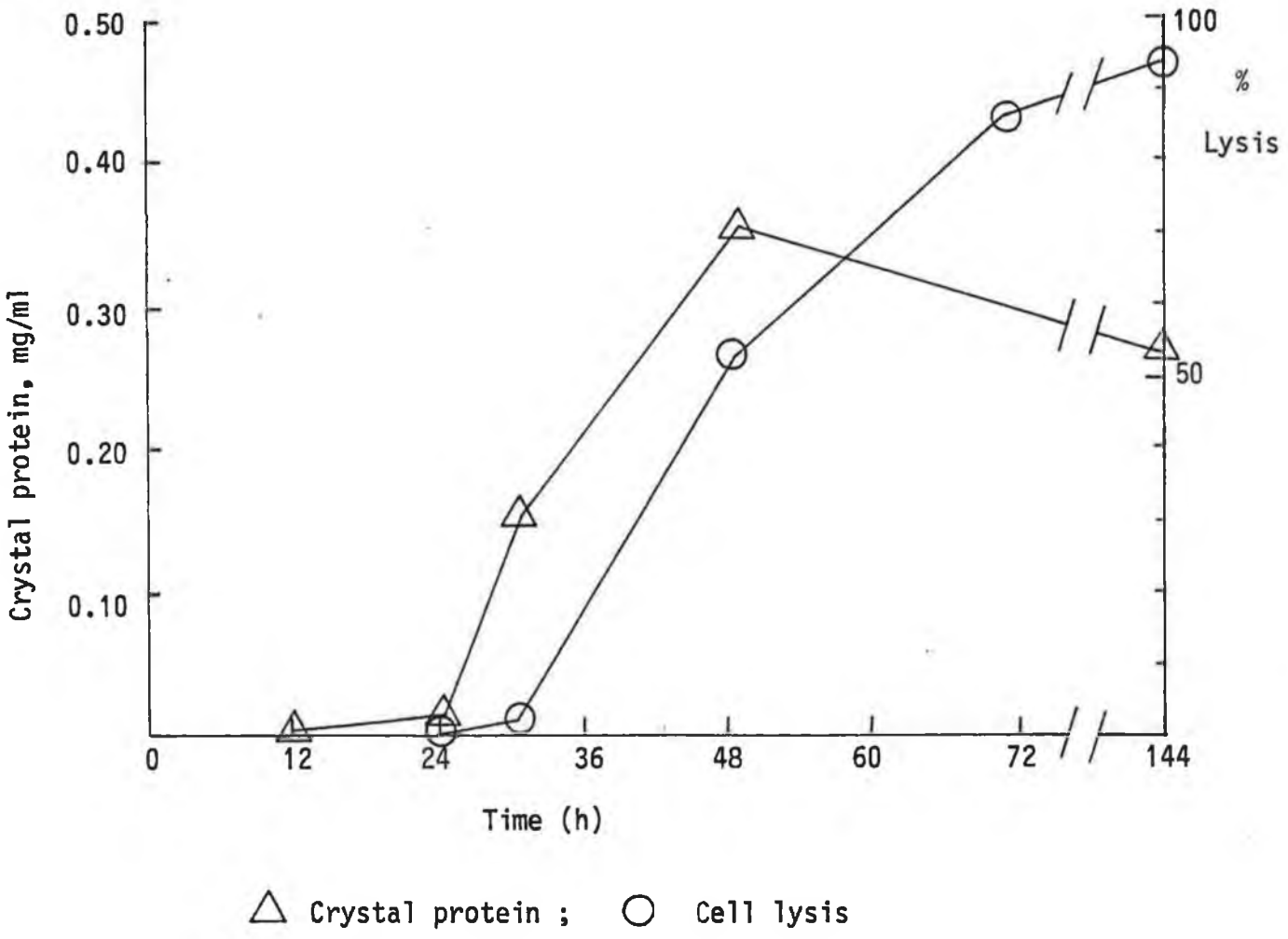
Thus, in terms of an industrial fermentation, it appeared that levels of cell lysis could be used to predict when maximal bioactivity was produced in the fermenter broth. However, this was not a method which was able to indicate what the maximal values were, rather it was limited to predicting when they occurred.

3.1.4.2 Determination of maximum bioactivity levels in production broths

Having established how to predict when the highest levels of bioactivity occurred in the fermentation broths it was then necessary to determine how high these levels were.

FIGURE 3.1.5

Bioactivity production (in terms of crystal protein concentration) and cell lysis during flask cultures of B. thuringiensis in the Megna medium.



See Table 3.1.6 for medium formulation.

FIGURE 3.1.6

Bioactivity production (in terms of crystal protein concentration) and cell lysis during flask cultures of B. thuringiensis in the Dulmage medium (Table 3.1.6).

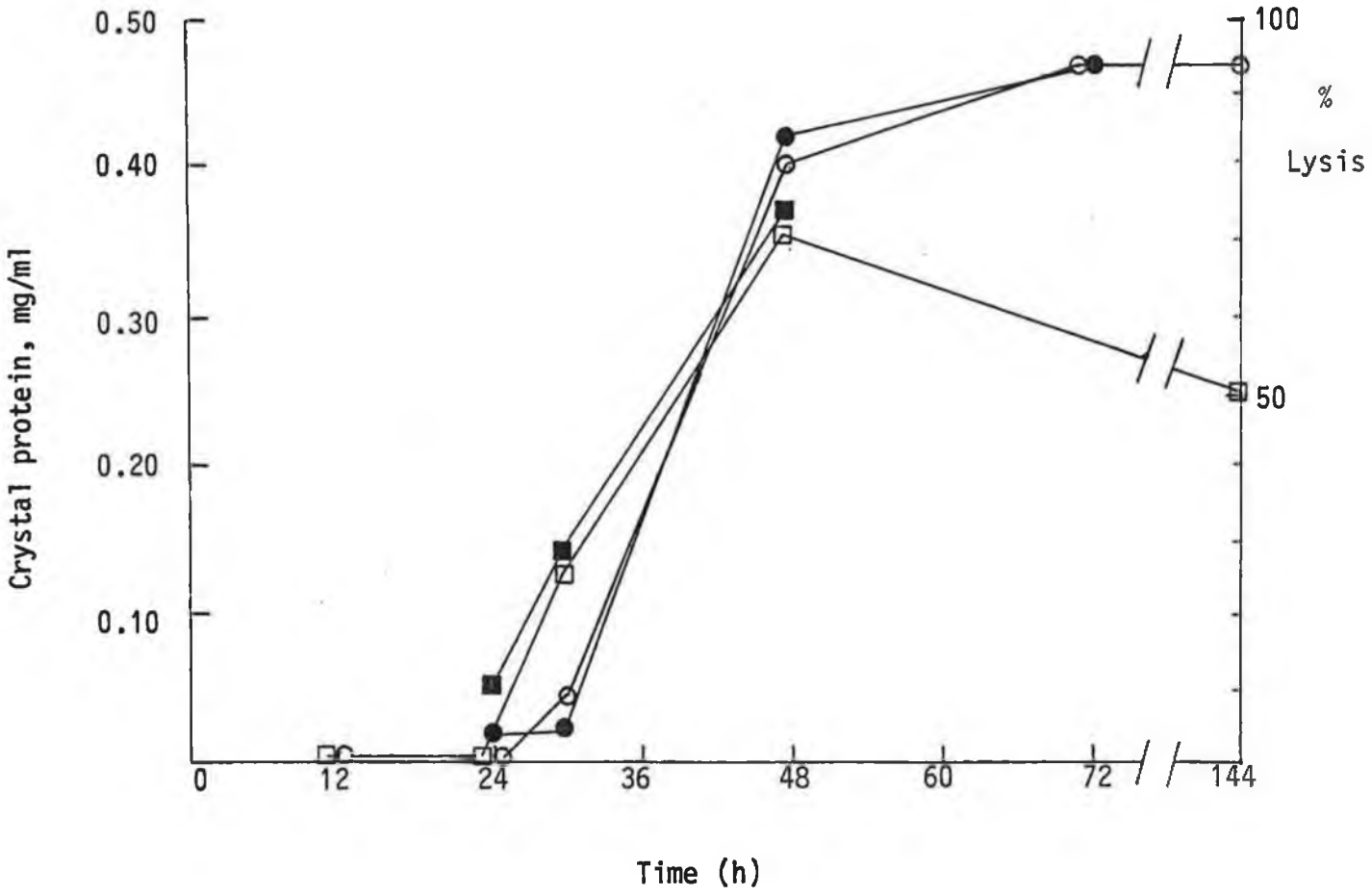
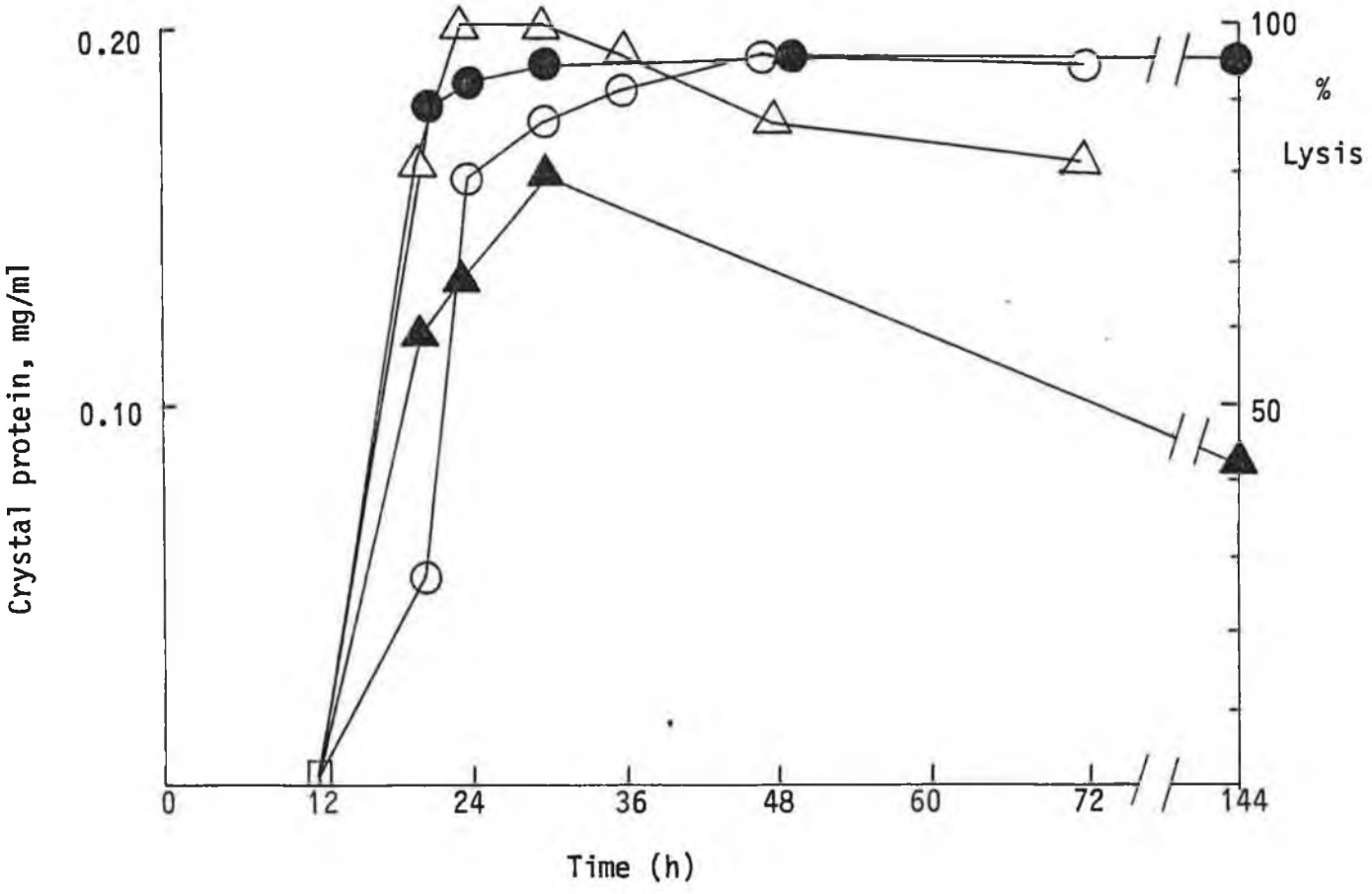


FIGURE 3.1.7

Bioactivity production (in terms of crystal protein concentration), and cell lysis during flask cultures of B. thuringiensis in the CRC Molasses medium (Table 3.1.6).



△ ▲ Crystal protein ; ○ ● Cell lysis

△ ○ Run 44 results ; ▲ ● Run 47 results.

FIGURE 3.1.8

Bioactivity production (in terms of crystal protein concentration), and cell lysis during flask cultures of B. thuringiensis in the CRC Starch medium (Table 3.1.6).

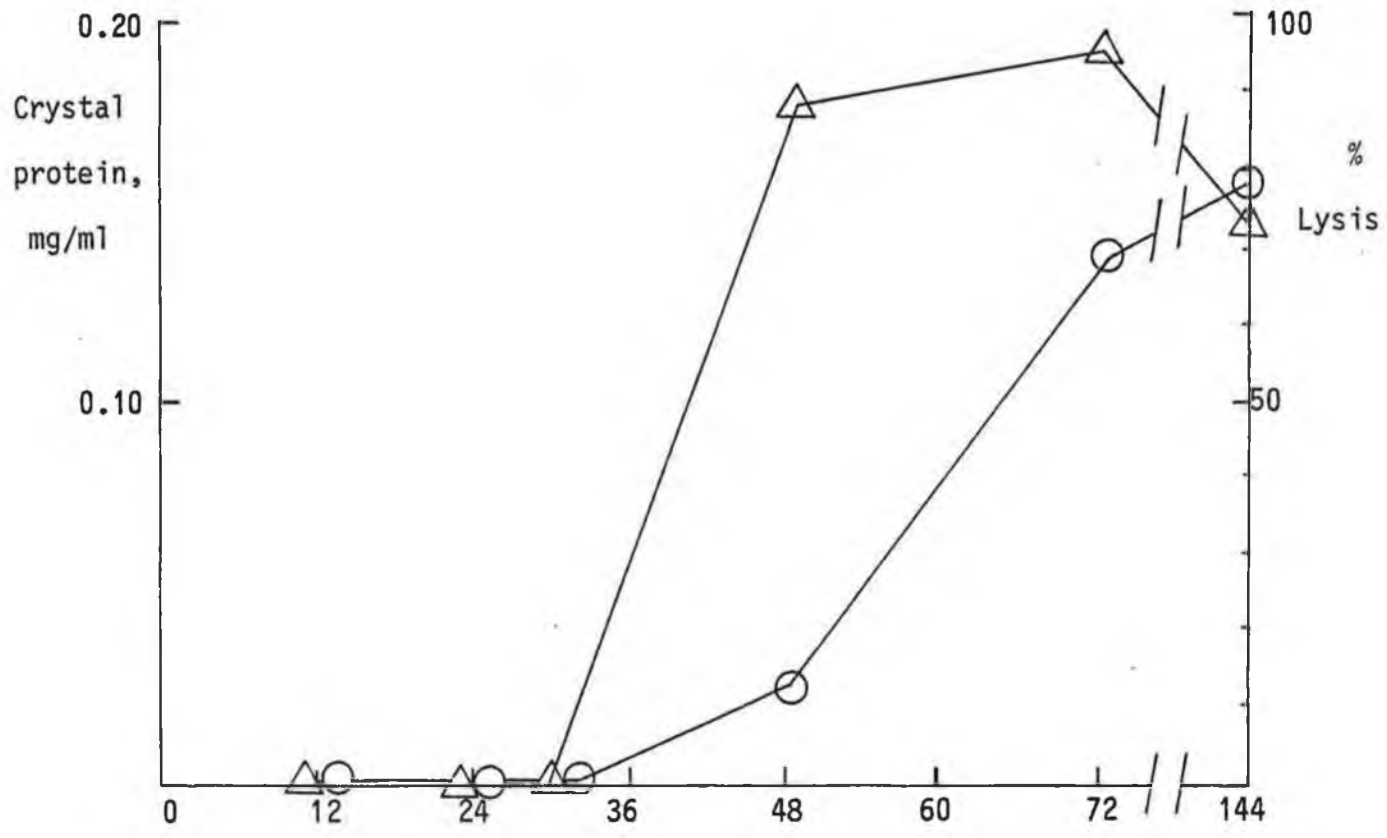
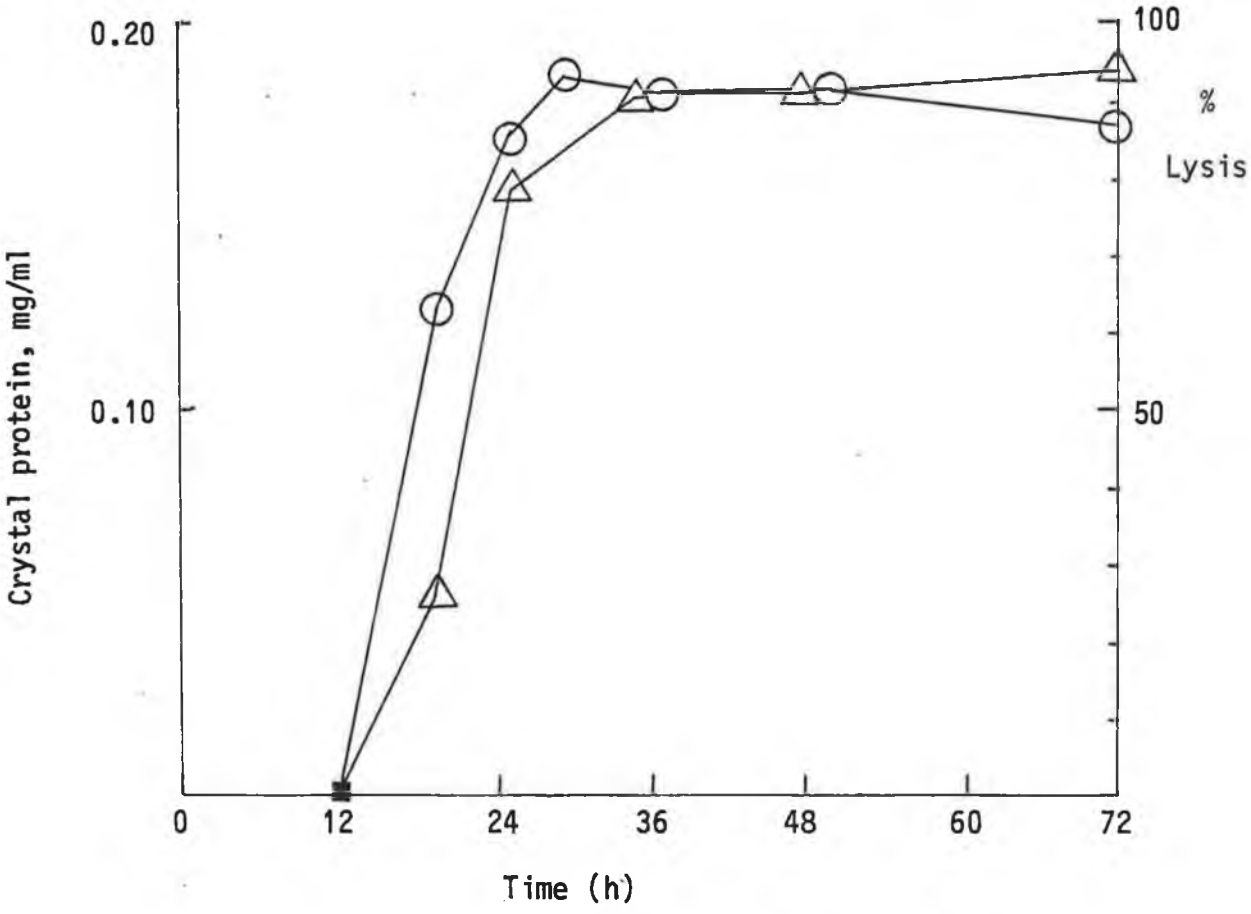


FIGURE 3.1.9

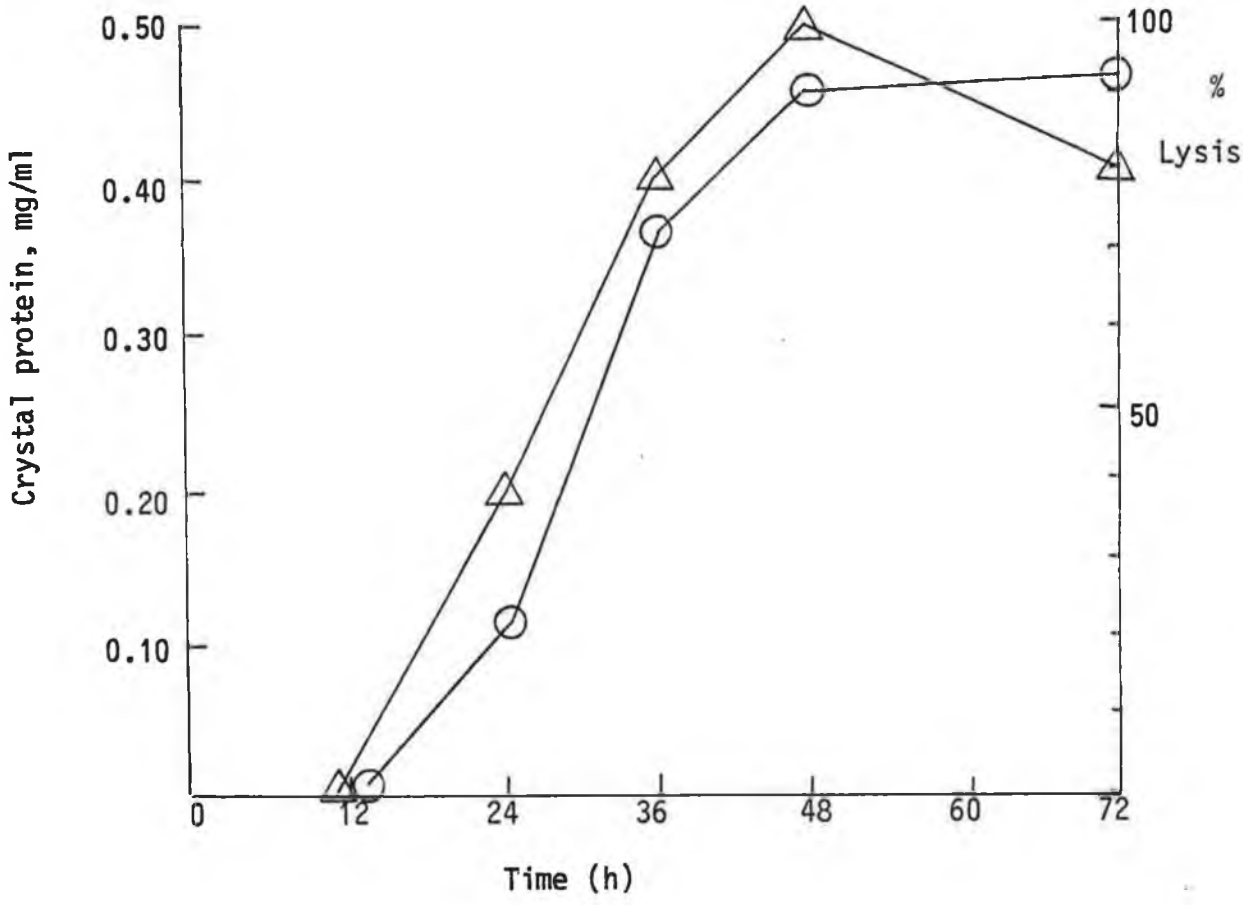
Bioactivity production (in terms of crystal protein concentration), and cell lysis during flask cultures of B. thuringiensis in the Suc 15 medium (Table 3.1.7).



○ Crystal protein ; △ Cell lysis

FIGURE 3.1.10

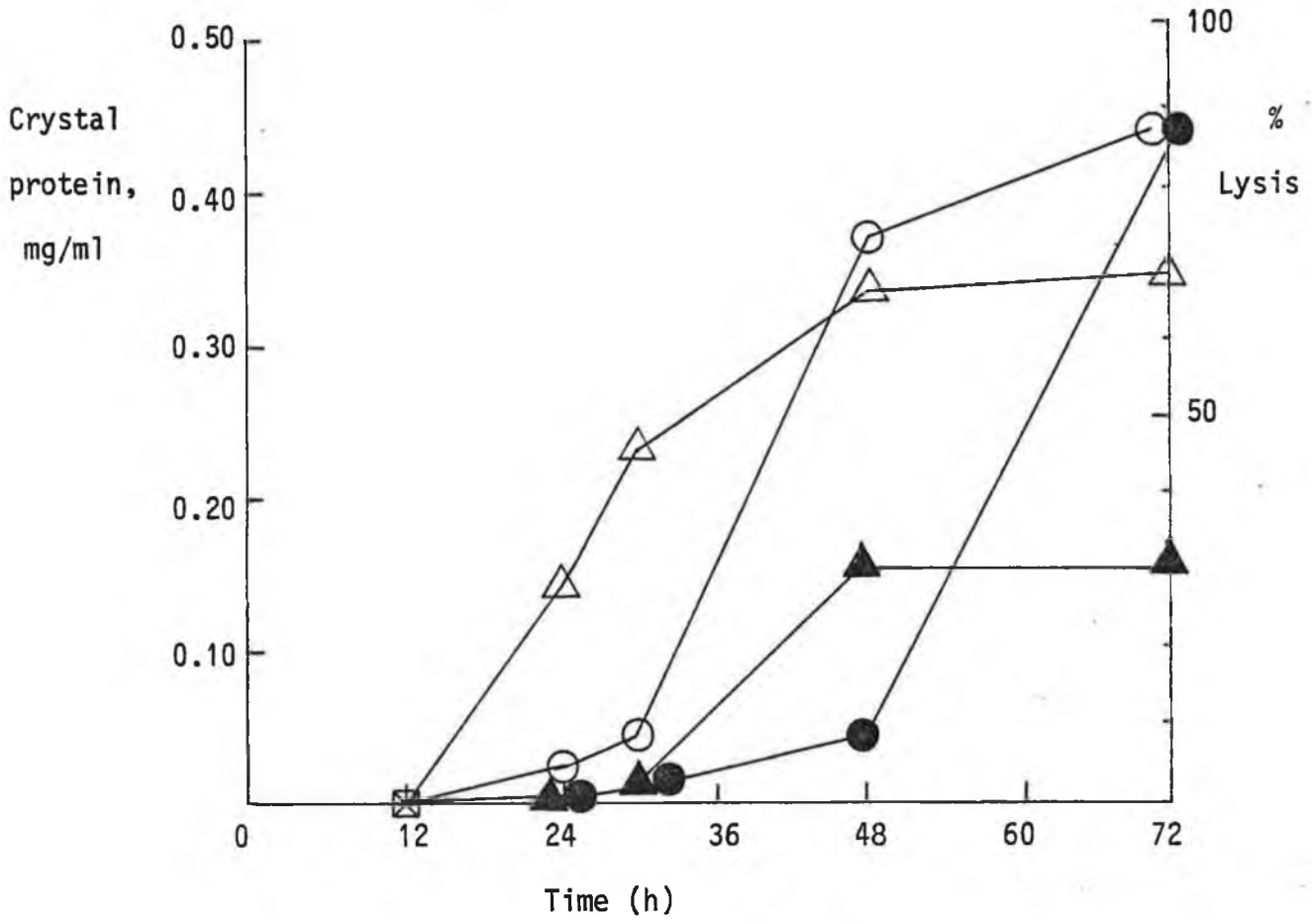
Bioactivity production (in terms of crystal protein concentration), and cell lysis during flask cultures of B. thuringiensis in the SM medium (Table 3.1.8).



△ Crystal protein ; ○ Cell lysis

FIGURE 3.1.11

Bioactivity production (in terms of crystal protein concentration), and cell lysis during cultures of B. thuringiensis in the FFSF and SP media (Table 3.1.8).

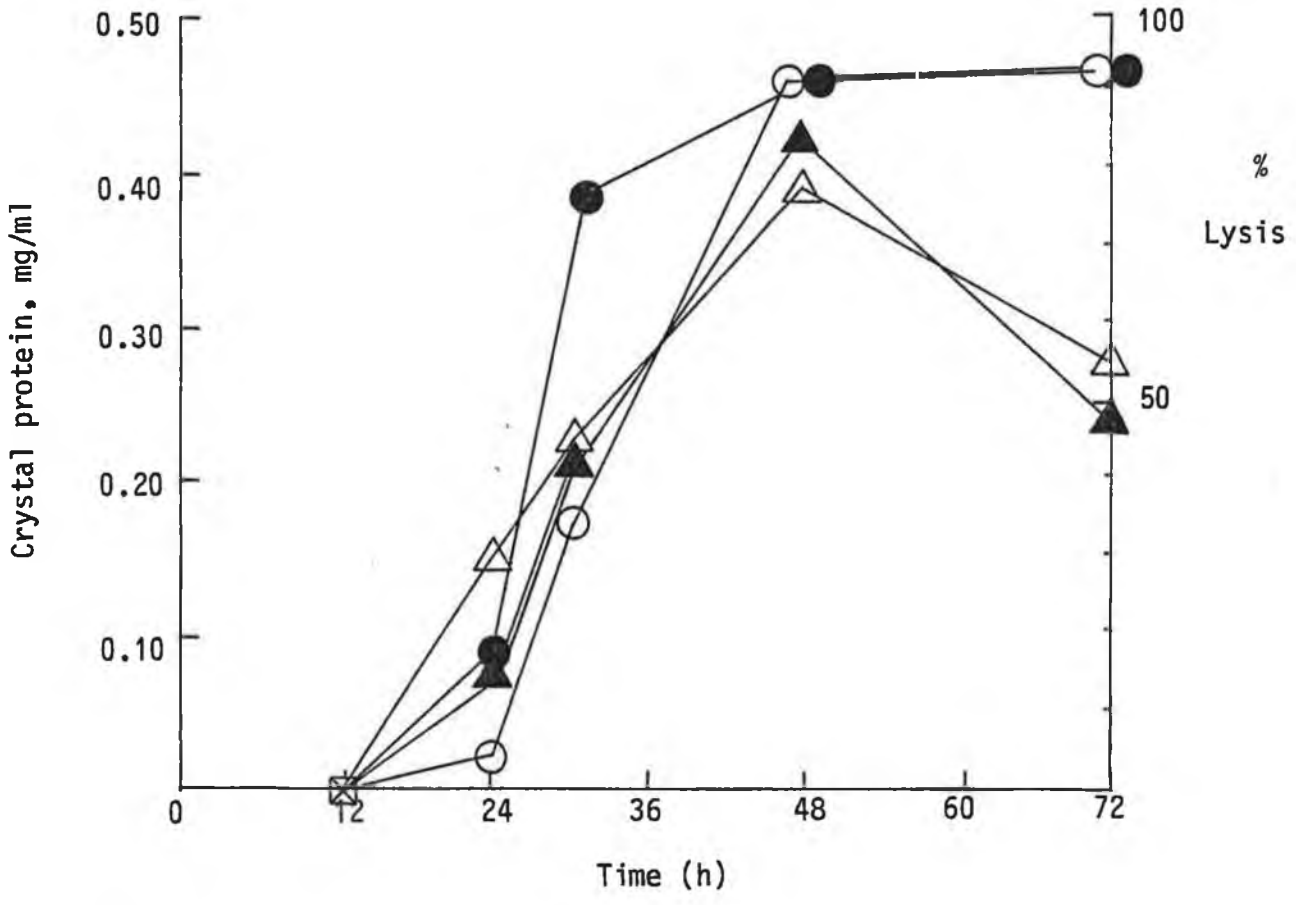


△▲Crystal protein ; ○●Cell lysis

△○FFSF medium ; ▲●SP medium

FIGURE 3.1.12

Bioactivity production (in terms of crystal protein concentration), and cell lysis during flask cultures of B. thuringiensis in the SBM and SM media (Table 3.1.8).



Cultures of B. thuringiensis grown in production media were produced in flasks as had been done in earlier experiments 3.1.4.1. The bioassay procedure was the same as had been used in 3.1.4.1 except that the international standard, IPS.82, was used as a standard material, in addition to the pure crystal suspension which was employed in 3.1.4.1.

Thus, bioactivity values were determined in terms of both of these reference materials. This meant that bioactivity results were quoted in terms of mg crystal protein/ml as well as in terms of international toxic units/ml fermentation culture. The two reference materials were used to compare maximal bioactivity levels in various broths because the use of more than one standard preparation gave the bioactivity data a greater degree of reliability, 1.7.3.

During the fermentations described in these experiments viable counts and bioactivities were determined only at, or after, maximal cell lysis. The results obtained for cultures using the literature media are presented in Table 3.1.12. Table 3.1.13 shows the results for some of the variant media described in Tables 3.1.7 - 9.

The results showing bioactivity levels in the literature media clearly demonstrated the Dulmage medium to be the most active of those studied, Table 3.1.12. The Megna medium produced similar viable count levels to the Dulmage, and was approximately as active. The Drake and Smythe medium never produced significant levels of insect toxicity, which was expected in view of the fact that B. thuringiensis var israelensis was never seen to sporulate in this medium. The Molasses and Starch production media produced lower viable counts and significantly lower bioactivities than the Megna and Dulmage media, Table 3.1.12.

TABLE 3.1.12

Maximal bioactivity and viable count levels observed during flask cultures of B. thuringiensis in literature media.

MEDIUM	MAXIMUM LEVELS			TIME OF MAX. BIOACTIVITY
	VIABLE COUNTS ($x10^9$/ml)	CRYSTAL PROTEIN (mg/ml)	INSECT POTENCY ITU$x10^4$/ml	(h)
Drake and Smythe	2.0-2.5	<0.01*	<0.1*	-*
Megna	2.0-2.5	0.30-0.40	8.6	72
Dulmage	1.2-2.5	0.30-0.40	10.5	48-72
CRC Molasses	0.3-1.1	0.10-0.20	4.0	24
CRC Starch	0.7-1.5	0.15-0.25	ND	48-72

* Significant bioactivity levels never detected in these broths

+ ITU: international toxic units

ND: not determined

Results from more than one fermentation run.

Of the various literature media variants tested the SM medium, derived from the Dulmage, was the most active of all media examined, Table 3.1.13. Indeed, it was the media which used the crude, ground soyabean meal, as apposed to the commercially prepared soyaflours, which produced the highest levels of bioactivity in these investigations. The Megna media, which all contained defatted soya flour, consistently produced lower bioactivities than the SM medium, even though similar levels of viable counts were recorded in each. The Suc 15 (V3) variant of the Drake and Smythe medium was the only variant of this medium to be bioassayed. It was found that this Suc 15 medium gave poor viable counts, and low bioactivities, Table 3.1.13.

At the end of the literature and variant media assessment phase, a single medium which produced high levels of bioactivity and viable counts had been developed. This was the SM variant of the Dulmage medium. Table 3.1.14 shows a comparison of the original Dulmage and the optimized SM variant.

The advantages of the SM medium over the Dulmage were that, firstly, it was less than half the price of the original. Secondly, it produced significantly higher levels of bioactivity in a slightly shorter cycle time. When compared on a cost per 10^{10} ITU's it was revealed that the SM medium was almost three times as cost effective than the Dulmage, Table 3.1.14. On this basis it was concluded that a significant improvement in the original medium had been achieved.

3.1.4.3 Comparison of bioactivity levels in my fermentation broths with those of other workers

Comparison of the levels of bioactivity achieved during this series of experiments with results of other workers was usually not possible for

TABLE 3.1.13

Maximal bioactivity and viable count levels observed during flask cultures of B. thuringiensis in literature-variant media.

MEDIUM	ORIGINAL DERIVATION	MAXIMAL LEVELS			TIME OF MAXIMAL
		VIABLE COUNTS ($x10^9$/ml)	CRYSTAL PROTEIN (mg/ml)	INSECT POTENCY ITU$x10^4$/ml	BIOACTIVITY (h)
SM	Dulmage	2.0-3.0	0.45-0.55	11.5-13.0	48
FFSF	"	1.9	0.35	8.7	72
SP	"	1.2	0.15	4.4	72
SBM	"	2.3	0.40	10.8	48
V1	Megna	2.1	0.22	5.5	48
V2	"	2.0	0.22	5.4	48
V3	"	3.3	0.23	6.3	72
V4	"	2.1	0.23	5.7	48-72
V5	"	2.5	0.30	7.6	72
V6	"	2.6	0.30	8.0	72
V7	"	1.9	0.16	4.0	72
V3	Drake & Smythe	1.0	0.18	4.0	30

Results from a number of fermentation runs
ITU: international toxic units

TABLE 3.1.14

Comparison of the Dulmage and SM media

PARAMETER	MEDIUM	
	DULMAGE	SM
Cost/m^3 IR£	14.3	6.6
Total Solids (% w/v)	3.0	3.3
Max. viable counts ($x10^9$/ml)	1.2-2.5	2.0-3.0
Time of max. cell lysis/bioactivity (h)	48-72	48
Max. crystal protein (mg/ml)	0.30-0.40	0.45-0.55
Max. insect potency (ITU $x10^4$/ml)	10.0-10.5	11.5-13.0
Cost/10^{10} ITU (IR£)	1.40	0.52

Results are from a number of fermentation runs

a number of reasons. Firstly, bioactivity data simply were not reported in the Drake and Smythe, Megna and CRC patents, and in any case these processes were published before the discovery of B. thuringiensis var israelensis. This would have meant that bioassay data, had they been reported, would have referred to activity against lepidopteran insects using an entirely different bioassay protocol and reference material, which would have been totally incompatible with results presented during this work. Secondly, other authors used the Acetone/Lactose coprecipitation procedure of Dulmage et al. (1970) to produce powders for bioassay from the fermentation broths without indicating the yield of powder from a unit broth volume (Obeta and Okafor, 1984). Thirdly, Margalait et al. (1983) did not describe their bioassay protocol, nor did they report insecticidal potencies in terms of international units per volume of broth. Fourthly, a totally different assay system, where a standard material was not used, was employed in one instance, meaning that the results reported were not comparable to any other system (Smith, 1982).
Lastly, results obtained by workers engaged by industrial concerns, myself included, were not at liberty to report results pertaining to bioactivity levels obtained in their fermentations.

However, despite these difficulties it was possible to establish that the bioactivity in my broths was at least of the same order as the bioactivity in some reported media.

Margalait et al. (1983) reported that for one of their media (which was not described) the maximum toxicity was an LC_{50} of 7 ng/ml. However, this group neither compared to toxicity of these broths to a standard material, nor did they report any other data which might have been useful for estimating insecticidal potencies. The LC_{50} values of my fermentation broths were typically in the 10-20 ng/ml range, when

the SM medium was used.

Smith (1982) did not use a recommended bioassay procedure, as was done during this work, nor did he use a standard material in his assays, therefore, his results were not directly comparable to mine. However, this author reported spore counts of 2×10^5 - 9×10^8/ml for his cultures after 72 hours growth. Furthermore, it was stated that the culture dilutions used to obtain suitable larval mortalities were 10^{-5} to 10^{-4} (Smith, 1982). In the bioassays done during this work 10^{-5} dilutions of the SM medium invariably caused 100% mortality. This indicated that the insect toxicities in my broths were as much as 10 times as high as had been reported by Smith (1982); this correlated well with the low viable counts observed in the published work.

Despite the limited published data with which to compare the bioactivities in my culture broths, the results which have been reported can be used to indirectly verify that the potencies obtained in my broths were at least as high as has been observed by other workers.

3.1.4.4 Investigation into possible relationship between viable counts and bioactivities in whole cultures

It nas been mentioned, 3.1.4, that previous reports discounted any significant correlation between viable counts and bioactivity. However, these reports referred to powder materials prepared by the Acetone/Lactose Coprecipitation procedure of Dulmage et al. (1970). The bioassay protocol used during this work used diluted whole-cultures with no intermediate step between fermentation and sample bioassay, apart from the dilution.

In addition early bioactivity results obtained during this work with

the literature media appeared to indicate some link between viable counts and insect toxicities, Table 3.1.12. It was therefore decided to analyse closely the data obtained using a range of media in a number of flask fermentations which had been conducted as described in 3.1.3.

The analysis of the data is presented in Table 3.1.15. The results presented in this table show that there is no strict correlation between bioactivities and viable counts. However, the variation in the biomass/bioactivity ratio for the crystal protein data was 0.14 - 0.33 mg crystal protein/10^9 cells. For the insecticidal potency data a variation of 4.6 - 6.8 ITU x $10^4/10^9$ cells was found, with only one data point lying outside the 4.6-5.8 ITU x $10^5/10^9$ cells range. With the crystal protein data all biomass/bioactivity ratios, except one, were in the 0.14 - 0.25 mg crystal protein/10^9 cells range.

While these biomass/bioactivity ratio variations were certainly significant, they were far less than those reported by Dulmage (1971) where the ratios were found to vary over three orders of magnitude. Dulmage's results were obtained using a number of isolates of a single leptox variety of B. thuringiensis in two different media. Therefore, the biomass/bioactivity ratios were caused not only by the use of different media, but also by the use of a range of bacterial isolates. Nonetheless, in most cases, when using a single isolate in just two media, biomass/bioactivity ratios were seen to vary by one or two orders of magnitude with neither medium consistently giving the higher bioactivity.

TABLE 3.1.15

Assessment of possible correlation between viable counts and bioactivities in whole flask fermentation cultures.

MEDIUM	RUN	VIABLE COUNT $x10^9$/ml	CRYSTAL PROTEIN mg/ml	INSECT POTENCY ITU$x10^4$/ml	BIOMASS/BIOACTIVITY RATIO	
					mg crystal/ 10^9 spores	ITU$x10^4$/ 10^9 spores
Dulmage	44	1.00	0.33	ND	0.33	ND
	49	1.90	0.37	11.0	0.19	5.8
SBM	49	2.30	0.39	10.8	0.17	4.7
SM	44	2.00	0.50	13.7	0.25	6.8
	49	2.40	0.45	11.6	0.18	4.8
Megna	47	2.50	0.36	ND	0.14	ND
CRC Molasses	44	0.90	0.20	4.1	0.22	4.6
	47	0.56	0.10	ND	0.18	ND
Suc 15*	44	0.90	0.19	4.3	0.21	4.8

* V3 of the Drake and Smythe variants

B. thuringiensis inoculated and incubated under standard conditions. 1l or 2l flasks containing 100ml or 200ml of medium, respectively, were used.

With regard to the results presented here, it would appear that viable counts, while not being a reliable index of bioactivity, certainly seemed to be a general indication of insect potency levels in fermentation broths, Tables 3.1.12,15. These data reveal that media which produced high viable counts, such as the SM, Dulmage and Megna media, tended to give the highest bioactivities. The reverse could be stated for media which gave low viable counts, such as the CRC Molasses and Suc 15 media. This generalization would apply only to the use of a single isolate of B. thuringiensis, because it has been conclusively shown that different isolates of a given B. thuringiensis variety vary considerably in their bioactivity production even when cultured in a single medium (Dulmage, 1971; Dulmage and Cooperaters, 1981; Salama et al., 1983; Smith, 1982).

The reason why biomass/bioactivity ratios were found to remain relatively constant using bioassay data from whole cultures, while the same parameter was reported to vary considerably in powder preparations is not known. It is possibly related to the fact that the powders are treated with acetone during their preparation and various media may differ in their ability to "protect" the parasporal crystal from the effects of the solvent, due to the presence of one or more of the medium's components. It is also possible that the acetone precipitation procedure causes variable clumping of the spores which may be dependent on the presence of medium constituents which may increase or decrease the degree of clumping.

3.1.5 Process Scale-up

Having developed the media to be used for the inoculum production stages it was then necessary to transfer the process from flasks into laboratory-scale fermenters, and thence to pilot-scale. For this purpose 5l, 10l, and 40l fermentations were carried out for the production stage using the fermenters and culturing conditions described in 2.3.5.

The fundamental aim of a scale-up operation is to ensure that the process and productivity results obtained in flasks can be transferred to the larger scale while maintaining yields, and, if possible, improving them. Therefore, the inoculum development and production stage fermentation had to be run in fermenters to verify the results on the larger scale.

The main consideration in effecting a scale-up operation have already been discussed in section 1.8.1.2, and in the recent reviews of Banks (1979) and Lilly (1983). All that will be said at this stage is that the classical scale-up problems of medium sterilization, large-scale inoculum production, and the mixing/aeration requirements of the production stage fermentation were given particular attention.

The initial step in scaling-up the flask process was to grow the organism in the casein inoculum medium using fermenters. Having achieved satisfactory growth at the inoculum stage the production medium was run in Laboferm and Microgen fermenters (2.3.5) using varying culture conditions. Finally, a pilot-scale fermenter was used for the production stage using the conditions optimized for the laboratory scale vessels. The effect of medium sterilization was encountered in both inoculum and production stages due to the extended

time period required for the sterilization of the Laboferm and pilot fermenters (2.3.5).

3.1.5.1 Growth of second inoculum stage in fermenters

Having successfully grown the Inoc. 2 stage in flasks as described earlier (3.1.2), it was necessary to ensure that similar growth of the organism could be achieved in laboratory-scale fermenters.

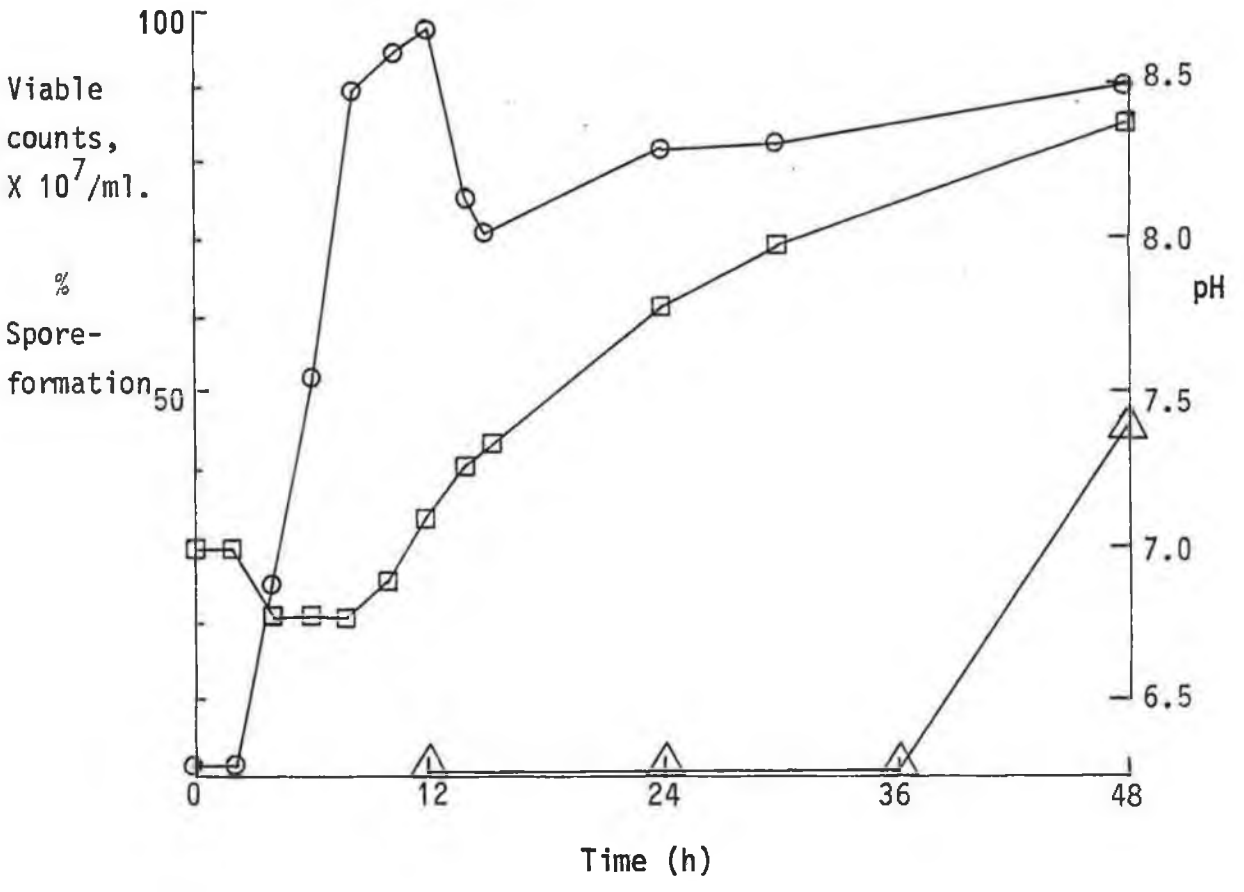
This was done by inoculating a 5l batch of casein inoculum medium in a Laboferm fermenter with a 5% volume of a 24 hour TW flask culture prepared under standard conditions. The fermenter culture was then incubated at 30^{o}C, aerated at 1l/l/min, with agitation set at 400 RPM. Foaming was controlled using 20-30 ml of a 1:10 emulsion of PPG antifoam, 2.3.3.2. During growth in the fermenters viable counts, pH and microscopic observations were done and the result of two fermentations are shown in Figure 3.1.13.

Camparison of the results shown in this figure and of those in Figure 3.1.3 show that similar results were obtained for the growth, and inhibition of sporulation in both types of vessel. In each case sporulation was not detected before 24 hours, and the pH profiles were almost identical. Viable count determinations were again affected by clumping of the cells, but after lysis and clump breakdown a true indication of biomass was obtained which was similar for flask and fermenter cultures at about 1.0 - 1.5 x 10^{9} cells/ml.

It was therefore concluded that the results obtained for the growth patterns of B. thuringiensis in flasks could be readily transferred to fermenter-scale cultures. In addition, it should be noted that the longer sterilization times required for the Laboferm fermenters did not appear to adversely affect the growth of the organism in the

FIGURE 3.1.13

Growth of B. thuringiensis in a 5l culture of the casein inoculum medium using Laboferm fermenters.



○ Viable counts ; □ pH ; △ % Spore-formation.

Medium was inoculated with a 5% volume of a 24 hour TW culture and incubated under standard conditions. Lysis was not observed within 48 hours of culture inoculation.

fermenters. This point would be of increased importance when much larger inoculum volumes, of up to 500l, would be required on a full production-scale, based on the premise that larger broth volumes need longer sterilization times (Banks, 1979).

3.1.5.2 Growth of production stage in laboratory-scale fermenters

In a manner analgous to the scale-up study done for the second inoculum stage, the growth of B. thuringiensis in the production medium laboratory scale fermenters was also investigated.

Throughout this series of fermentations the SM medium was used (3.1.3.2), with 5l batches in Laboferm fermenters, or in Microgen vessels containing 10l of broth. The production fermenters were inoculated with 5% volumes of the casein inoculum medium grown in fermenters according to standard procedures (2.3.3). Following inoculation the fermenter temperature was controlled at $30^{o}C$ and aerated and agitated under varying conditions. During growth of the organism in the fermenters pH and microscopic examinations were done routinely. In addition, for some of the Microgen fermentations dissolved oxygen (DO) was measured using a galvanic DO probe. Viable counts were done only at the end of the fermentations when high levels of lysis and clump-breakdown had occurred. The results of these fermentations are presented in Table 3.1.16 and Figure 3.1.14.

The fermenter cultures invariably produced high viable counts of between 2-3 x 10^{9} cells/ml by t48, at which stage maximal levels of cell lysis had been achieved. Usually, the cultures were aerated at 1l/l/min, but when aeration rates were increased to try and increase yields of biomass good growth was still observed, but levels of sporangial lysis were significantly decreased, Table 3.1.16. This

TABLE 3.1.16

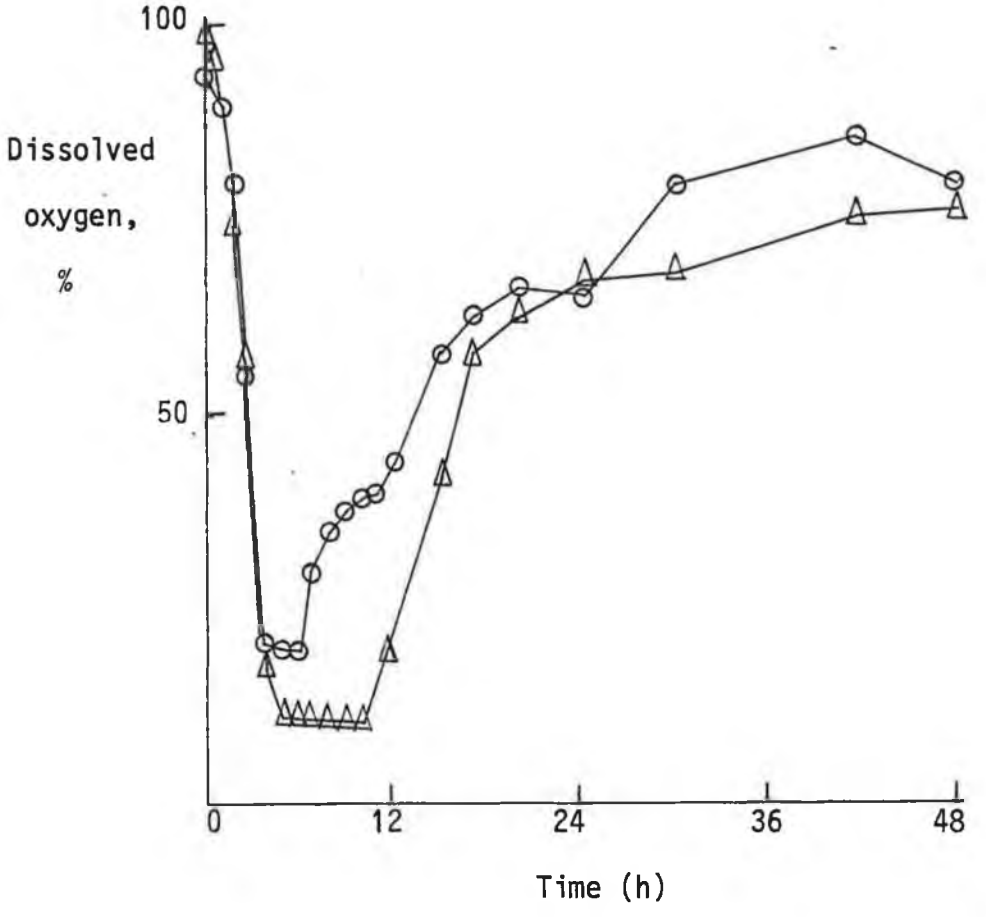
Results obtained during fermenter culturing of B. thuringiensis during the production stage using laboratory-scale fermenters and the SM medium.

VESSEL TYPE	CULTURE VOLUME (l)	RUN NO.	VESSEL NO.	CULTURING CONDITIONS		VIABLE COUNTS AT t48 ($x10^9$/ml)	% LYSIS AT t48
				AERATION l/l/min	AGITATION RPM		
Laboferm	5	34	1	1.0	400	2.5	90
			2	1.5	400	2.2	75
Microgen	10	30	1	1.0	400	2.4	85
		32	1	1.0	400	2.3	85
		33	1	1.0	400	2.4	90
			2	1.5	500	2.8	70
		34	1	1.0	400	2.9	95
		55	1	1.0	400	2.1	85

All cultures controlled at 30^oC, and 5% inoculated from Inoc. 2 cultures grown in Laboferm fermenters.

FIGURE 3.1.14

Dissolved oxygen patterns during growth of B. thuringiensis in Microgen fermenter cultures.



Δ Run 30 ; O Run 34

The organism was grown in the SM medium after 5% inoculation by a 24 hour, fermenter-grown inoculum culture. Following inoculation the production medium was incubated at 30°C, 1l/l/min, 400 RPM for 48 hours. Dissolved oxygen was measured with a galvanic dissolved oxygen probe.

indicated that, although it had been shown in flasks that low levels of aeration slowed down the rate of growth of the organism (3.1.1), excessively high levels of aeration decreased the sporulation and lysis efficiencies without affecting viable counts. This phenomenon may have been related to the culture regeneration alluded to previously, 3.1.3.3. If at the end of the fermentation where lysis may increase the nutrient level in the broth, high levels of aeration may enable a greater degree of regeneration to occur due to the combined effects of adequate levels of nutrients and oxygen. This regeneration would not be expected to reach the same extent where lower levels of aeration were used, due to a lower oxygen availability.

The dissolved oxygen profiles were measured during a number of fermentation runs and were found to follow roughly similar patterns. Early in the fermentation DO levels dropped sharply after a short lag phase to between 10% and 25% of the initial values. Thereafter, DO values increased steeply again till about t24 when the increase became more gradual, Figure 3.1.14. However, despite the broad similarities in DO profiles in the Microgen fermenters, the operation and results obtained using this equipment were found to be variable, even to the extent of two probes in the same pot giving markedly different patterns of dissolved oxygen. Thus it was felt that these data could only be used to indicate that during vigourous vegetative growth oxygen was quickly consumed by the organism, so that the oxygen level in the fermenter cultures was extremely low. After this stage, the amount of oxygen in the medium increased significantly, indicating that sporulation did not require as much oxygen as vegetative growth.

This increase continued for some time and reached a plateau after t24, or sometimes began to decrease again, possibly due to some culture

regeneration.

The DO profiles suggest that regeneration might be prevented by decreasing, or ceasing altogether the aeration, but this would only be attractive if rates of sporulation and lysis were unaffected by such a change in culturing conditions.

It was concluded that the growth of B. thuringiensis in fermenters was at least as good as had been observed in flasks. Again, the long sterilization period required for the Laboferm fermenters was not found to give results significantly different from those in the quickly sterilized Microgen vessels. The optimal aeration rate was taken as 1l/1/min which would allow acceptable growth while not affecting sporulation and lysis levels at the end of the fermentation.

3.1.5.3 Growth of the production stage in a pilot-scale fermenter

In the final stage of the scale-up process, using the experience gained during the fermentations described in sections 3.1.5.1,2, B. thuringiensis was cultured in a pilot-scale fermenter.

A 40 l volume of SM production medium was inoculated with a 5% volume of a 16 hour casein inoculum medium culture grown in a Microgen fermenter according to standard procedures, 2.3.3.2. The production fermenter temperture was controlled at 30°C and aerated with 1l/1/min of compressed air. Agitation was set at 500 RPM and foaming controlled using Silcolapse 5000 antifoam. Viable counts, pH and microscopic examinations were used to monitor the culture, which was run for 48 hours.

This pilot-scale fermentation gave a viable count of 6.5×10^9 cells/ml with 95% spore release at t48, and a pH profile similar to

what had been observed previously using this medium, Figure 3.1.15. It was therefore concluded that the growth of the organism in this type of vessel was superior to that observed in Microgen fermenters.

The reason that these especially good results were obtained by the use of this type of fermenter may have been attributed to the different fermenter design. as this factor is crucial to the suitability of a vessel to a given fermentation process (Banks, 1979).

Regardless of the reasons underlying the good performance of the fermentation in this type of vessel it was once again demonstrated that the process worked out in flasks was readily transferred to the pilot-scale.

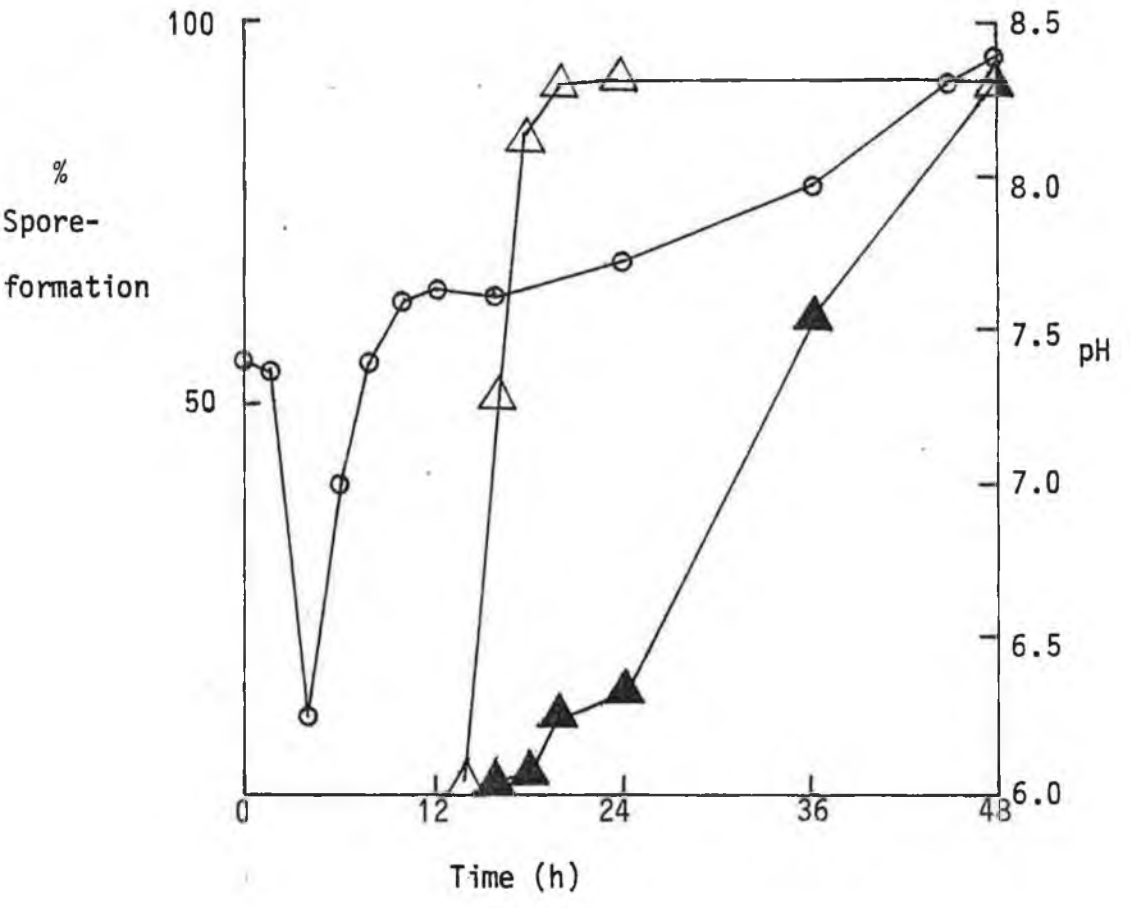
An additional point which should be made at this stage is the fact that the pilot-scale fermentation was unaffected by the prolonged sterilization time required to achieve sterility in this vessel (2.3.5.3). This indicated that the medium used for the production stage fermentation was likely to be suitable for use on a full industrial scale because of this medium sterilization factor, and also because the flask results were found to be reproduced on the larger scale used here.

3.1.6 Occurrence and Investigation of Strain Deterioration

Fermentation studies up to this point had been carried out using stock cultures which had been stored at $4^{o}C$ on nutrient agar slants for up to three months. To determine the effect of prolonged storage and repeated subculturing on the growth of the organism, a series of experiments was conducted. During this work stock cultures were diluted and streaked onto agar to obtain isolated colonies and the isolated colonies examined to check for spore and crystal production.

FIGURE 3.1.15

Growth of B. thuringiensis in the SM medium during a pilot-scale fermentation.



o pH ; △ % Spore-formation ; ▲ % Lysis

The fermenter medium was inoculated from a 24 hour inoculum culture produced in a Microgen fermenter. Following 5% inoculation, the production medium was cultured at 30°C, 1l/l/min, 500 RPM. Viable counts were determined only at t48 and were found to be 6.5 X 10^9/ml.

One fermentation run was conducted using a stock culture which had been stored and subcultured for over a year to see the effect on the growth and sporulation of B. thuringiensis when cultured in the SM and Dulmage media.

3.1.6.1 Spontaneous mutant isolation from stock cultures

The spontaneous mutation of B. thuringiensis var israelensis was investigated by streaking spore suspensions (2.2.3) from various stock cultures onto nutrient agar for single colony isolation. Following inoculation, the nutrient agar plates were incubated at 30°C for 48 hours. After this stage, individual colonies were examined by phase contrast microscopy to check for the presence of spores and parasporal crystals.

The stock cultures examined were:-

1) B. thuringiensis var israelensis freshly subcultured from freeze dried IPS.82 (2.2.1)
2) A four month old slope of strain 1884 initially isolated and subcultured directly from a freeze dried vial (2.2.2)
3) A six month old slope which had been subcultured from a 9 month old slope which in its turn was derived from an 18 month old slope which was inoculated from a spore suspension prepared from IPS.80 (2.2.1).

Microscopic examination of isolated colonies from the plates streaked with the stock cultures described above revealed the presence of mutants lacking crystals or lacking both crystals and spores. Mutants failing to produce spores alone were not detected.

The acrystalliferous (cry^-) mutants and the acrystalliferous,

asporogenic (spo$^-$, cry$^-$) mutants were most frequently detected on plates derived from the IPS.80 stock culture described above. Of 20 colonies chosen at random 2 were found to be spo$^-$,cry$^-$ and 5 spo$^+$,cry$^-$ mutants were isolated. The acrystalliferous mutants sporulated normally were found to form colonies which were indistinguishable from the wild type. However, the spo$^-$,cry$^-$ colonies after 72 hours appeared much less opaque than the wild type colonies. Microscopic examination of the spo$^-$,cry$^-$ cells at this stage revealed that the cells had themselves become transluscent, almost "ghost-like" by comparison to the normal cells, which appeared as dark rods. The size and shape of the mutant cells were unaltered.

By contrast to the old, frequently subcultured IPS.80 stock culture, the IPS.82 and 1884 isolates proved to be poor sources of acrystalliferous mutants. Twenty colonies of each were examined, but spo$^-$, cry$^-$ mutants were never detected. Of the colonies examined, only one spo$^+$cry$^-$ colony was isolated from IPS.82, and two from 1884. The fact that cry$^-$ mutants could be isolated at all from the freshly subcultured IPS.82 suggests that either this material alone had an innate spontaneous mutant population or that any culture source, regardless of age or subculture number might be expected to have a detectable level of mutant cells.

It therefore seemed likely that the most probable reason for the ease of isolation of spontaneous mutants from the IPS.80 culture was that it had been subcultured and stored for a considerable length of time without making any effort to exclude the mutants during the subculturing.

These limited investigations, while not giving accurate data on the rate of spontaneous mutation on the organism, provided strong indications to suggest that prolonged storage and/or frequent

subculturing of B. thuringiensis var israelensis was detrimental to its crystal producing capacity.

Previous reports had made reference to the possibility of isolating spontaneous mutants of B. thuringiensis (Faust et al., 1983; Gonzalez et al., 1981; Gonzalez and Carlton, 1984; Kamdar and Jayaraman, 1983; Ward and Ellar, 1983). Most of these mutants were isolated as was described here. However, Gonzalez and Carlton (1984) claimed that when B. thuringiensis var israelensis was streaked onto Difco starch agar, the cry$^-$ colonies had a distinctive morphology and were thus isolated without the use of phase contrast microscopy.

In another study, again using B. thuringiensis var israelensis, the frequency of spontaneous mutation increased as the organism was allowed to grow in a nutrient medium, and then used to inoculate another flask of the same broth and allowed to achieve sporulation (Kamdar and Jayaraman, 1983). The frequency of cry$^-$ mutant isolation increased as the passage number increased. Thus, after the first transfer 30% of the cells were cry$^-$ mutants, and after four transfers 75% of the colonies studied were cry$^-$ with 16% being spo$^-$ cry$^-$ and only 9% were wild type. This increase in the occurence of spontaneous cry$^-$ mutants was also accompanied by a decrease in the insect toxicity of the cultures as the passage number increased.

The results obtained during my investigation into strain deterioration were never as dramatic as those outlined by Kamdar and Jayaraman. Nonetheless, the same general trend where increased subculturing precipitated mutation of the population was confirmed.

3.1.6.2 Effect of prolonged storage and/or frequent subculturing of B. thuringiensis var israelensis on the growth and sporulation of the organism in a fermentation culture.

In the previous section it had been established that the ability of B. thuringiensis to produce parasporal crystals could be affected by storage conditions and/or subculturing frequency. During this stage, the effect of these parameters on the growth and sporulation of the organism was investigated to establish if these physiological characteristics could be similarly affected.

A fermentation run was conducted by inoculating a series of 21 flasks containing 200 ml of either the SM or the Dulmage medium with Inoc. 2 cultures of varying ages. For comparative purposes, two of the Dulmage flasks were inoculated with a spore suspension (2.2.3) to give an initial viable count similar to that obtained using the vegetative cell inocula from the casein inoculum medium cultures.

The initial culture source was a slant from the IPS.80 subculture described in section 3.1.6.1 which had been found to be seriously deteriorated with respect to crystal producing capacity. Spore suspensions from the subculture were used to inoculate the TW flasks, and the spore-inoculated Dulmage production flasks.

A series of TW flasks was inocualted on a staggered basis over 12 hours and incubated for 24 hours as in sections 2.3.1,2. After 24 hours growth these cultures were used to provide 5% inoculum volumes for a series of casein inoculum medium flasks. The Inoc.2 flasks were seeded on a staggered basis also, so that by the time they were used to inoculate the production flasks, cultures of 10, 12, 14, 16, 18, 20 and 26 hours had been produced.

Inoculum cultures of varying ages were used to verify that inocula of

a range of ages could be used to seed the production stage, and to demonstrate that any effects observed on the growth and sporulation of the organism were not due to the use of an unsuitable inoculum source.

Once 5% inoculated the production flasks were incubated as in 2.3.1 for 48 or 72 hours. Viable counts were only made at 48 and/or 72 hours, while the VC:SC:FS ratio (section 2.3.6.3) was determined only at 48 hours. The results obtained for the production flasks during this run are presented in Table 3.1.17.

The data obtained clearly demonstrated that poor levels of growth and sporulation were achieved in all flasks, regardless of inoculum type. It was therefore concluded that the poor performance of the organism was not related to the inoculum preparation, but rather, strain degeneration was strongly suspected. This conclusion seemed appropriate in view of the spontaneous mutant isolation from slants of this subculture, noted in 3.1.6.1.

The implication of the results obtained here, and in section 3.1.6.1 was that strict attention had to be paid to the subculturing frequency and the conditions of storage used for the stock bacterial cultures. Up to this point, during these investigations, nutrient agar slants of B. thuringiensis var israelensis were not used after being stored for three months at $4^{o}C$. In addition fresh subcultures were made on a frequent basis from the original IPS.80 freeze-dried powder, and therefore the possibility that strain deterioration had occurred in some of the stock slants during the course of this work was greatly diminished.

TABLE 3.1.17

Results obtained during a flask fermentation of B. thuringiensis in the SM production medium using a degenerated strain.

MEDIUM	INOCULUM AGE (h)	pH AT tx			VC:SC:FS at t48	VIABLE COUNTS ($x10^9$/ml)	
		24	48	72		t48	t72
SM	10	6.88	7.78	ND	40:10:50	1.3	ND
	12	6.88	7.50	ND	20:5:75	1.8	ND
	14	6.68	7.70	ND	45:45:10	1.2	ND
	16	6.93	7.73	ND	50:40:0	1.2	ND
	18	6.60	7.75	ND	45:25:30	1.4	ND
	20	6.68	7.77	ND	45:55:0	0.7	ND
	26	7.36	7.73	8.45	55:45:0	0.7	1.5
Dulmage	26	7.22	7.95	8.48	35:60:5	ND	1.2
	spores*	7.80	7.87	8.20	25:5:70	ND	1.3

* Flasks inoculated with a spore suspension to give a similar initial viable count to that in the vegetative cell - inoculated flasks of about $5x10^7$/ml.

21 production flasks containing 200ml medium were 5% inoculated with casein inoculum cultures of varying ages, and then incubated under standard conditions.

3.1.6.3 Recommendations on a subculturing protocol

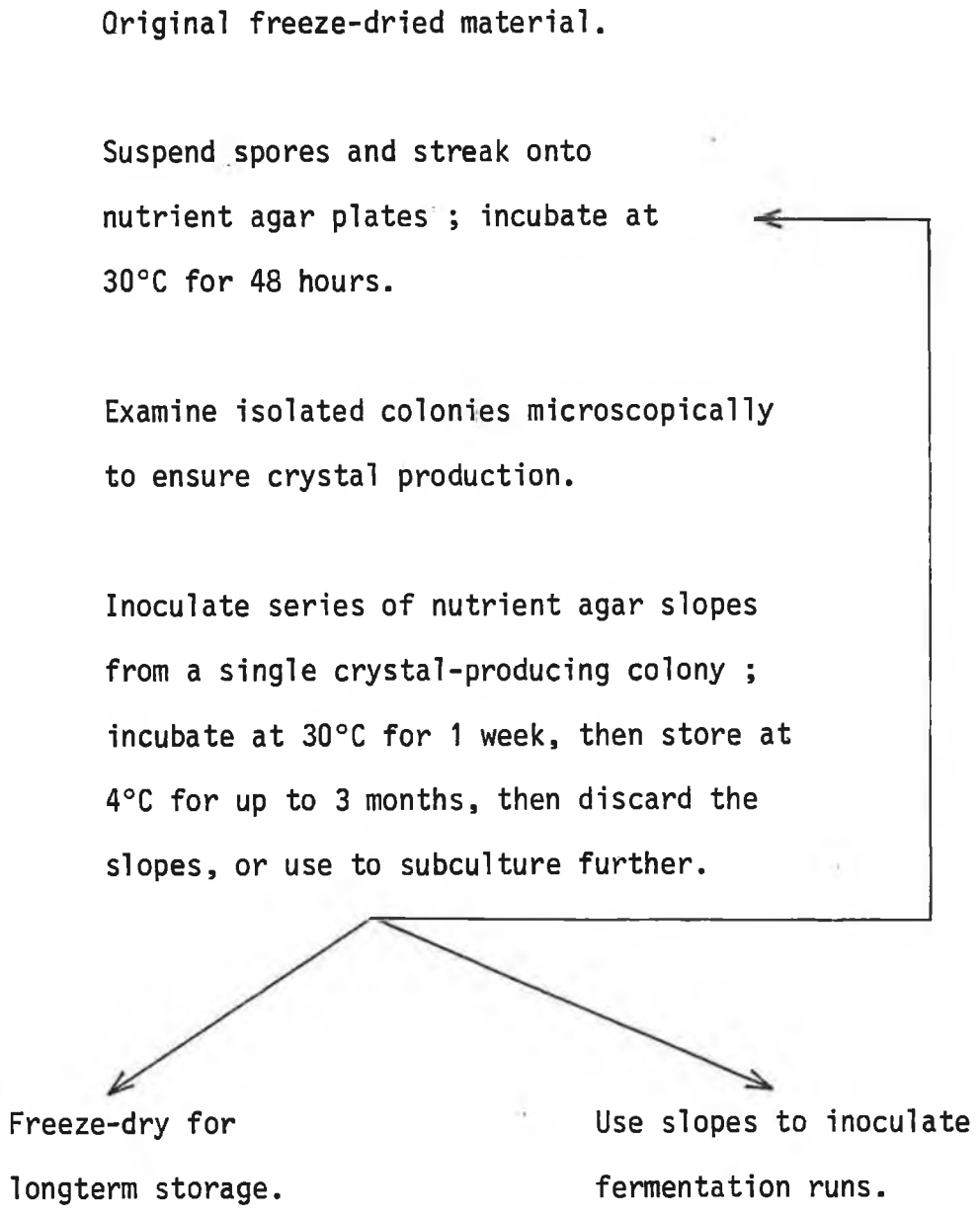
The experience gained during the investigation described above suggested that for longterm implementation of an industrial fermentation programme, or a laboratory-based study of B. thuringiensis var israelensis, a rigourously controlled subculturing protocol would be required. In addition, freeze-drying of useful isolates would also be highly desirable for longer term storage of the organism.

With these considerations in mind, a subculturing protocol was developed whose essential feature was the microscopic verification of crystal production in isolated colonies which could then be used in subsequent operations, Figure 3.1.16. It is expected that the use of such protocol would minimize the risk of strain deterioration, but such problems could be almost completely eradicated by freeze-drying high product-yielding isolates.

Strain productivity could be more stringently tested by inoculating a given production medium with a given isolate and incubating under standard conditions. Bioassay of these broths and comparison to results previously obtained with other isolates in this medium would enable the strain productivity to be ascertained with certainty. The disadvantage of this system would be the time required for ensuring strain productivity. Therefore the technique of microscopic examination to establish that an isolate is producing crystals is better suited as a quality control procedure for use during subculturing. Bioassay of a standard culture broth would be more suitable for use in ensuring strain productivity on a less frequent basis, say once a subculturing protocol has been completed on the three monthly basis recommended here.

FIGURE 3.1.16

Recommended subculturing procedure for ensuring strain productivity of B. thuringiensis var israelensis isolates.



3.1.7 Summarizing Comments on Process Design and Scale-up

During this section on the fermentation of B. thuringiensis a process has been developed, first of all in flasks and then in laboratory-scale and pilot-scale fermenters. This entailed the design of an inoculum production procedure devised to produce a suitable inoculum for the seeding of the final production stage.

The levels of biomass and sporulation of the organism in a range of literature-reported production media were then determined. Following this these levels were improved by the use of variants of the literature media. Bioactivity investigations into the levels of insect toxin produced by B. thuringiensis var israelensis in both the literature media and their variants selected a single medium which yielded product levels optimized with regard to medium costs and production cycle time.

Scale-up of both the inoculum and production stages was successfully achieved and finally, strain stability and productivity checking procedures were developed.

A full discussion of the results obtained during this development programme is given in Section 4.

3.2 PERFORMANCE OF THE CONVENTIONAL BIOASSAY

A detailed description of the conventional bioassay system is presented in section 2.4.3. In summary, the procedure involved selection larvae of a specific size from a population which had been grown for five days. Twenty five selected larvae were counted into waxed paper cups and made up to 100 ml with distilled water. Aliquots of insecticide of between 50-100 ul were added to the cups, and larval mortality was recorded 24 and 48 hours later. Median lethal response levels of the analytical and standard materials were compared and the bioactivity of the test preparations calculated. Because two different standard materials were used which were quantified in different units, bioactivity data were determined either as mg crystal protein/ml culture broth when compared to the pure crystal preparation (2.5), or, as International Toxic Units per mg or ml of material when the IPS.82 formulation was used as the standard.

The conventional bioassay system was set up at the NIHE following a period of training at the London School of Hygiene and Tropical Medicine under the supervision of Dr. Graham White. The method established was based on that learned in London and on a standard procedure recommended by the WHO (Rishikesh and Quelennec, 1983). The bioassay work formed a central part of the fermentation studies as this is the only universally acceptable method for the estimation of the bioactivity of an insecticidal material (Rishikesh and Quelennec, 1983).

On a given day, one conventional bioassay was set up, during which 100-150 cups were typically counted out. This was sufficient to bioassay approximately 8 samples, at least one of which had to be a standard material. This proved to be a very cumbersome technique

which required 6-8 hours to enumerate the 2500-5000 larvae needed, and then to add an insecticidal aliquot to each cup.

3.2.1 Experience with Larval Rearing and Preparation for Bioassay

As was expected, after five days of growth, a certain proportion of all four larval stages (1.7.1) were found in the larval colony. It was impossible to get a batch of larvae which were all at the early fourth instar (L4) stage, and the best that was achieved was fairly synchronous development where as many as 50-60% of the larvae were suitable for bioassay purposes. This was most easily done wnen the larval density and food availability were controlled as strictly as possible, as described in Section 2.4.2.2.

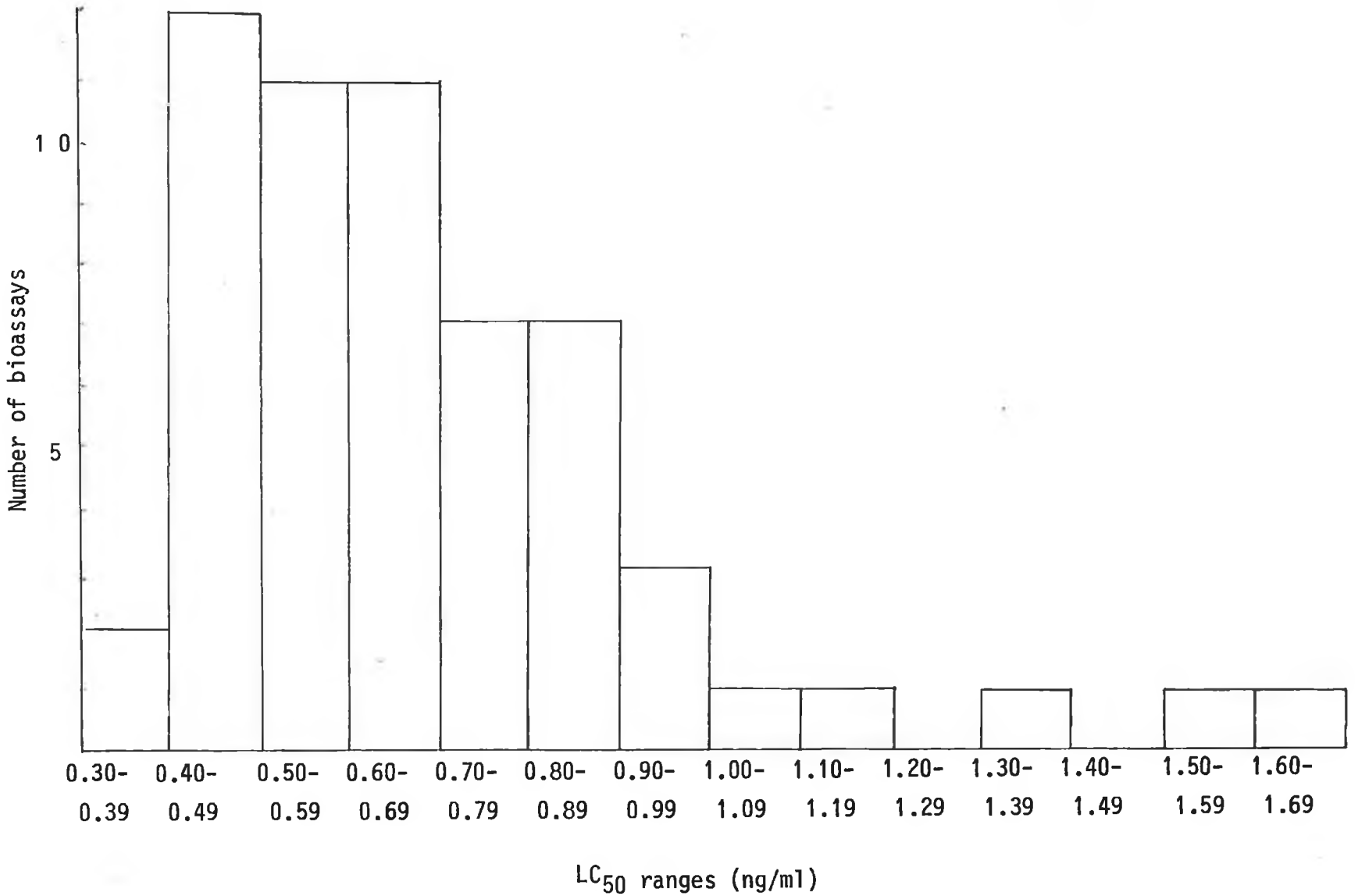
Following rearing of the larvae, thorough cleaning was necessary in order to remove as many food particles as possible because of the reported interference of particulate matter with the activity of B. thuringiensis var israelensis (Rishikesh and Quelennec, 1983; Ramoska et al., 1982; Sinegre et al., 1981 b). This step visibly removed large quantities of particulate matter from the larvae and undoubtedly contributed to the reproducibility both with a given assay, and between assays done on different days.

3.2.2 Reproducibility of Results Obtained Using the Standard Materials

In order to ascertain how reproducible the bioassay results obtained for the standard materials were, the LC_{50} values after 48 hour for bothstandards were collated. The number of bioassays which gave LC_{50} values in the 0.30-0.39 ng/ml range, and in similar incremental ranges up to 1.70 ng/ml, for the pure crystal suspension was determined and presented in a bar chart, Figure 3.2.1. A similar collation was done

FIGURE 3.2.1

Ranges of LC_{50} values obtained for the pure crystal suspension when used as a standard material in the conventional bioassay ; and, number of bioassays giving LC_{50} values in the given ranges.



for the IPS.82 material and the results of these findings are presented in Figure 3.2.2. Finally, the range of values for the LC_{50} IPS.82 to LC_{50} Pure Crystals ratio were determined and the number of results falling into particular ranges were plotted, Figure 3.2.3.

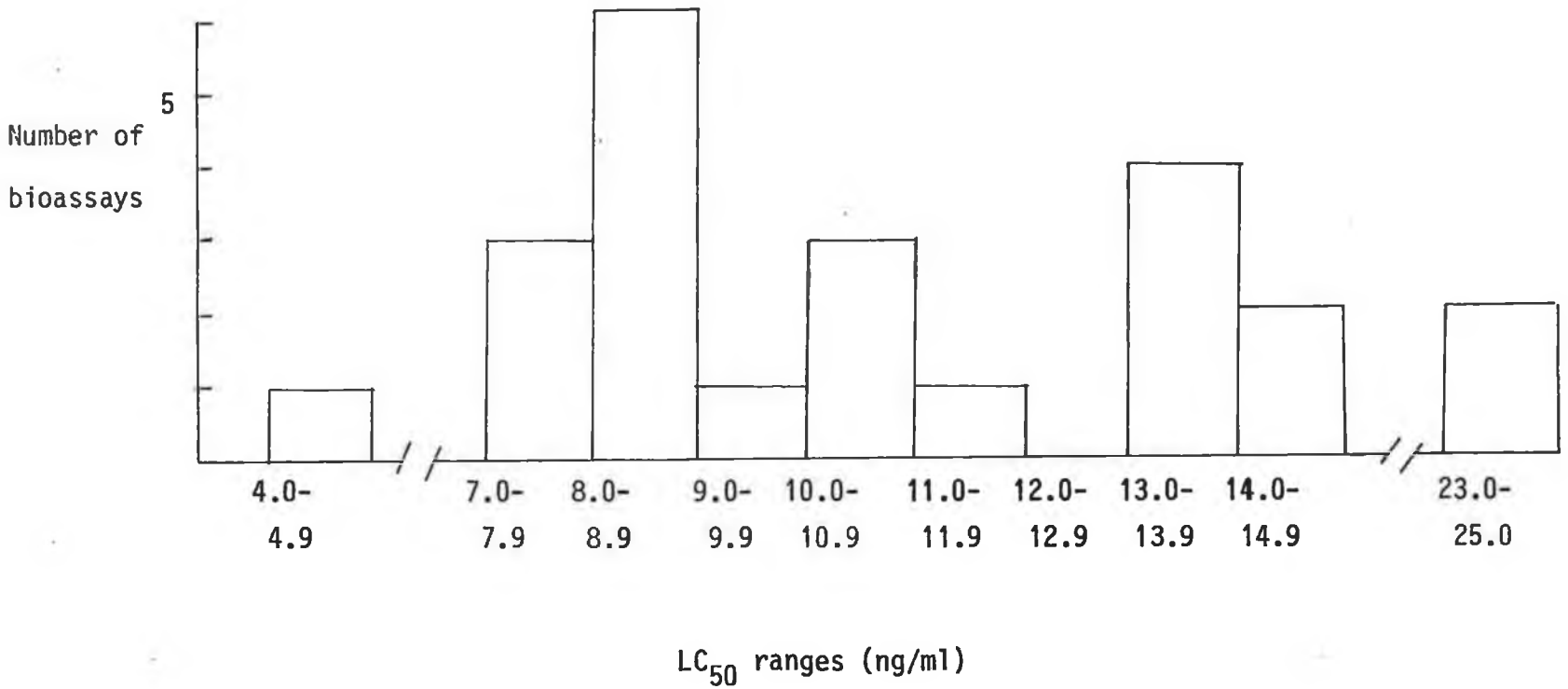
Bioassay results for the two standard materials gave a range of LC_{50} values for the individual preparations. During the entire bioassay programme the pure crystal preparation was used 58 times, and the IPS.82 formulation was tested on 23 occasions. The range of LC_{50} values for the pure crystal suspension was 0.35-1.65 ng/ml with over 90% of the results in the 0.40-1.00 ng/ml range. The IPS.82 formulation gave LC_{50} results between 4.5 and 24.0 ng/ml, with 87% of the values between 7.0 and 14.0 ng/ml.

This variation in LC_{50} values is largely a function of the variation in larval susceptibility due to the difficulty in obtaining synchronous development of the insects, mentioned in 3.2.1. However, given that variation in larval susceptibility was inevitable, a very high proportion of the LC_{50} values did fall into narrow ranges. This was especially apparent for the pure crystal suspension, which vindicated its selection as a standard material, even though the use of this type of reference preparation had not been previously reported. Thus, the good agreement in LC_{50} data for the standard materials was taken as a strong indication that the larval rearing and selection procedures used (2.4.2,3) did produce insects within a small range of susceptibilities in a reproducible fashion on a day to day basis. This was confirmed by Dr. G. White when he inspected the mosquito rearing-bioassay facility in Dublin.

When the LC_{50} values of the IPS.82 and pure crystals preparation for a given assay were compared, it was found that the LC_{50} ratios varied

FIGURE 3.2.2

Ranges of values obtained for IPS.82 when used in the conventional bioassay as a standard material ; and, number of bioassays giving LC_{50} values in a given range.



over a small range, Figure 3.2.3. All the results from only 22 bioassays were between 10.0 and 21.0 with 13 of the ratios between 15.0 and 18.0. Such consistent results were to be expected on the basis of the consistency of the individual LC_{50} values of the two standard materials described above.

Some of the actual dose-response plots for the two standard materials are presented in Figure 3.2.4,5. It can be seen from these graphs that the bioassay system described here could be used to obtain useful and reliable data relating to the bioactivity of the materials examined.

3.2.3 Reproducibility of Results Obtained for Fermentation Broths

The reproducibility of data obtained for fermentation broths was examined by collating the crystal protein results. The data for the crystal protein content of the broths was used because a wide range of broth samples had been repetitively bioassayed using the standard material, much more so than had been done in conjunction with IPS.82.

The data obtained for a number of borth samples which had been bioassayed up to five times, and the variation of the crystal protein are presented in Table 3.2.1.

Table 3.2.1 shows that the percentage variation for the materials when bioassayed on a minimum of three occasions was always less than 20%. Considering the difficulties in rearing, preparing and selecting larvae for bioassay it was felt that this variation was highly acceptable. These variation figures compare favourably with values reported in the literature. Nugud and White (1982) found their results gave 10% variation over 3 assays using experimental industrial

FIGURE 3.2.3

Range of values obtained for LC_{50} IPS.82 / LC_{50} Pure Crystals ratio obtained during conventional bioassays ; and, number of bioassays giving results in a given range.

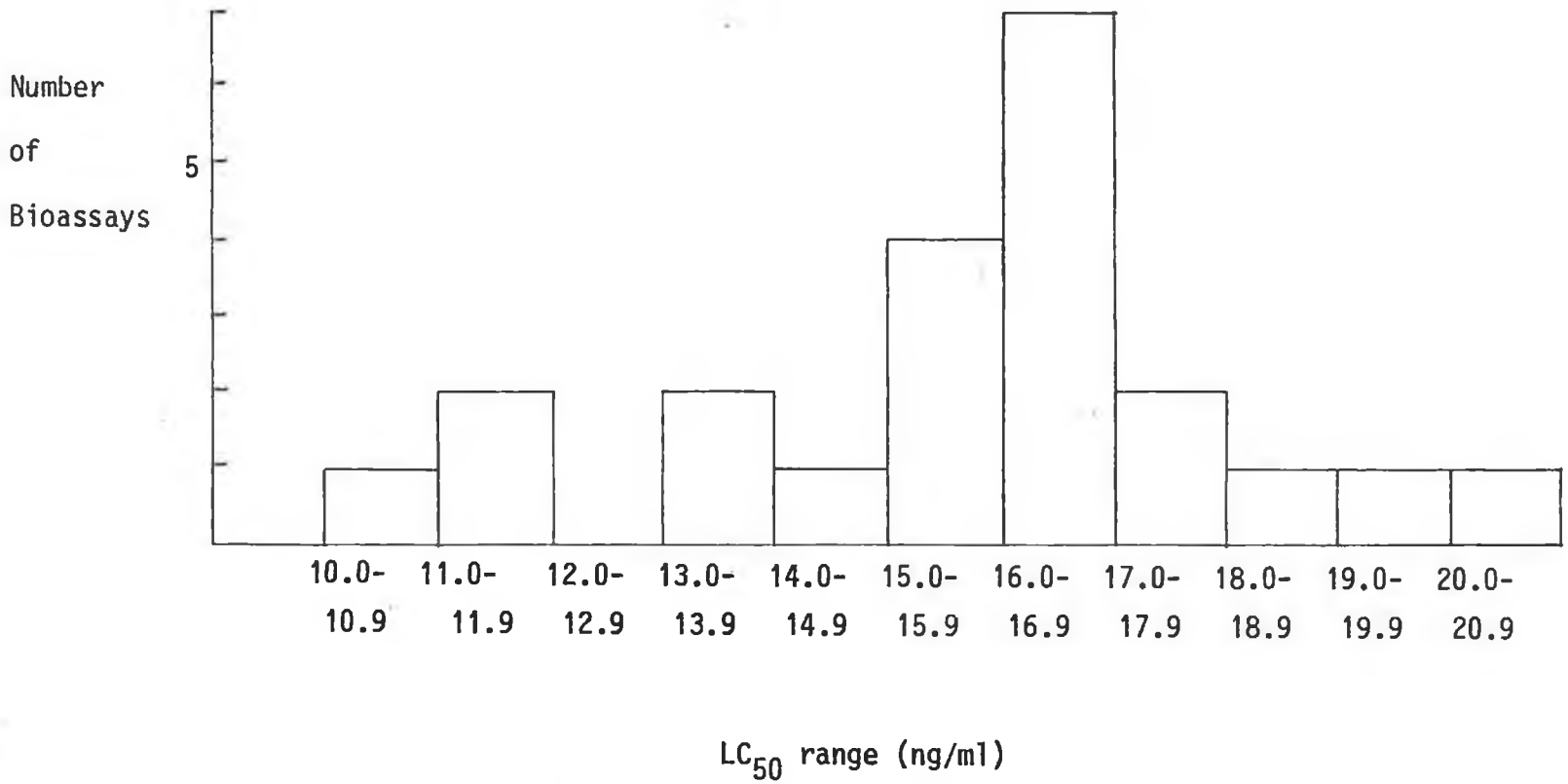


FIGURE 3.2.4

Typical results from the Mortality vs. Concentration Bioassay using the pure crystal suspension as the insecticidal material.

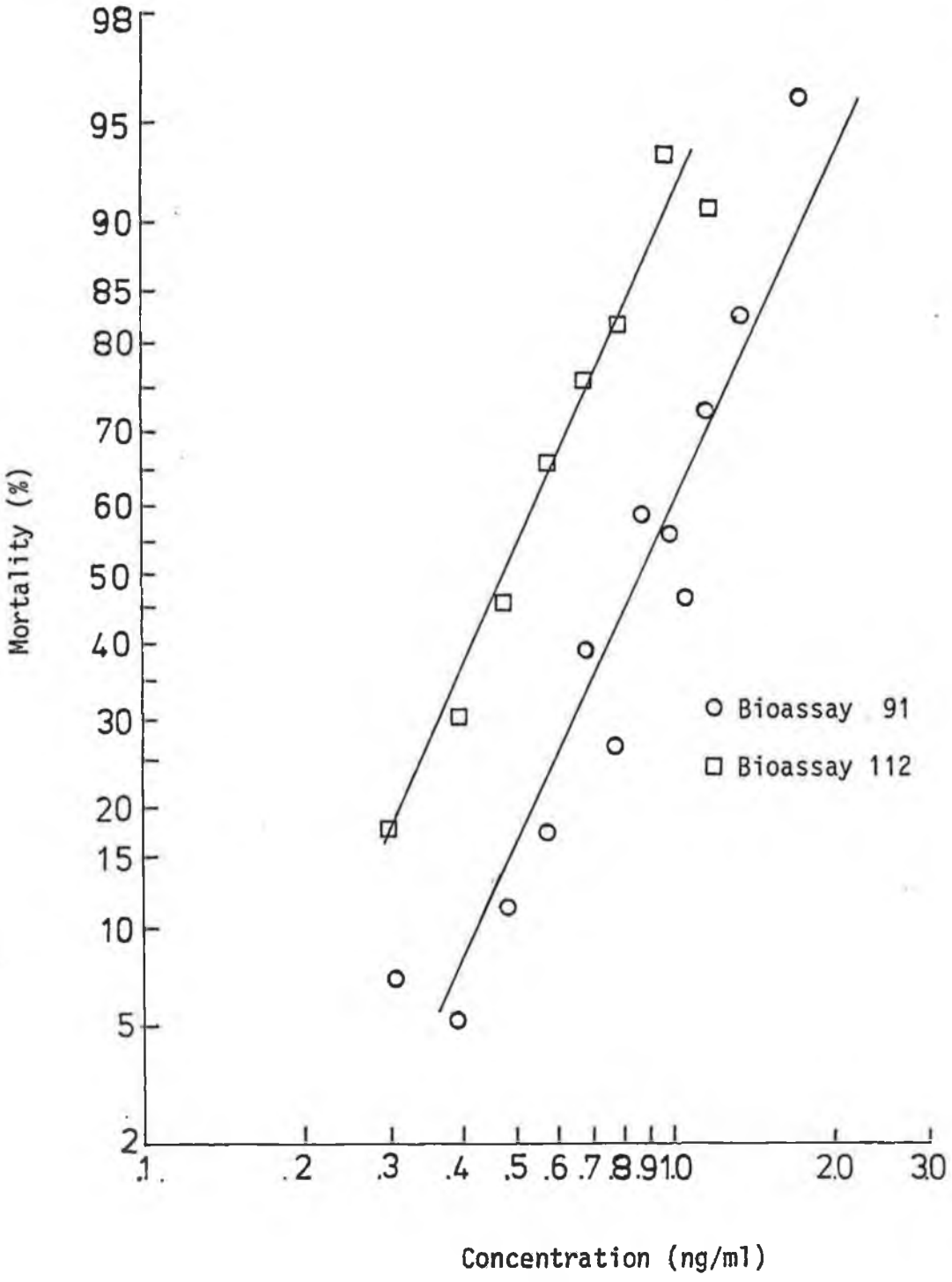


FIGURE 3.2.5

Typical dose-response plots obtained using IPS.82 in the conventional mortality vs. concentration assay.

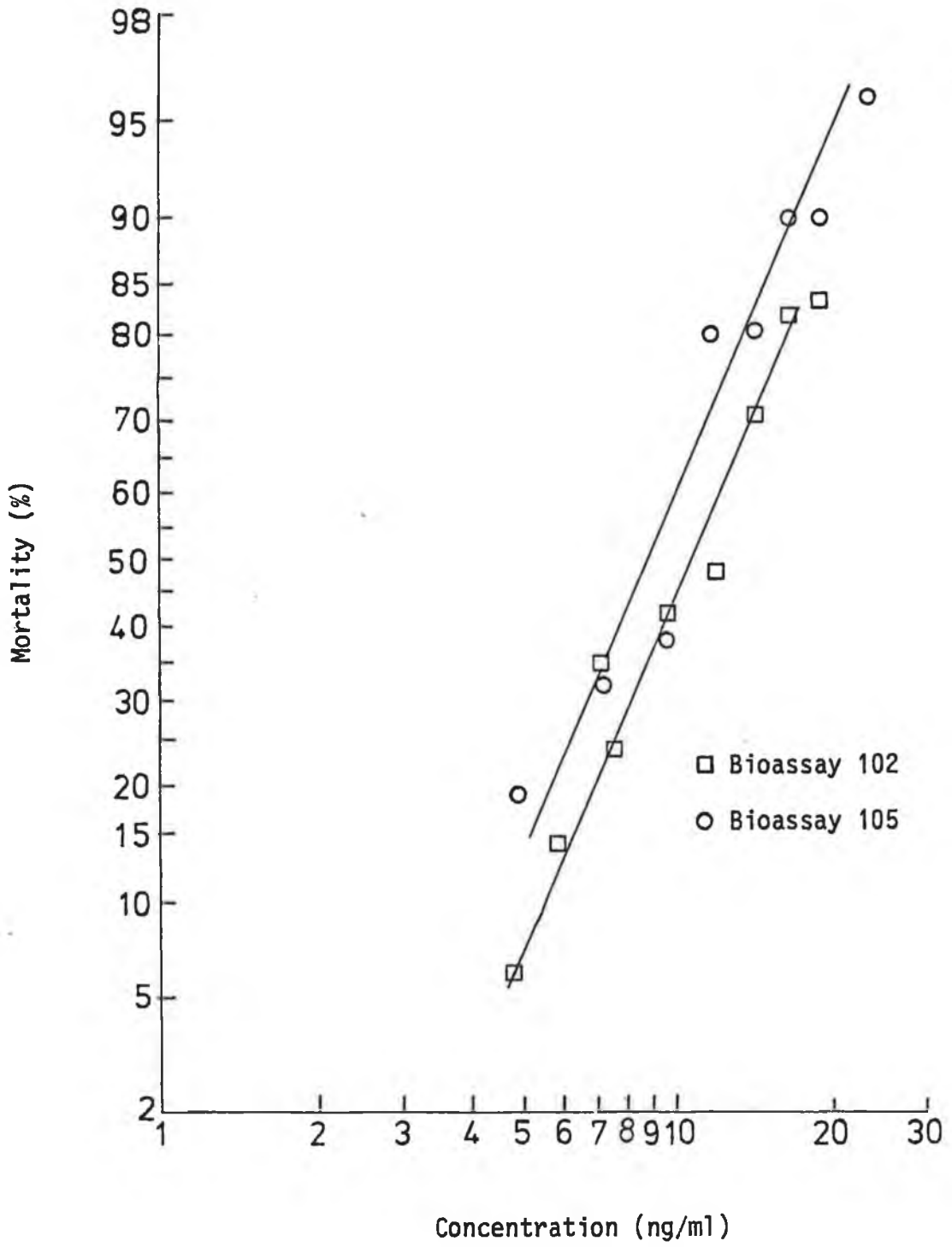


TABLE 3.2.1

Results obtained for a number of fermentation broths when bioassayed on at least 3 different occasions.

MATERIAL	*CRYSTAL PROTEIN, mg/ml						
	A	B	C	D	E	MEAN	% VARIATION
SM. 44.1.48	0.53	0.67	0.54	-	-	0.50	11.7
MOL. 44.1.24	0.21	0.19	0.22	0.18	-	0.20	10.0
Dul. 47.1.48	0.30	0.31	0.38	-	-	0.33	11.8
Mol. 47.2.24	0.10	0.12	0.14	-	-	0.12	16.7
Meg. 47.1.48	0.40	0.30	0.39	-	-	0.36	14.3
Str. 47.2.48	0.18	0.15	0.22	0.15	-	0.17	18.9
Dul. 49.1.48	0.35	0.38	0.35	0.41	0.37	0.37	7.9
FFSF. 49.1.48	0.33	0.35	0.29	0.34	-	0.33	9.4
FFSF. 49.1.48	0.38	0.37	0.41	-	-	0.39	5.1
SBM. 49.1.48	0.38	0.37	0.41	-	-	0.41	17.9
Meg. 50.1.48	0.18	0.26	0.21	-	-	0.22	18.1
V3. 50.1.48	0.29	0.22	0.24	-	-	0.25	13.7

* The letters refer to the crystal protein concentration result obtained on the 1st., 2nd., etc, time the broth was bioassayed. The results were obtained from at least 10 bioassays.

preparations. In another report, published by WHO, variations for an experimental formulation using IPS.78 as the standard material over three experiments were typically in the 30% - 40% range (de Barjac and Larget, 1979).

In conclusion, it was accepted that the data obtained for the bioactivity of the standard materials and the fermentation broths using the assay system described in 2.4 were reliable. Furthermore, the use of the pure crystal suspension proved to be as reproducible, if not, more so, than the use of IPS.82 as a standard reference material.

3.3 INVESTIGATION OF THE PARASPORAL PURIFICATION TECHNIQUE

A purification of the parasporal crystals of B. thuringiensis var israelensis was undertaken for two reasons. Firstly, to prepare a pure crystal suspension for use as a standard material in the bioassay systems, 2.4. Secondly, to quantitate the efficiency of the system with a view to the usefulness of such a process on an industrial scale in preparing an organism-free formulation.

There is considerable interest in organism-free formulations (Luthy et al., 1982) because such a product would enable treatment of insect habitats without exposure to high numbers (up to 10^{13} spores per acre; Luthy et al., 1982) of bacteria, however harmless. In addition, the developer of a virulent B. thuringiensis strain could protect their invention by the use of such a formulation. At the moment, commercial insecticides based on B. thuringiensis contain spores and crystals, and thus the organism is easily isolated from the industrial materials. For these reasons the introduction of an organism-free formulation would represent a considerable step forward in the technology of biological insecticides.

The parasporal purification method used was based on that of Goodman et al. (1967). The method is described in detail in section 2.5.2 but by way of summary it will be briefly described here.

A spore/crystal suspension prepared from a soluble medium was introduced into an aqueous two-phase system based on the mutually incompatible polymers, polyethyleneglycol (PEG) and the sodium salt of dextran sulphate. The system was shaken in a separating funnel and the phases allowed to separate. Due to the differing partition coefficients of the two types of particle, separation was achieved on

the basis of the spores preference for the upper PEG layer, and the affinity of the crystals for the interface and lower dextran sulphate layer. This principle has been widely investigated and applied to the separation of particles, and even of proteins and nucleic acids (Albertsson, 1971; Sacks and Alderton, 1961). After partition the PEG layer was removed, fresh PEG added and the process repeated. A number of passes were done and samples of fractions were taken in order to quantitate the efficiency of the process. A diagrammatic representation of the crystal purification appears in Figure 3.3.1.

Prior to this, no known studies of this kind had been reported; so it was felt that the data obtained should be of considerable use in assessing the potential industrial application of the technique.

3.3.1 Quantitative Description of Fractions Produced During the Crystal Purification Procedure.

The technique used for purifying the crystals is described in detail in 2.5.2 and summarized above in 3.3. Initially, the original spore/crystal primary suspension was quantitated with respect to viable count, volume, optical density of 600mm, and dry weight. Following completion of the separation the final crystal suspension was examined in the same way, but in addition the protein content was determined as described in 2.7.2. Then the primary spore/crystal suspension and the final pure crystal suspension were bioassayed in the same way as the fermentation broths to establish the crystal protein content of the original spore/crystal mixture. The results of these analyses are presented in Table 3.3.1.

During the purification, the waste PEG layers were measured with respect to volume, viable count, OD600 and crystal protein content by

FIGURE 3.3.1

The parasporal crystal purification process

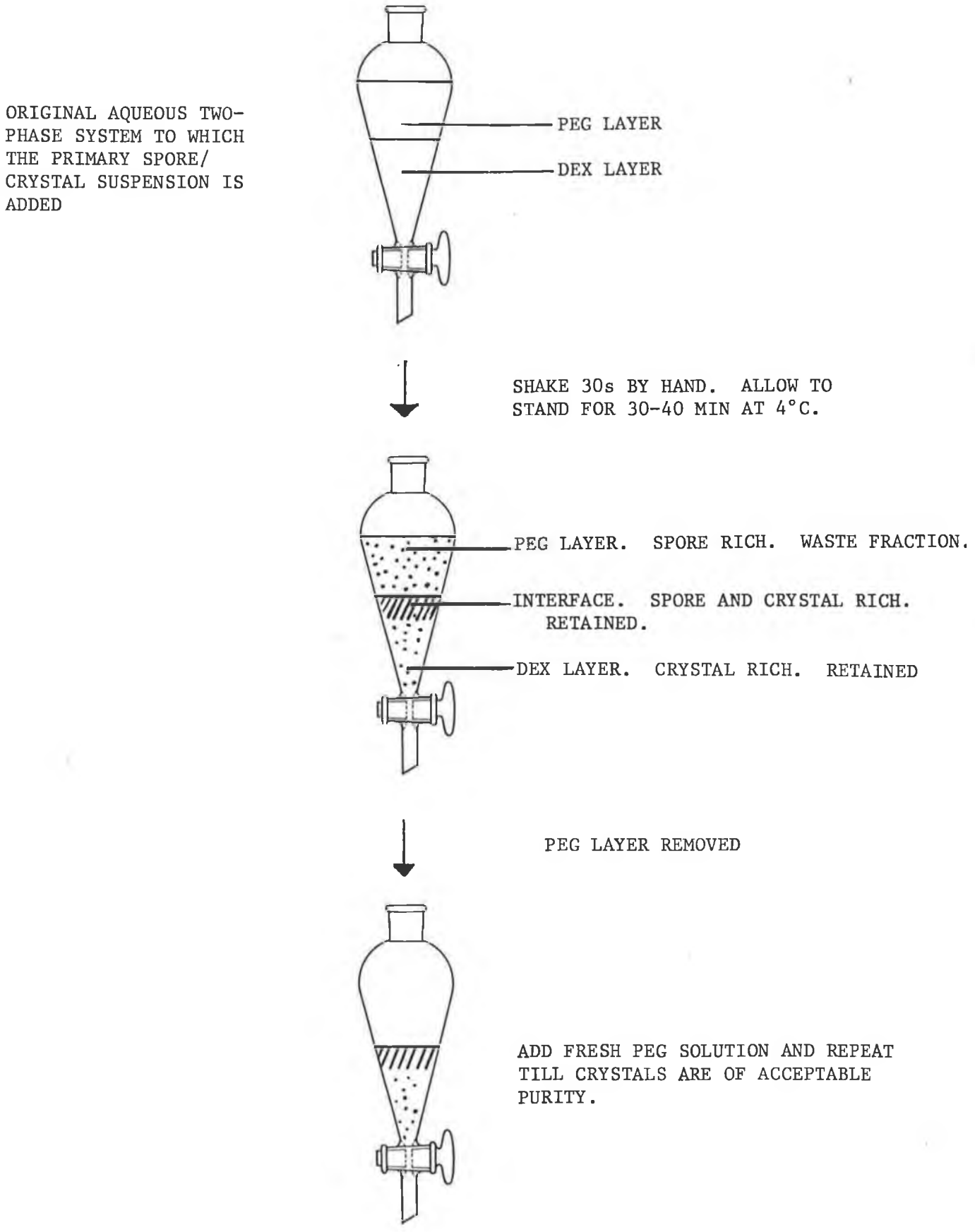


TABLE 3.3.1

Quantitative description of the primary and final crystal suspensions from the crystal purification process.

PARAMETER	SUSPENSION	
	PRIMARY	FINAL
Volume (ml)	5.0	10.0
TVC (x 10^{10}/ml)	2.1	0.01
Total Spores (x 10^{10})	10.5	0.10
% Initial Spores	100.0	0.9
OD600	325.0	20.7
Dry Weight (mg/ml)	58.8	2.3
Protein* (mg/ml)	ND	1.04
Crystal Protein (mg/ml)	5.5+	1.04+
Total Crystal Protein (mg)	27.5+	10.4*
% Original Crystal Protein	100.0	38.0

* Determined by Coomassie Blue Dye-Binding Method

+ Determined by comparative bioassay to final crystal suspension

comparative bioassay to the pure crystal suspension. The data obtained during these analyses are presented in Table 3.3.2.

From the data in Table 3.3.1 it can be seen that the final crystal suspension contained less than 1% of the original number of spores and 38% of the initial amount of crystal protein material. Thus, it is immediately evident that this aqueous two-phase system exhibited considerable ability to purify crystals form a spore/crystal mixture. The efficiency of this process may even have been higher had a spillage of the dextran sulphate/interface layers not occurred after pass number four.

The spillage occurred late in the separation process when over 90% of the spores had already been removed. And, because it only affected the non spore-containing phases, the loss of crystal material was proportionately greater than of spores. Thus, the overall spore removal figures were unaffected, but the recovery of pure crystals was markedly altered.

This assertion is substantiated by the data in Table 3.3.2 where the combined PEG layers could only account for 8.7% of the crystal material. This left a shortfall of 53% which remained unaccounted for, and further supported the view that the potential separation efficiency was significantly greater than the 38% found here.

At a given pass the PEG layer removed in the region of 40-50% of the spores present, but only 1-2% of the available crystal material, Table 3.3.2. This indicated the strong preference of the spores for the PEG layer by comparison to the crystals, and admirably demonstrates the reason why the technique was so successful in separating the two particle types.

TABLE 3.3.2

Quantitation of the PEG layers removed during the crystal purification process

PASS NO.	OD 600	SPORE REMOVAL				CRYSTAL REMOVAL			
		DURING PASS ($X 10^{10}$)	TOTAL AFTER PASS COMPLETE ($X 10^{10}$)	% OF INITIAL SPORE NO.	% OF AVAILABLE SPORES	DURING PASS (mg)	TOTAL AFTER PASS COMPLETE (mg)	% OF INITIAL CRYSTAL AMOUNT	% OF AVAILABLE CRYSTALS
1	2.34	3.86	3.86	37	37	0.62	0.62	2.2	2.2
2	2.64	2.58	6.44	61	40	0.43	1.05	3.7	1.6
3	2.14	2.13	8.57	82	52	0.52	1.57	5.7	2.0
4	0.81	1.02	9.59	91	53	0.22	1.79	6.5	0.8
5	0.675	0.37	9.96	95	41	0.35	2.14	7.8	1.4
6	0.285	0.05	10.01	95	9	0.22	2.36	8.6	0.9
7	1.40	0.06	10.07	96	12	0.04	2.40	8.7	0.2
Overall	-	-	10.07	96	-	-	2.40	8.7	-

* Available spores/crystals = Initial number/amount of spores/crystals in two phase system before pass 1 - number/amount of spores/crystals removed by previous PEG layers. In other words it was the number/amount of spores/crystals present in the system before a pass begun.

3.3.2 Possible Scale-up Potential of Crystal Purification Process

The separation technique used here is highly likely to be economical because of the high cost of the dextran sulphate which costs approximately IR£1 per gram. However, aqueous two-phase separation systems need only employ this component at all, and indeed I found that the cheaper form, dextran T500 could also be used for separating spores and crystals. However, this latter material was also costly. Despite these cost disadvantages, the use of these materials could be made more attractive by recovery and reusing the dextran layer which would simply require a centrifugation to remove particulate matter.

Alternatively, a number of biphasic systems which do not use any form of dextran have been described (Sacks and Alderton, 1961; Albertsson, 1971). These systems would obviously be much cheaper than the dextran utilizing protocols because of the low cost and industrial availability of PEG.

Given that a protocol could be developed using the biphasic PEG/buffer system, then its economic attractiveness would depend on how efficient the process was. In addition, the cost and ease with which it could be done on an industrial scale, and the advantages which could be accrued by the development of a virulent strain and introducing it into the market would also affect the implementation of such a process on a commercial scale.

It may already have occurred to the reader that a far easier way to develop an organism-free formulation would be to use a $spo^{-}cry^{+}$ mutant, and indeed such mutants have been isolated and described (Luthy et al., 1982; Wakisaka et al., 1982). However, such mutants are notoriously unstable with respect to not producing spores and frequently revert to the wild type phenotype, or lose the ability to

produce crystals, as well (Luthy et al., 1982). Therefore, at the moment the use of these mutants has failed to aid the introduction of organism-free formulations.

3.4 INVESTIGATIONS INTO THE DEVELOPMENT OF A NOVEL BIOASSAY SYSTEM

It was noted in section 3.2 that the conventional mortality vs concentration bioassay suffered from a number of disadvantages. These included the considerable length of time required to set the assay up, and the fact that it took two days to obtain results using the method.

This fact has received considerable attention and over the years a number of attempts have been made to develop biochemical methods for quantifying the amount of crystal protein in a fermentation broth. Many of these methods have been successful (Andrews et al., 1980; Smith and Ulrich, 1983; Wie et al., 1982, 1984). However, in the final analysis, bioactivity data must, at present, be derived from bioassay results by comparison with internationally recognized standards. Therefore, an investigation was conducted into the possibility of using fixed insecticide concentrations and determining the time required to achieve 50% mortality, as opposed to ascertaining the concentration required to cause the same response, which is used in the conventional mortality/concentration bioassay.

The advantages of such a system would be:

1) smaller number of larvae would be required because only one concentration (as opposed to 8-10 concentrations in the conventional system) need by used for a given material.
2) the assay would be much quicker because the insecticide concentration could be chosen to cause 50% mortality in 1-3 hours, requiring that a total exposure period of 8 hours or less be used.
3) Good assay data replication would be expected because fewer larvae would be needed and therefore larval preparation and selection could be done on a more stringent basis.
4) Because fewer larvae would be needed the work involved in setting

up the assay would be appropriately reduced.

Thus the potential for this type of assay was investigated using the same pure crystal suspension employed as the standard material in the conventional bioassay, 2.4.3.

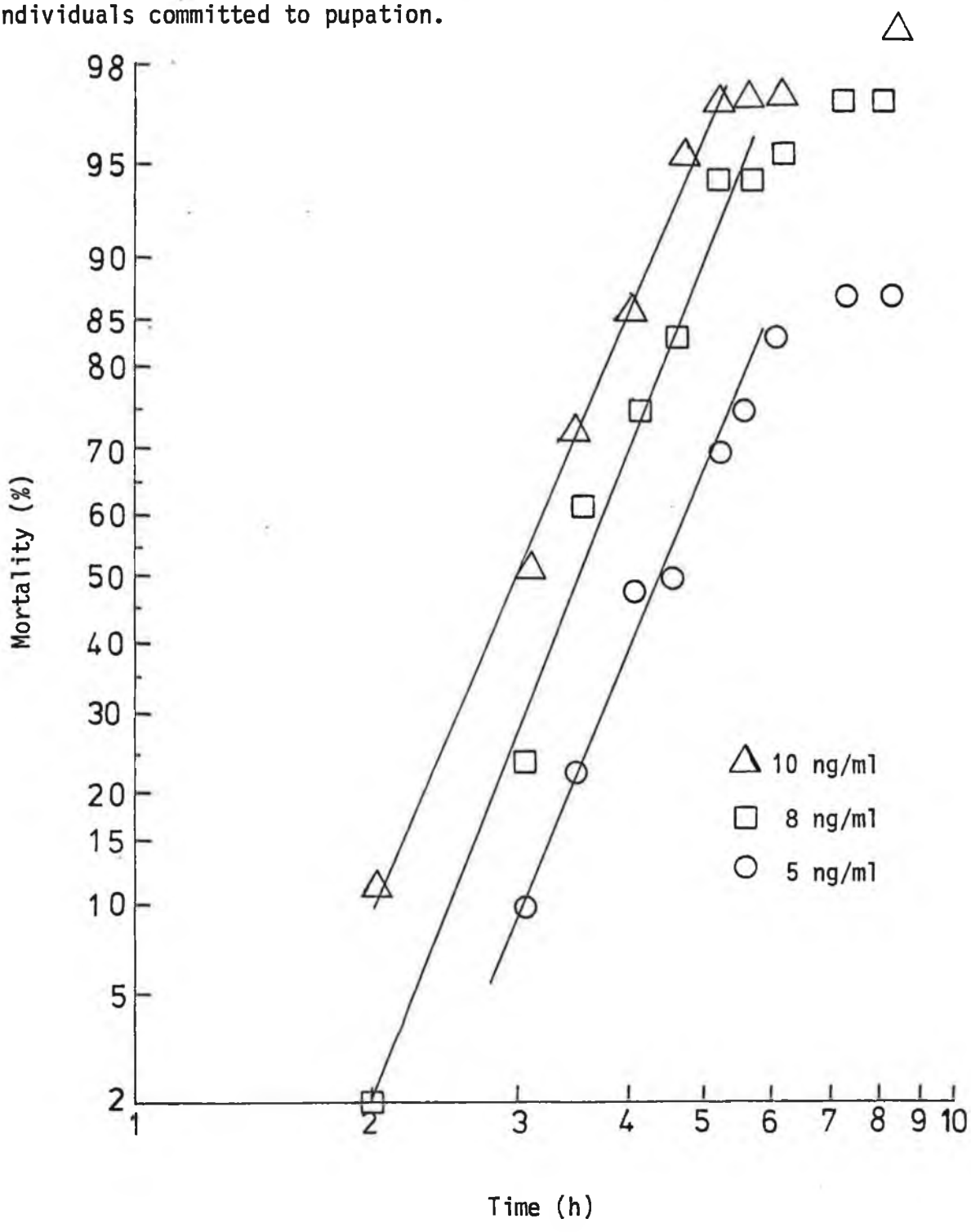
3.4.1 Results Obtained Investigating the Mortality vs Time Bioassay

During this study only the pure crystal suspension used for the conventional M/C bioassay was used. Firgures 3.4.1.,2 show the dose-response curves obtained when various crystal concentrations were used during two mortality vs time (M/T) bioassays. The curves show linear change in mortality with time which allowed the time for 50% mortality (LT_{50}) to be easily calculated from the data presented. It will be immediately noted tnat the mortality in Figure 3.4.1 began to level out after about 5-6 hours into the bioassay, whereas in Figure 3.4.2 this did not happen. This levelling out in the mortalities was caused by the fact that a certain proportion of the population was committed to pupation and therefore had stopped feeding, resulting in a lack of susceptibility to the crystal. The larvae were not killed by 24 hours exposure and would certainly have been transformed into pupae had the test been carried on beyond that point.

In addition, the LT_{50} values for the bioassay in which mortality did not reach 100%, Figure 3.4.1, were considerably higher than the $LT_{50's}$ in Figure 3.4.2. In the case of the 10ng/ml concentrations the difference in $LT_{50's}$ was a factor of 2.2. Thus the larvae used in Figure 3.4.1 were considerably less susceptible than those in Figure 3.4.2, so much so that a certain proportion of them were completely resistent to the insecticide by virtue of their commitment to pupation.

FIGURE 3.4.1

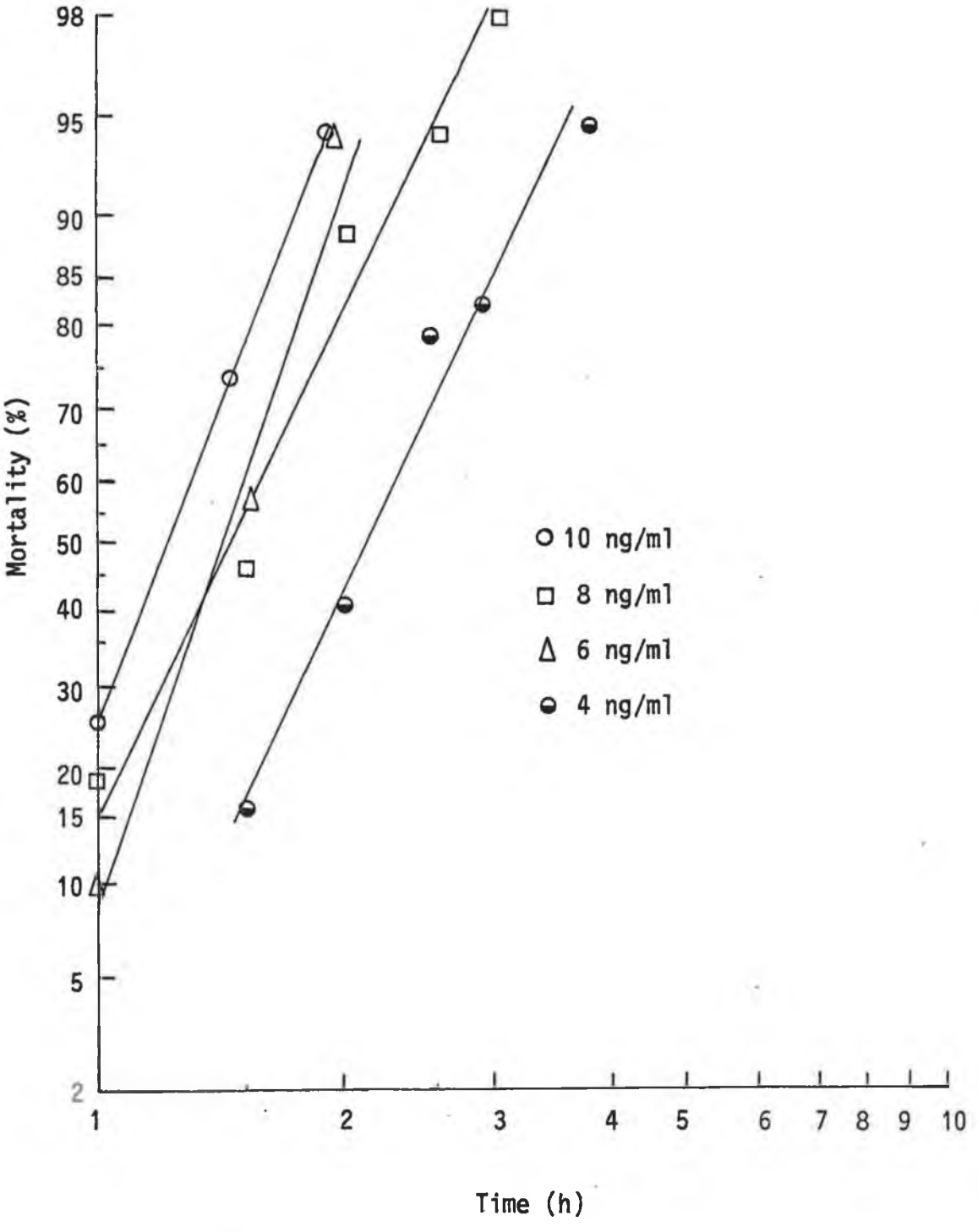
Mortality vs. Time bioassay results using the pure crystal suspension as the insecticidal material, and a larval population with some individuals committed to pupation.



All data obtained during a single bioassay.

FIGURE 3.4.2

Mortality vs. Time bioassay results using the pure crystal suspension as the insecticidal material, and larvae with a high degree of susceptibility to the crystal toxin.



All data obtained during a single bioassay.

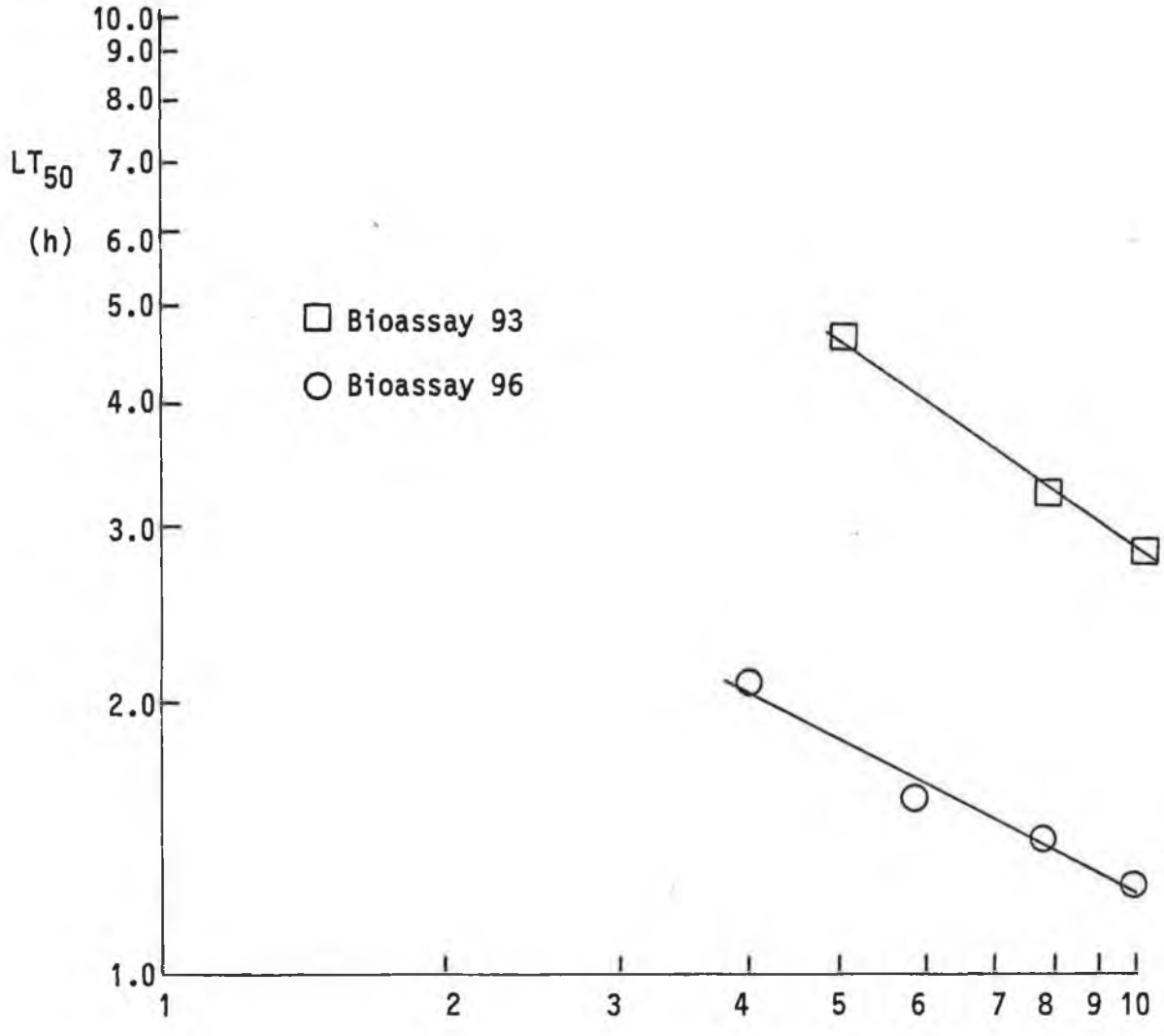
This difference in results further emphasizes the variability in larval susceptibility when using different populations prepared on different days, and the necessity to use standard materials allow to for this variation.

Because a number of crystal protein concentrations was used for each bioassay it was essential to know of the relationship between LT_{50} and crystal concentration was linear for results obtained during the same bioassay. If a linear relationship was not found then this assay system could not be useful as this would mean that the LT_{50} value obtained would be a function of the dilution of the material used as well as its concentration.
Figure 3.4.3 show that the plots of LT_{50} values against the crystal protein concentrations were linear for the two bioassays.

Thus it was shown that, by using given concentrations of a pure crystal suspension, a linear response of mortality with time was observed. In addition, the relationship between LT_{50} values obtained for various crystal concentrations in the same bioassay and the concentrations of crystal material used was also linear. Therefore, on the basis of these results, it would seem possible that the mortality vs time bioassay could be used to determine the bioactivities of fermentation broths. The introduction of such a system would allow determinations of bioactivity in a considerably shorter time than can be achieved at present using the conventional bioassay system.

FIGURE 3.4.3

LT_{50} values for given crystal concentrations, vs. crystal concentration for two mortality vs. time bioassays.



3.5 A STUDY OF PROTEASE ACTIVITIES IN CULTURE BROTHS OF B. THURINGIENSIS VAR ISRAELENSIS

As was shown in 3.1.4, crystal protein concentration in culture brotns of B. thuringiensis var israelensis followed cell lysis and reached a maximal level when lysis was approximately 90%. Thereafter, the level of crystal protein in the broths declined, so that by 72h only 60-70% of the maximal bioactivity remained in the SM medium.

During sporulation of bacteria of the genus Bacillus high levels of protein turnover and proteolysis occur (Doi, 1972; Maurizi and Switzer, 1980). Indeed, reports have been published on studies of proteases from cultures of B. thuringiensis (Chestukhina et al., 1978, 1979, 1980; Egorov et al., 1982, 1983; Epremyan et al., 1981; Lecadet et al., 1977; Li and Yousten, 1975; Stepanov et al., 1981). In addition, Monro (1961) presented evidence that most, if not all of the crystal protein is synthesized during sporulation from recycled protein. It therefore seemed likely that the decrease in bioactivity of the culture broths was due to proteolysis of the crystals. An investigation on the protease activities was therefore carried out with a view to:

1) Characterizing the protease activities with respect to pH optimum, effect of protease inhibitors and effect of divalent metal ions,
2) Determination of the time of protease production in fermentation cultures and an evaluation of the protease activity levels in various media,
3) Purification of protease activity from culture supernatants in order to determine the number of enzymes present and to perform a partial characterization of these proteinases.

In the literature, a number of studies on proteases produced by B. thuringiensis have been reported and a summary of the results obtained during these investigatins is presented in Table 3.5.1. A more detailed discussion of these investigations, and of microbial proteinases in general, has been given in section 1.6.

3.5.1 Determination of pH Optima of the Protease Activities Investigated Using Crude Culture Supernatants

The choice of which assay procedures to be used for the quantification of protease activity in culture supernatants was determined by what had been used by previous workers investigating B. thuringiensis proteases, and which types of proteases were expected on the basis of what had been reported for the bacilli in general by Maurizi and Switzer (1980).

3.5.1.1 pH optimum of caseinase activity

The digestion of casein was chosen as a widely reported, general purpose assay which could be used in a number of applications in the slightly acid to highly alkaline pH range. The substrate was cheap, readily available and the assay was easily performed. In addition, the comparison of the A280 of the acid-soluble digestion products with a standard tyrosine curve meant that the procedure had a basis on which to make activity comparisons on a day to day basis.

The pH optimum of crude supernatant protease preparations was determined for a range of broths harvested at the end of the fermentation when full lysis had occurred. A number of determinations

TABLE 3.5.1

Summary of literature reports describing proteases from *B. thuringiensis*

Bacillus thuringiensis variety	Stage of growth	Location of protease	Protease pH optimum	Molecular weight	Effective inhibitors	Reference
thuringiensis	early sporulation	intracellular	ND	23,000	PMSF, 0.5mM EDTA, 5mM	Lecadet et al., 1977
kurstaki	stationary	extracellular	7.0	37,500	orthophenanthroline, 1.0mM	Li and Yousten, 1975
galleriae	ND	extracellular	8.5	29,000	PMSF, 0.1mM pCMB, 0.1mM EDTA*	Epremyan et al., 1981 Stepanov et al., 1981
israelensis	ND	purified parasporal crystals	8.5, 10.0	ND	ND	Chilcott et al., 1981

ND: not determined. * Inhibitor concentration not stated

were made and Figure 3.5.1 shows a typical pH profile derived using the Dul. 52.1.72 sample (See 2.3.6.1 for section on sample labelling). The result in this figure showed, as did the result in all other determinations a pH optimum of 8.0.

3.5.1.2 pH optimum of the azocaseinase activity

The reasons for using this assay were largely the same as those for using the casein digestion technique. However, in the absence of a standard proteinase preparation, or product standard curve the method had no inherent device for comparison of assays on a day to day basis. Despite this, activity values obtained for given samples using this method were found to be acceptably reproducible from assay to assay (data not shown). The main incentive for using this method was for the rapid estimation of fractions from the gel filtration experiment (3.5.5.2) by using microlitre quantities of sample.

The pH optimum of a crude culture supernatant from SM.51.2.72 was determined on three occasions and the combined results are shown in Figure 3.5.2. As with the casein digestion assay the pH optimum for this activity was 8.0.

3.5.1.3 pH optimum of the leucine p-nitroanilidase (leunase) activity.

This activity used a cheap, readily available substrate and had been reported to occur in association with the crytals of leptox strains of B. thuringiensis (Chestukhina et al., 1980). In contrast to the two assays described above, this substrate is specifically hydrolysed by aminopeptidases (Pfleiderer, 1970) and results obtained using it would allow more concrete conclusions to be drawn as to the type of enzyme present.

FIGURE 3.5.1

pH optimum of the caseinase activity in a crude culture supernatant from the Dul.52.1.72 broth.

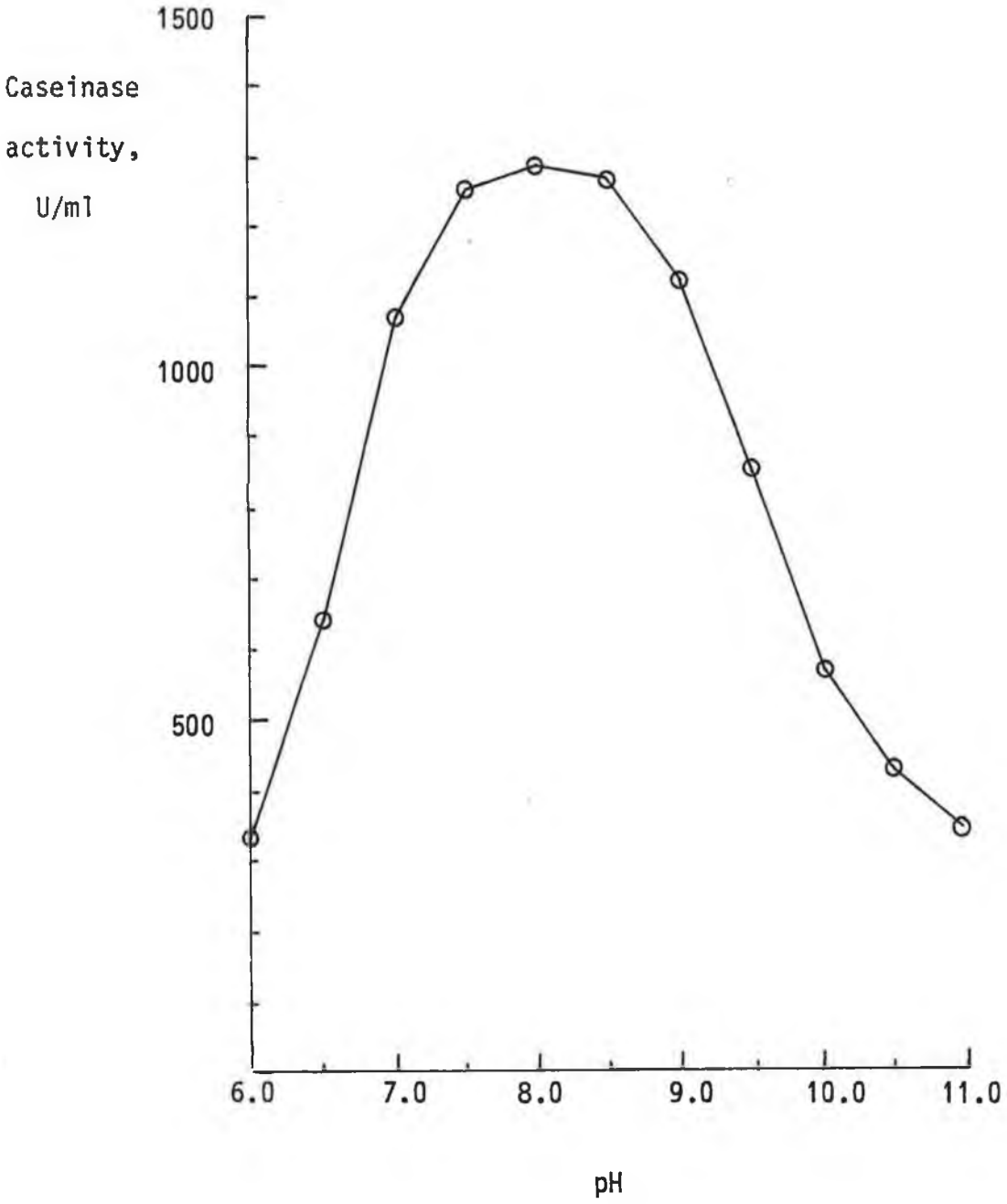
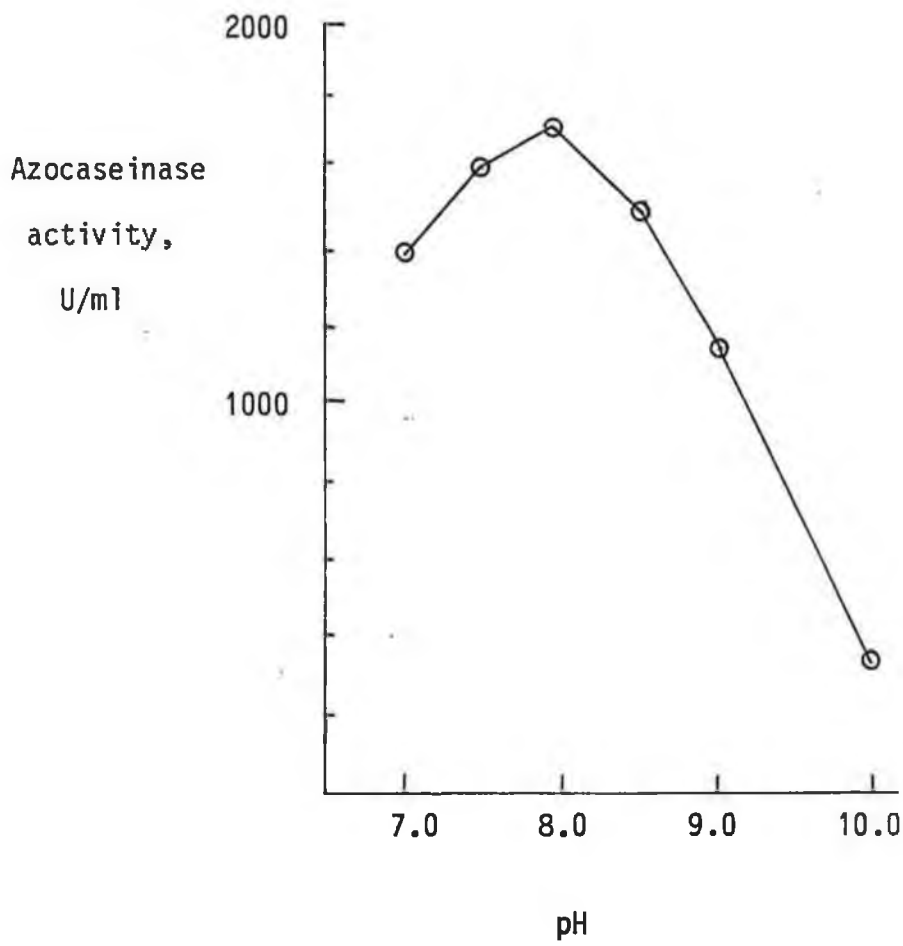


FIGURE 3.5.2

pH optimum of the azocaseinase activity in a crude culture supernatant from the Dul.52.1.72 broth.



The pH optimum of the same culture supernatant as had been used to determine the casein digestion optimum, Dul 52.1.72, was examined by assaying between pH7.0 and pH11.0, as described in 2.6.3. The results of the determinations are present in Figure 3.5.3. and indicate maximal activity at pH 10.25.

This pH optimum was clearly different from that observed for the two protein-digestion protease assays. Although variation in pH optimum for a single enzyme with different substrates has been previously reported for the subtilisins (Ottesen and Svendsen, 1970), it was felt that the significant difference in these pH optima suggested the presence of more than one proteinase.

3.5.2 Effect of Protease Inhibitors on the Casein Digestion and Leunase Activities.

The effect of a range of protease inhibitors on casein digestion and leunase activities of crude supernatants from cultures of B. thuringiensis var israelensis were investigated. The results are presented in Table 3.5.2.

It was found that the inhibition patterns for the two activities were dissimilar both qualitatively and quantitatively. A strong inhibition of both acitivities by EDTA was the most significant overlap in the results obtained, which suggested that a divalent metal ion was required either for enzyme activity and/or stability.

Phenylmethylsulphonyl flouride (PMSF) inhibited only the casein digestion activity, but at a higher concentration than was found to cause inhibition by other workers. Stepanov et al. (1981), using a purified enzyme preparation, found that their material was totally inhibited using 0.1 mM PMSF, when assaysed against a synthetic

FIGURE 3.5.3

pH optimum of the leunase activity in a crude culture supernatant from the Dul.52.1.72 broth.

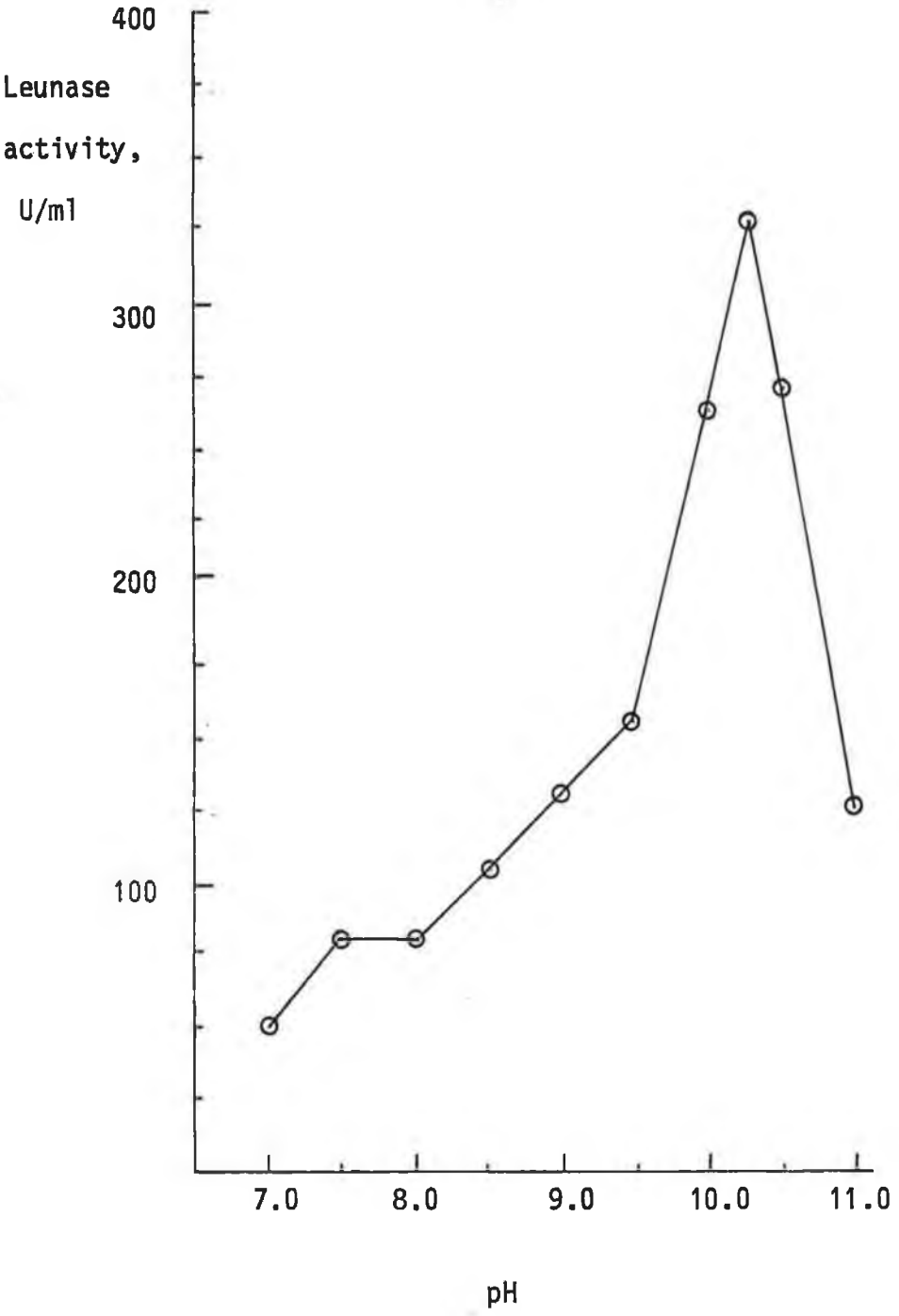


TABLE 3.5.2

Effect of inhibitors on the caseinase and leunase activities of a crude protease preparation.

INHIBITOR	ENZYME-TYPE INHIBITED	CONCN. mM	RESIDUAL ACTIVITY (%)	
			CASEINASE	LEUNASE
EDTA	Neutral metallo-proteases	0.02	50	ND
		0.15	12	ND
		1.00	9	2
		5.00	9	2
		10.00	ND	2
PMSF	Serine alkaline proteases	0.50	85	ND
		1.00	65	100
		5.00	2	100
pCMB	Sulphydryl proteases	0.15	85	ND
		0.80	ND	20
		4.20	ND	3
pHMB		0.15	90	ND
		0.80	ND	20
		1.00	196	ND
		4.20	ND	3

ND: not determined. A single protease preparation was used throughout, Dul.52.1.72, where 95% lysis had occurred. Inhibitor concentrations refer to those in the final reaction volume.

substrate specific for serine proteinases.

Inhibitors of sulphydryl proteases, p-chloromercuribenzoic acid (pCMB) and its sodium salt p-hydroxymercuribenzoate (pHMB) gave markedly differing effects on the two activities. Strong inhibition was observed on the leunase activity, while low levels of inhibition to strong stimulation of the casein digestion activity occured.

Chestukhina et al. (1980) found that leunase activity in association with parasporal crystals was inhibited by EDTA, but these workers did not investigate the effect of sulphydryl enzyme inhibitors. However, Stepanov et al. (1981) purified an extracellular protease from culture broths of B. thuringiensis and found it was inhibited by both PMSF and pCMB. On the basis of this evdience, and of another report which described similar inhibition patterns for a protease from Streptomyces rectus (Mizusawa and Yoshida, 1976), Stepanov and coworkers proposed the recognition of a subfamily of microbial serine proteinases which were also sensitive to sulphydryl inhibitors.

The results obtained here do not support the assertions of Stepanor et al. (1981) in that neither activity was inhibited strongly by both PMSF and pCMB. However, the results of this investigation were obtained using a crude culture supernatant which probably contained a number of proteinases. Therefore, the two sets of data are not necessarily directly comparable; nonetheless no evidence was obtained to support the notion of the Soviet group on the basis of the most easily detected protease activities.

3.5.3 Effect of Dialysis and Divalent Metal Ions on the Casein Digestion and Leunase Activities.

It was decided to investigate tne effect of metals on the protease activities to obtain additional data in an attempt to determine the number and types of proteinases present in supernatants from cultures of B. thuringiensis var israelensis.

As a preliminary step to investigating the effect of metals on the protease activities the effect of dialysis was first studied. This was done because it would be necessary to use materials in which as much excess metal had been removed so that more concrete conclusions could be drawn from the results of how metal ions altered the protease activities.

3.5.3.1 Effect of dialysis on the protease activities

During this series of experiments a cell-free supernatant was prepared from SM.51.1.72, a sample in which full sporulation and lysis was complete. The supernatant was dialysed as described in 2.8.2, against 100 volumes of dialysis buffer (10mM NaCl, 50 mM Tris, 0.04 mg/ml NaN_3, pH 8.0) containing a) no additive, or b) 1mM Ca^{++}, or c) 1mM EDTA. Residual caseinase and leunase activities were determined and the results presented in Table 3.5.3.

The most striking feature of these results was the strong inhibition of both activities when dialysed against 1mM EDTA. Dilutions of 1:50 were used to assay these materials which meant that the EDTA concentration was 0.02 mM in the diluted enzyme solutions, and even less in the final reaction volumes. Thus, the levels of inhibition observed in the EDTA-dialysed materials were not due to residual inhibitor.

TABLE 3.5.3

Effect of dialysis on caseinase and leunase activities in a crude culture supernatant.

MATERIAL	RESIDUAL CASEINASE ACTIVITY (%)		RESIDUAL LEUNASE ACTIVITY (%)
	A	B	
Undialysed	100	92	100
Dialysed Against 0 Ca^{++}	92	75	120
Dialysed Against 1mM Ca^{++}	102	ND	115
Dialysed Against 1mM EDTA	5	5	12

A: Protease diluted for assay in buffer containing 1mM Ca^{++}

B: Protease diluted for assay in metal-free buffer

All results obtained using SM.51.1.72

Dialysis in the presence of 1mM Ca^{++} caused no loss to either activity, and in the leunase case, a slight increase in residual activity was observed. This slight increase in the leunase activity was also noted after dialysis against buffer containing no added calcium, and may, therefore, have been due to removal of a low molecular weight inhibitory substance. Caseinase activity was slightly decreased on dialysis against the unsupplemented buffer. This activity reduction was worsened when the dialysed enzyme was diluted in metal-free buffer for activity determination, Table 3.5.3. As was mentioned above, leunase activity was actually increased by dialysis in the presence and absence of calcium. This difference in the response of the two activities to dialysis in the absence of calcium again suggested the possibility of more than one proteinase being present.

3.5.3.2 Effect of metals on the protease activities

Using a culture supernatant preparation which had been dialysed against 10mM NaCl, 50 mM Tris, 0.4 mg/ml NaN_3, pH 8.0 the effect of divalent metal ions on the caseinase and leunase activities was studied. The supernatant had been prepared from SM.51.1.72 and was assayed as described in 2.6.1 using only chloride salts of the metal ions. The results of this study are presented in Table 3.5.4.

As with the protease inhibitors, the response of the two protease activities was found to differ significantly for most of the metals tested. Only in the case of Mg^{++} were both activities found to be largely unaffected at the three metal concentrations used. Calcium ions tended to give a slight stimulatory effect to the caseinase activity, as did the highest magnesium concentration. No other metal stimulated either activity although Mn^{++} had no effect whatsoever on

TABLE 3.5.4

Effect of metal ions on protease activities of a culture supernatant dialysed against metal-free buffer.

METAL	CONCN. uM	RESIDUAL ACTIVITY, %	
		CASEIN DIGESTION	LEUNASE
Ca^{++}	1000	123	55
	500	110	88
	100	104	
	96		
Co^{++}	1000	70	26
	500	65	64
	100	85	35
Cu^{++}	1000	20	3
	500	40	0
	100	88	3
Mg^{++}	1000	120	92
	500	100	85
	100	100	95
Mn^{++}	1000	100	0
	500	100	0
	100	100	48
Zn^{++}	1000	50	85
	500	66	71
	100	100	75

Chloride salts of metals used. Minerals added to substrate solution before addition of enzymes diluted in metal-free buffer. Metal concentrations refer to final reaction volume. 100% value was the activity of undialysed material.

the casein digestion. Cobalt, copper and zinc ions tended to cause a decrease in the residual caseinase activity with increasing metal concentration, but all levels of these metals, except 100 uM Zn^{++}, gave measurable inhibition of the digestion of casein.

In general, the leunase activity was more strongly inhibited by the presence of the metal ions. Each metal at the three concentrations used, except for Mg^{++}, caused marked inhibition of this activity. This was especially apparent for copper and manganse ions.

In most cases the degree of inhibition increased with increasing metal concentration, except for 100 uM Co^{++} where residual activity was only about half that of the 500 uM level. The significant difference in the response of the two activities studied provided further evidence to suggest the presence of at least two proteinases.

3.5.3.3 An attempt to restore activity to an EDTA - dialysed broth.

It has already been mentioned that dialysis of a culture supernatant against ImM EDTA caused significant loss of both casein and leucine p-nitroanilide hydrolysis activities, 3.5.3.1. Such a preparation was assayed, in the presence of various metal ions at concentrations significantly greater than that of the inhibitor in the diluted enzyme solution, to determine if the activity loss caused by EDTA could be reversed. The results of these emperiments using the supernatant from SM.51.1.72 are presented in Table 3.5.5.

The data obtained during the investigation showed that neither activity was restored using any of the six metals beyond approximately 25% of the original levels.

TABLE 3.5.5

Investigations into restoration of activity of a culture supernatant dialysed against 1mM EDTA.

METAL SUPPLEMENT	RESIDUAL CASEINASE ACTIVITY (%) FOR METAL CONCENTRATION, uM			RESIDUAL LEUNASE ACTIVITY (%) USING 500uM METAL
	100	500	1000	
Ca^{++}	-	-	5	7
Co^{++}	27	20	7	7
Cu^{++}	-	-	7	0
Mg^{++}	-	-	5	5
Mn^{++}	-	-	5	0
Zn^{++}	22	27	8	21

Culture supernatant used was SM.51.1.72. Activity of EDTA-dialysed materials compared to broth dialysed in absence of added calcium, following adjustment to account for sample dilution during dialysis. Enzyme was diluted in buffer containing metal at a concentration such that when the enzyme and substrate were mixed, the resulting metal concentration was as quoted here. The dialysed broth was diluted 1:50 in the relevant metal-containing buffer for assay.

These results demonstrate that proteolytic activity appears to be irreversibly destroyed by EDTA.

The results suggest that a tightly bound divalent cation is required for enzyme stability and/or activity. Removal of this cation by dialysis against EDTA irreversibly destroyed both activities. The bound cation was not removed by dialysis against dialysis buffer resulting in the rentention of the activity of the enzyme/s.

3.5.4 Protease Production in Fermentation Cultures

During a number of fermentation runs, the production of protease activity in flask and fermentation cultures was followed. This was done in order to check if the protease pattern could be related to the sporulation of the organism as had been done for other bacilli (Maurizi and Switzer, 1980).

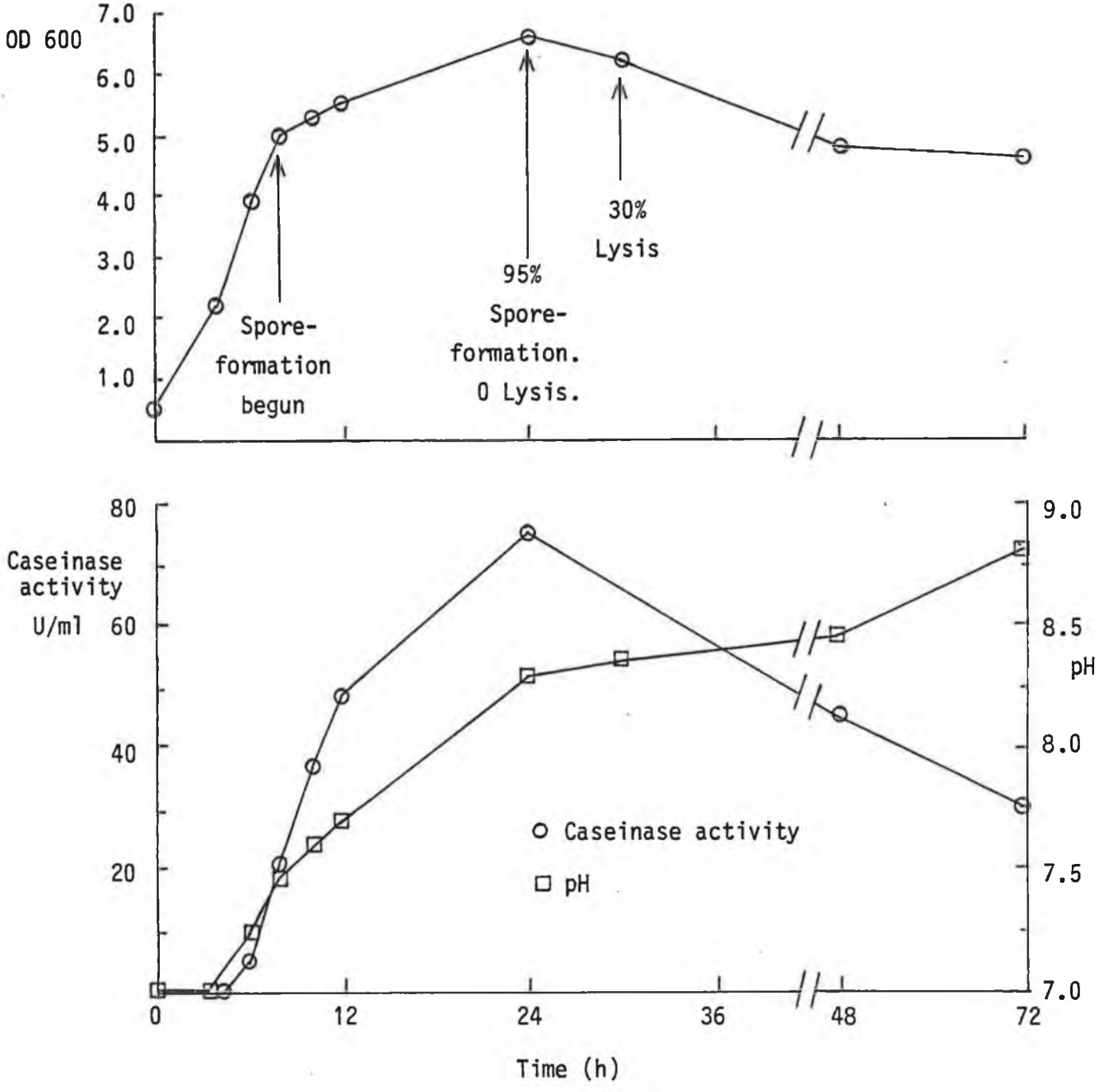
3.5.4.1 Extracellular protease levels during growth of B. thuringiensis var israelensis

In the first experiment, caseinase activity was followed in cell-free supernatants from shake flask cultures using the medium of Li and Yousten (1975). 200 ml of medium per 2l flask were inoculated with a 5% volume of the casein inoculum culture prepared under standard conditions. The production flasks were then incubated as recommended in 2.3.1. During growth of the organism OD 600, pH, Caseinase activity, and sporulation efficiencies were determined. The results for this run are presented in Figure 3.5.4.

It should be noted that during vegetative growth detectable protease activity was observed, and during this stage while cell density

FIGURE 3.5.4

Patterns of growth, pH, sporulation and protease production during flask culturing of B. thuringiensis in the Li and Yousten (1975) medium.



The organism was cultured in 2l flasks containing 200ml medium under standard conditions of inoculation and incubation.

increased, so too, did the medium pH and the caseinase activity. This increase continued till about 95% of the cells had formed spores, whereupon, the proteinase activity dropped sharply and continued to do so for the remainder of the culturing period, Figure 3.5.4.

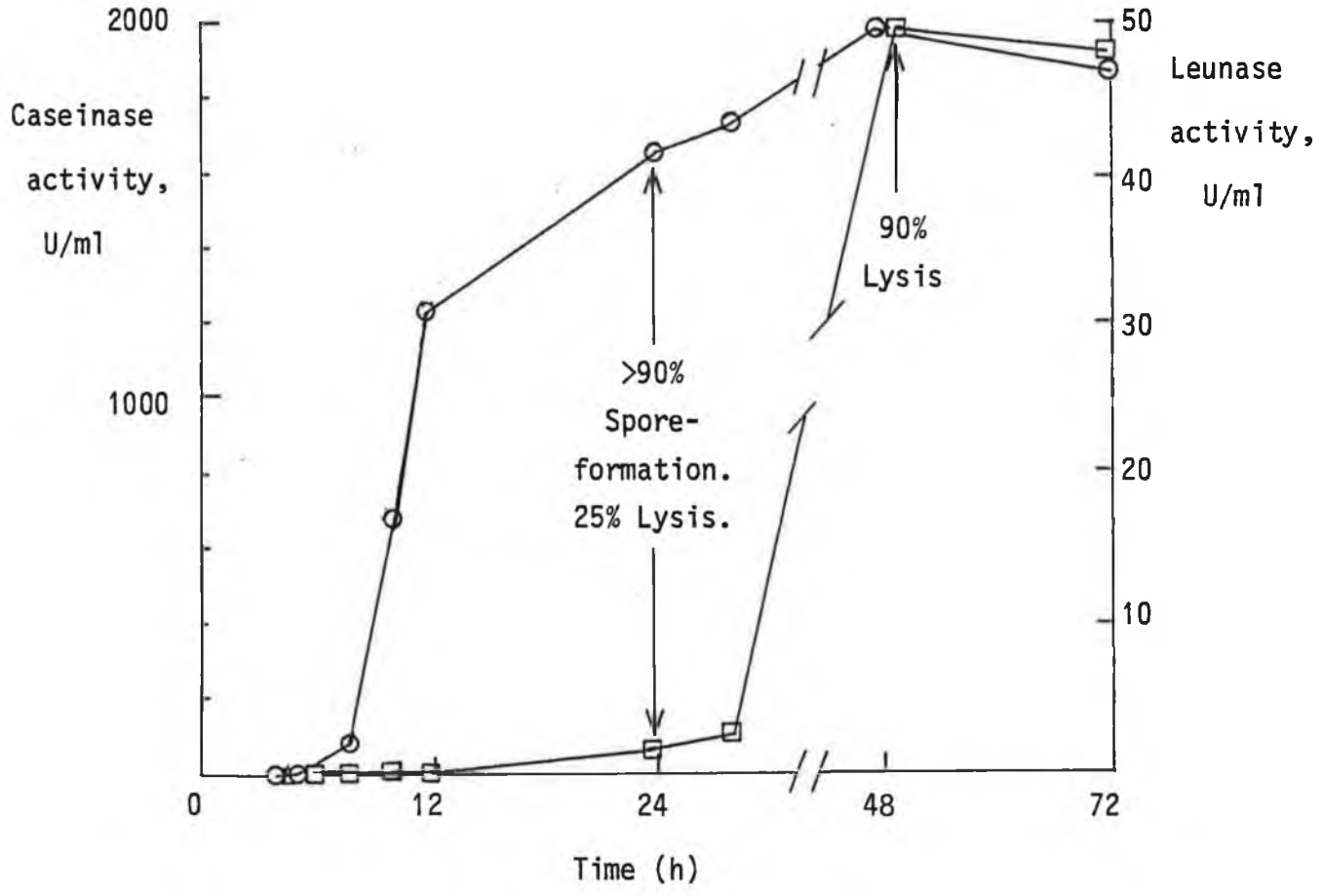
Caseinase and leunase activities were also followed in a Microgen fermenter culture, and in a Pilot- fermenter broth using the SM medium. The experimental conditions used during these runs were described in 3.1.3.2, 3. The results for these fermentations are presented in Figures 3.5.5. and 3.5.6.

The patterns of protease production during both these fermentations were broadly similar. Significant levels of extracellular caseinase activity were not detected till about 4-6 hours after inoculation. Thereafter, caseinase activity increased gradually till the onset of sporulation at about 12-14 hours into the fermentation where a plateau level was reached. This plateau level was maintained till a significant level of lysis occurred which caused a marked increase in the caseinase activity. Caseinase activity reached maximal levels at about t48 when lysis was complete, and following this began to decline slowly, Figure 3.5.5. By contrast, significant levels of leunase activity were not detected in cell-free supernatants till after 24 hours in the fermenter cultures. After this, leunase activity increased and reached a maximum value at t48. There would, therefore, appear to be a correlation between the appearance of leunase activity and cell lysis, suggesting that the leunase enzyme is intracellular.

In both the flask and fermenter runs, extracellular caseinase activity was observed before phase-bright spores were visible. Thus the presence of the enzyme could not be used as an event marker for the commencement of sporeformation. However, it did appear that two phases of caseinase activity production took place. If this was due to

FIGURE 3.5.5

Pattern of protease production during growth of B. thuringiensis in the SM medium in a 10l Microgen fermenter culture.

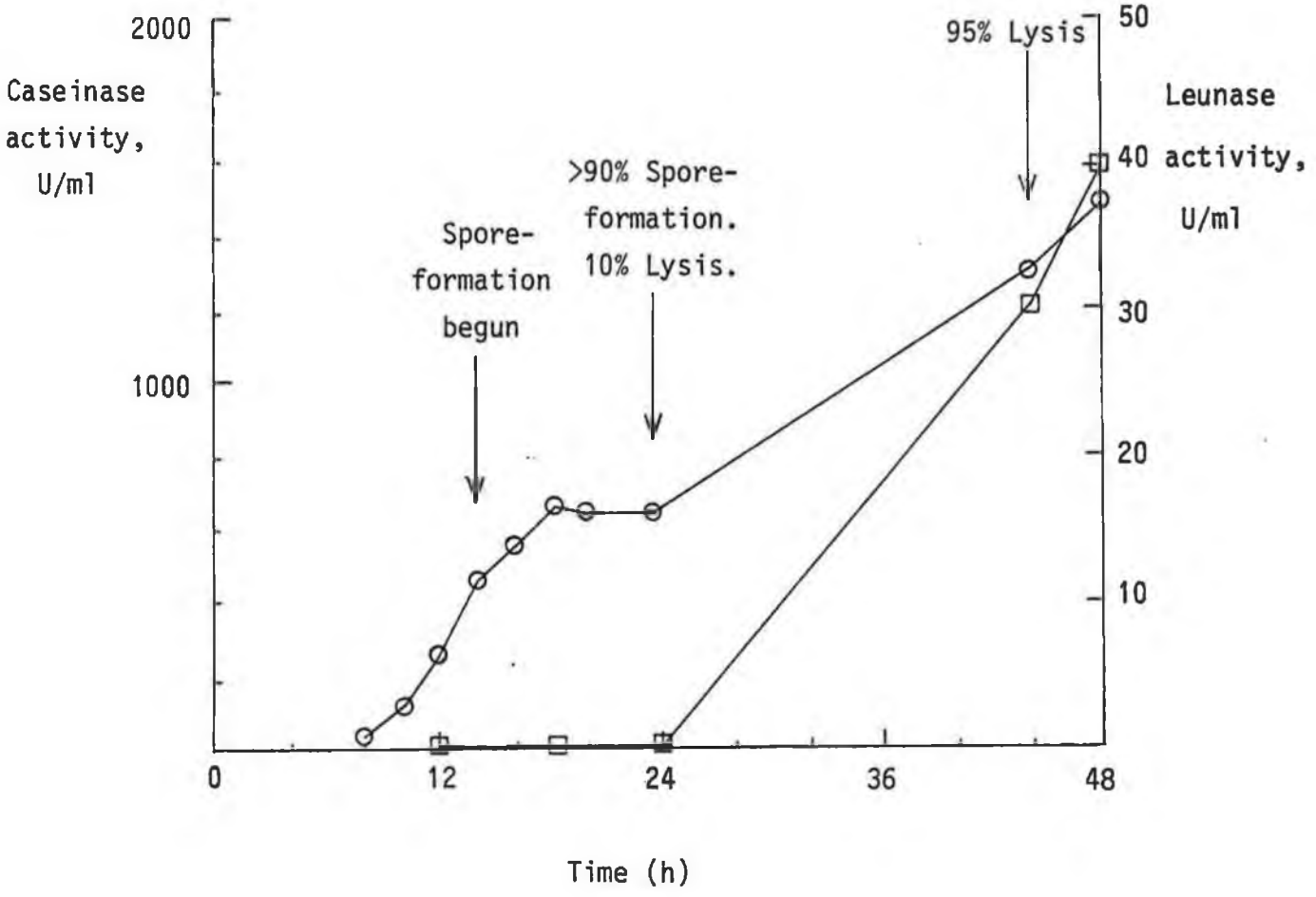


○ Caseinase activity ; □ Leunase activity.

The organism was cultured under standard conditions of inoculation and incubation.

FIGURE 3.5.6

Pattern of protease production during growth of B. thuringiensis in the SM medium in a pilot fermenter culture.



○ Caseinase activity ; □ Leunase activity.

the synthesis of two different enzymes, and if the two enzymes could be readily distinguished, then caseinase assays under distinguishing conditions could possibly be used as indicators of the fermentation progress.

The appearance of leunase activity in the cell-free supernatant following cell lysis could also be used as an indicator of the state of the culture. It is not yet known if the same enzyme is responsible for the increase in caseinase and leunase activites following lysis, but the evidence presented in 3.5.2 and 3.5.3 would suggest that more than one enzyme is involved.

Thedifferent observations on protease production following cell lysis in the two media used during this study suggest that the patterns of enzyme production by B. thuringiensis var israelensis may vary, depending on the properties of the medium in which it is cultured.

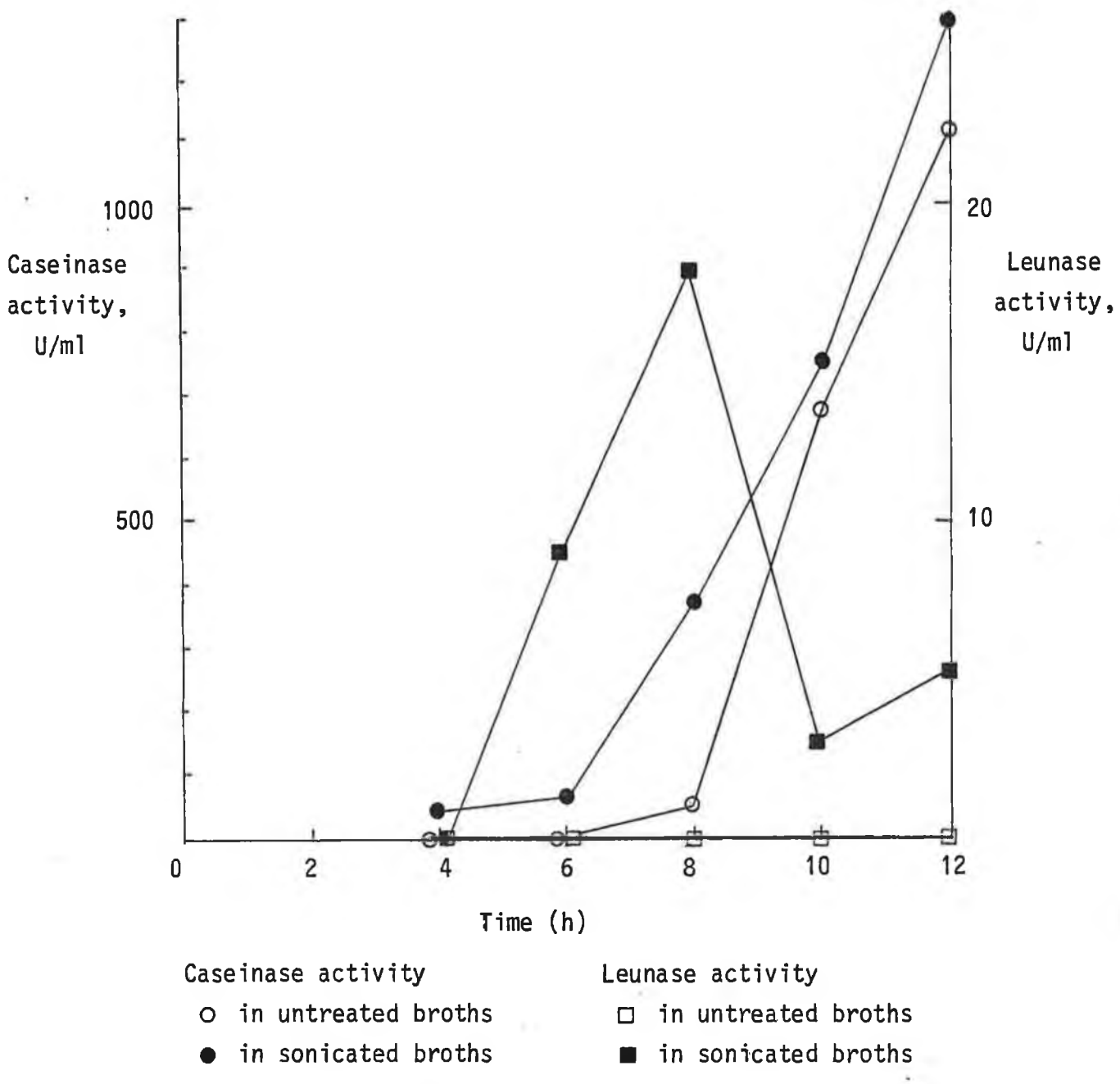
3.5.4.2 The effect of sonication on protease patterns during the early stages of growth of B. thuringiensis in a fermenter culture.

During the culturing of B. thuringiensis in the Microgen fermenter described in 3.5.4.1, aliquots of samples taken during the first 12 hours of growth were sonicated in an ice bath using an Ultrasonics sonicator to cause at least 95% cell disintegration. The sonicated samples were then centrifuged and the leunase and caseinase activities of the supernatants determined. These protease levels were taken to be equivalent to the total of the extracellular and intracellular pools produced in the cultures. The results of this investigation are presented in Figure 3.5.7.

It was shown that the protease levels in the sonicated broths were higher than in the untreated samples. This was not unexpected, as

FIGURE 3.5.7

Comparison of extracellular + intracellular protease with exclusively extracellular levels during the first 12 hours of a 10l Microgen fermenter culture of B. thuringiensis.



Aliquots of samples taken were centrifuged directly to produce cell-free supernatants containing only extracellular protease. The other sample aliquots were sonicated to cause >95% cell disruption before being centrifuged, and were then assumed to contain intracellular + extracellular proteases. No cell lysis occurred during the first 12 hours of the fermentation.

distinct pools in the intracellular and extracellular compartments have been previously reported in bacilli (Maurizi and Switzer, 1980). The enzymes comprising these two pools have been speculated to be the products of different genes (Maurizi and Switzer, 1980), and thus sonicated samples should be expected to contain at least two different proteases.

Of considerable interest was the detection of a peak of leunase activity in the sonicated materials while in the untreated samples leunase activity was absent. This peak in activity occurred before spores could be microscopically detected but would have roughly corresponded with the time at which vegetative growth was slowing down, and the cells were preparing to enter the sporulation phase. This is based on the assumption that refractile spores are not microscopically detectable till approximately 2-4 hours after completion of Stage I in the sporeformation process (Bulla et al., 1980).

This leunase activity peak may be an interesting physiological marker, as its appearance is relatively transient and is very possibly linked to the sporulation of the organism.

3.5.5 Purification and Characterization of the Protease Activities from a Crude Supernatant from a B. thuringiensis Culture

A culture supernatant from SM.51.1.72 was used as the primary enzyme source for investigating the number and types of proteases which could be described in such a material. This type of study was undertaken as an initial step in examining the proteinases from B. thuringiensis. It has already been mentioned that proteinases produced by the organism may be responsible for the decrease in bioactivity in the

fermenter (3.5). Apart from this, the role of proteinases in bacterial sporulation has been extensively investigated over the years (Doi, 1972; Holzer et al., 1975; Maurizi and Switzer, 1980) and the reports pertaining to B. thuringiensis have been few in number. Furthermore, these reports have not presented a consistent picture of the number and types of proteinases produced by this organism, in that different reports have described the presence of a number of enzyme types, but each individual group has not provided collaborative evidence for the findings of other workers. Therefore, this investigation was carried out with a view to obtaining a greater understanding of the number and types of proteinases produced by this organism, as well partially characterizing them.

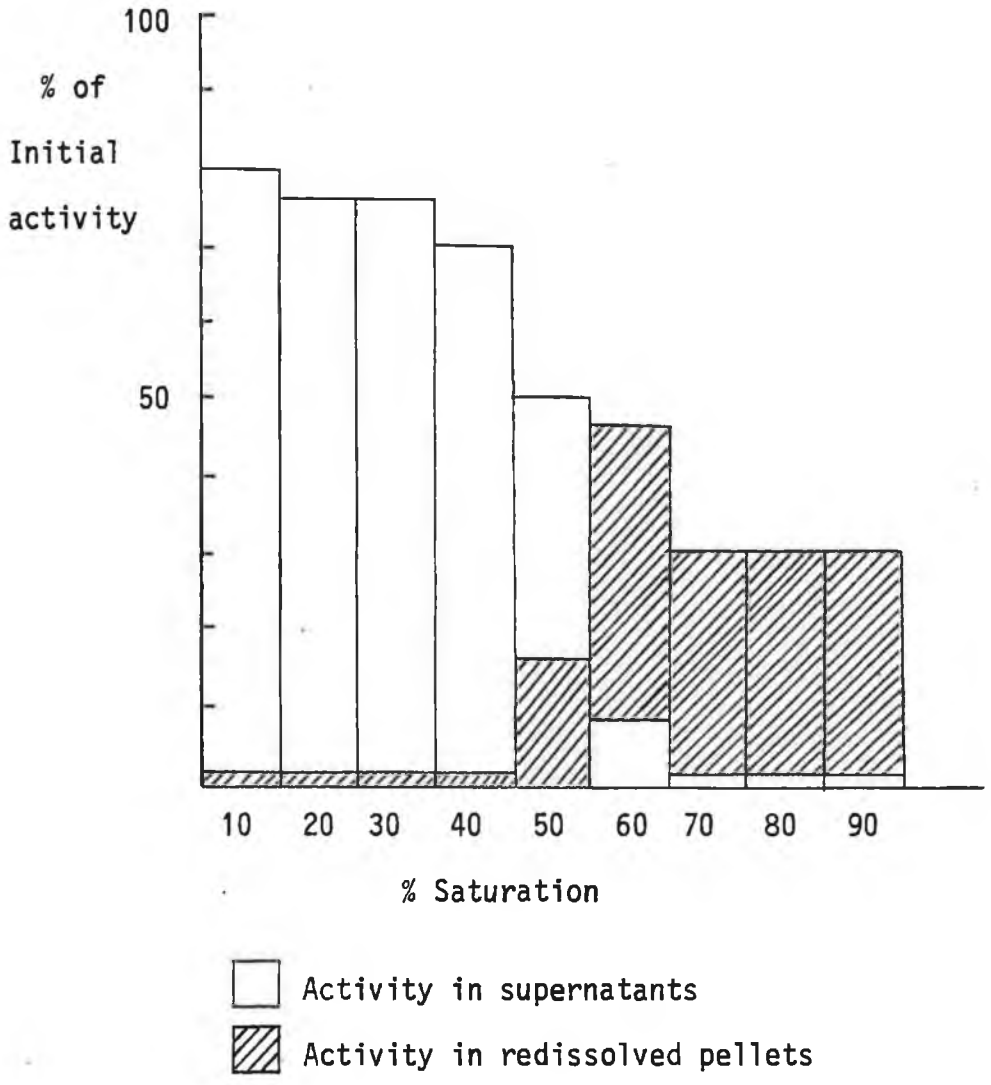
3.5.5.1 Ammonium sulphate fractionation

The supernatant from SM.51.2.72 was used to examine the precipitation of proteolytic activity by ammonium sulphate. This was the same material as had been used to investigate the effect of inhibitors, metal ions, and dialysis on proteinase activity, and therefore the experimental conditions used to produce the sample are described elsewhere. This precipitation study was made with a view to preparing a semi-purified protease sample which could be used in a gel filtration experiment.

To determine at which ammonium sulphate concentrations protease activity was precipitated, a number of supernatant samples were brought to a range of salt concentrations as described in 2.8.1. Following redissolution of the pellets, and dialysis of both pellets and supernatants, the caseinase activity of all fractions was determined. The results obtained are presented in Figure 3.5.8.

FIGURE 3.5.8

Precipitation of caseinase activity from a cell-free supernatant using ammonium sulphate.



This showed that little protease activity was precipitated at up to 40% ammonium sulphate saturation. However, up to 30% of the initial activity was lost at 40% saturation. Precipitation of the caseinase activity was highest at 60% salt saturation with higher salt levels causing considerable activity-loss. These data indicated that 60% ammonium sulphate most efficiently precipitated the caseinase activity. However, it should also be noted that significant denaturation of the enzyme occurred during the precipitation process which meant that recovered yields of the enzyme from the broth were low.

A further problem encountered during the ASF procedure was the precipitation of a viscous, spongy material at salt concentrations as low as 30% saturation. At higher concentrations a considerable amount of this material appeared in the pellets, so much so that the pellets were very difficult to redissolve. When they were redissolved, the solutions were so viscous that after storage at $4^{o}C$ for a few hours they could be turned upsidedown without being dispensed from the tube. Clearly, such a solution could not be successfully used in a gel filtration experiment. A series of investigations was therefore carried out to eliminate this material, whose molecular nature was unknown.

The use of protease, amylase and DNase preparations failed to achieve a significant reduction in the viscosity of the redissolved 0-60% pellets. However, when the broth was brought to 45% ammonium sulphate saturation, the pellet removed by centrifugation and discarded, and the supernatant adjusted to 60% salt saturation, the final 45-60% pellet was much smaller, and much easier redissolved than the 0-60% pellet had been.

This differential ASF procedure was found to conveniently eliminate

the viscous material, but it was also found that the yields of protease were poorer than the single step technique. By the differential precipitation only 10% of the original protease activity was recovered, which was significantly less than the 45% observed in the one-step process. However, an improvement of almost double the specific activity was obtained with the two-step precipitation over the one-step method, Table 3.5.6. This difference in yields may have been due to the prolonged exposure to ammonium sulphate, as two overnight precipitation steps were required in the two-step process, as opposed to the single overnight precipitation used in the one-step technique.

Having successfully resolved the sample viscosity problem, a protease preparation which had been produced by the differential ASF method was used for the gel filtration experiment.

3.5.5.2 Purification of the protease activities by gel filtration

A partial purification and increase in the specific activity of the caseinase activity by ammonium sulphate fractionation was achieved earlier, 3.5.5.1. Using this differential fractionation technique a further purification of a redissolved 45-60% pellet was attempted by gel filtration using Sephadex G-100. This was done in order to further purify the protease activity to determine

1) the number of proteases present and their pH optima
2) whether the caseinase and leunase activities were catalysed by the same, or a number of different enzymes.
3) the molecular weights of the proteases present.

A 45-60% ammonium sulphate pellet was prepared as described in 3.5.5.1

TABLE 3.5.6

Comparison of Specific Activities of 60% Ammonium Sulphate Pellets prepared by one-step and two-step procedures.

Precipitation Procedure	Specific Activity (Caseinase units/mg protein)	
	Original Broth	60% Pellet
One Step	1010	960
Two Steps	1030	1850

Supernatant from SM.S1.1.72

Protein determined by Folin-Lowry Method. Caseinase determined by standard technique. Pellets produced by either bringing supernatant directly to 60% saturation, or indirectly by first bringing it to 45% and then to 60% after removal of the 45% pellet.

from 200 ml of a supernatant from SM.51.1.72, redissolved in 15 ml protease diluting solution (1mM Ca^{++}, 10 mM NaCl, 50 mM Tris, 0.4 mg/ml NaN_3, pH 8.0), and dialysed against the same buffer. 10.0 ml of this solution was applied to a Sephadex G-100 column, 75 cm x 2.6 cm, as in 2.8.3. The column had been previously calibrated using standard molecular weight markers, and the protease was run with dextran blue and phenol red to determine the void and exclusion volumes, respectively. The A280 of the eluted material was measured and fractions of 5.0 ml collected.

The results for the molecular weight calibration of the column are presented in Figure 3.5.9 and Table 3.5.7. Figure 3.5.10 shows the elution pattern of the protease sample with respect to A280 and azocasein digestion activity.

The molecular weight calibration data for the column, when analyzed with a linear transformation programme on a Texas Instruments TI-55-II calculator, had a correlation coefficient of greater than 0.99. Thus it was concluded that there was an excellent correlation between molecular weight and elution volume for this column, under the conditions used.

When first examined at pH 8.0, the azocasein activity profile had a peak at 235 ml and a shoulder at an elution volume of 250 ml, Figure 3.5.10. In view of the fact that previous results had suggested the presence of more than one proteinase, the pH optima of two samples (Fractions 44, 54) on opposite sides of the main activity peak were determined. The results of these determinations are presented in Figure 3.5.11. From the data presented in this figure it was clear that at least two enzymes were involved, with markedly different pH optima of 8.0 and at pH 10.0. Significantly, these were the pH optima of the caseinase and leunase activities respectively.

TABLE 3.5.7

Elution volume results for standard materials used to calibrate the Sephadex G-100 column for the gel filtration of a protease preparation.

Standard Material	Molecular Weight ($x10^{-3}$)	Elution volume (ml) for gel filtration run		
		1	2	3
Dextran blue 2000	2000	111	115	114
Phenol red	0.5	450	455	454
Cytochrome c	12.4	283	280	ND
Chymotrypsinogen A	25.0	238	240	ND
Ovalbumin	45.0	ND	195	ND
Bovine serum albumin	68.0	ND	155	ND

ND: not determined

FIGURE 3.5.9

Calibration of the Sephadex G-100 column used for gel filtration of the ammonium sulphate-precipitated protease sanple.

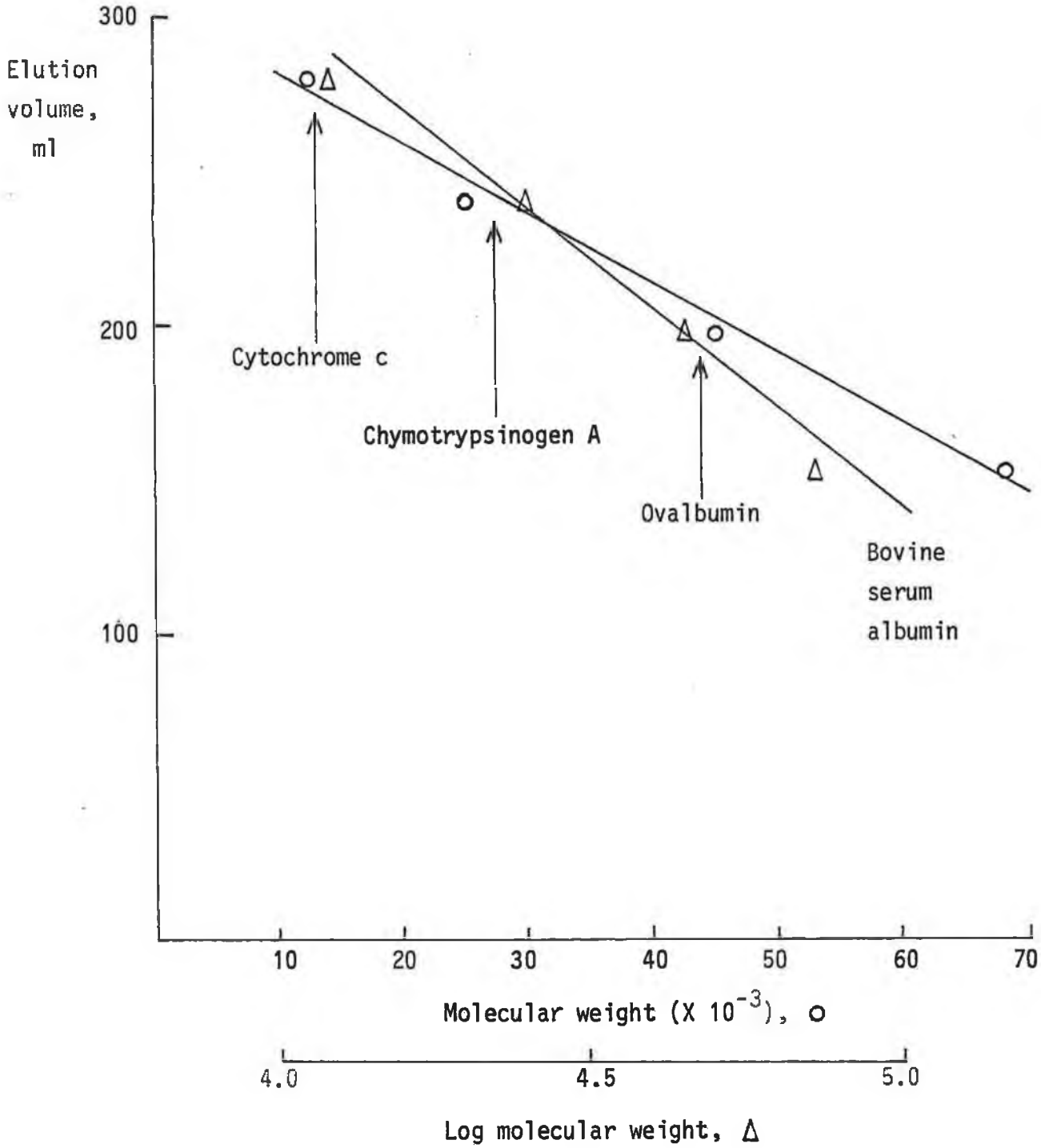
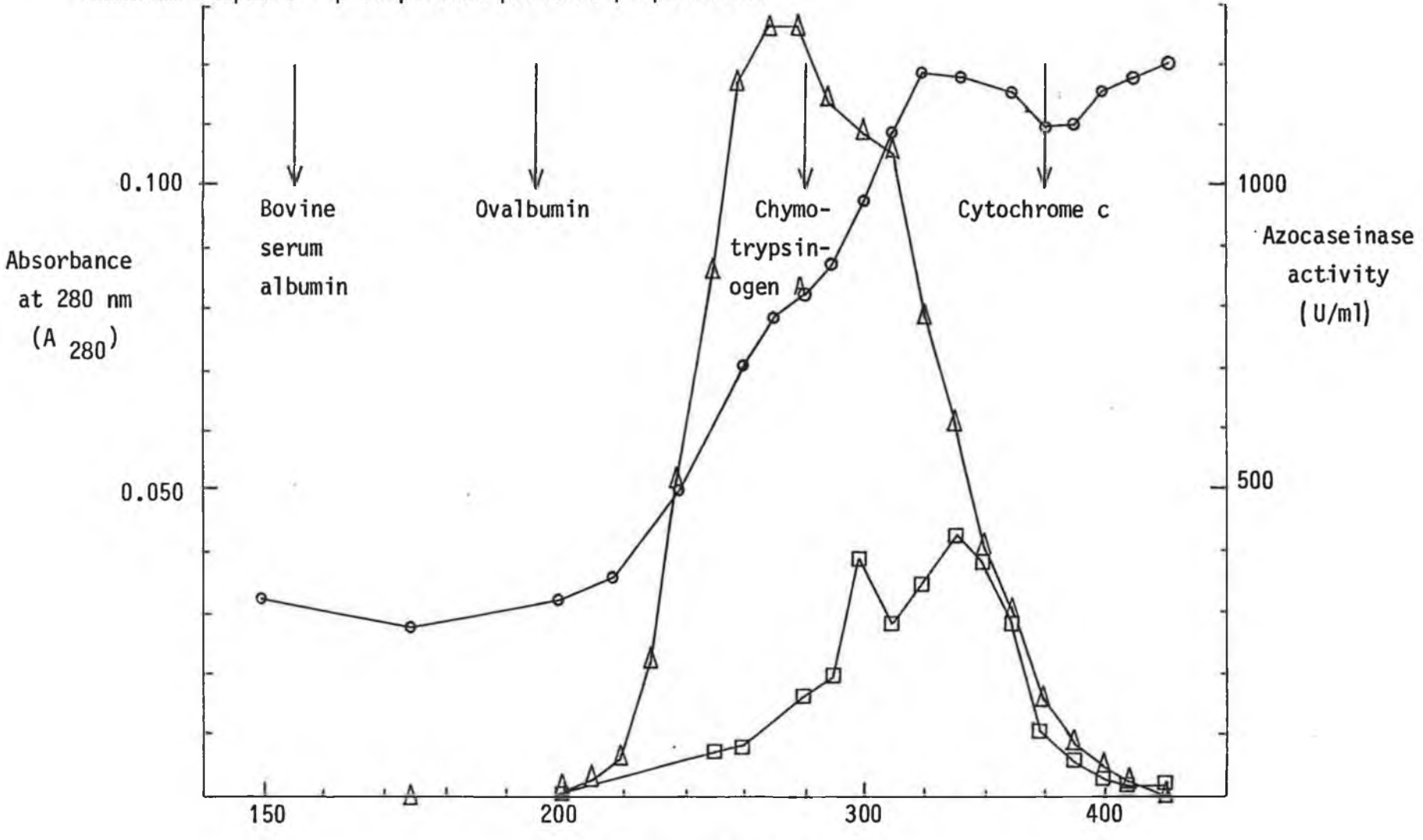


FIGURE 3.5.10

Elution pattern of the azocaseinase activity at pH8.0 and pH10.0 during the gel filtration of an ammonium sulphate - precipitated protease preparation.



Following on from this discovery, the azocasein digestion activity of fractions 40-60 were redetermined at pH 10.0, and the results of this redetermination are presented in Figure 3.5.10.

The data obtained provided evidence for the presence of at least three enzymes. One of these had a molecular weight of 29,500, was maximally active at pH 8.0, and had an elution volume of 235 ml. A second activity peak was detected at an elution volume of 265 ml corresponding to a protein of 16,100 daltons. This second enzyme was maximally active at pH 10.0, Figure 3.5.11. A third peak was detected using the azocasein assay at pH 10.0, and had an elution volume of 250ml, equivalent to a molecular weight of 22,900. However, the optimum pH of this enzyme was not determined.

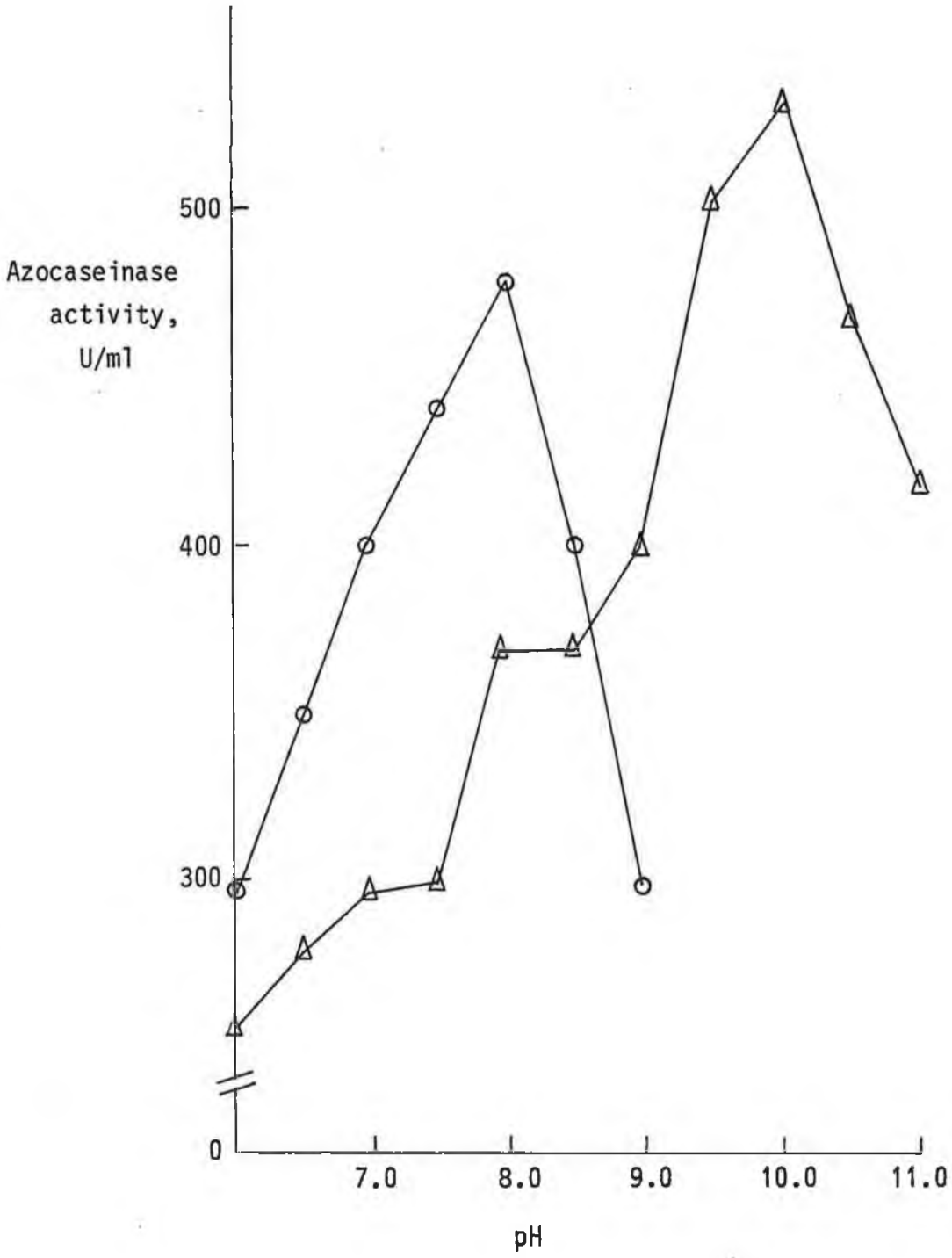
These gel filtration data verified previous results in showing the presence of a number of proteases in the supernatants of cultures of B. thuringiensis. However, due to the low gel filtration sample volume, it was not possible to test the effect of the protease inhibitors on the different fractions. This type of data would have provided further valuable information on the types of protease present.

3.5.6 Summarizing Comments on Protease Investigation

During these studies into the protease activities in culture supernatants of B. thuringiensis var israelensis two types of activity were examined. The first was the digestion of casein or azocasein and the second was the hydrolysis of the synthetic substrate leucine p-nitroanilide, which is catalysed by leucine aminopeptidases. The pH optima of these two activities were 8.0 and 10.25 respectively when crude culture supernatants were examined.

FIGURE 3.5.11

Azocaseinase pH optimum of two fractions from the gel filtration of an ammonium sulphate - precipitated protease preparation.



○ Fraction 44, elution volume 220ml

△ Fraction 54, elution volume 270ml

The production of these two protease activities during the fermentation of B. thuringiensis was determined. Evidence to suggest that the two activities were catalysed by different enzymes was obtained on the basis of effects of inhibitors and metal ions.

The protease activities were purified by ammonium sulphate precipitation and gel filtration. The gel filtration results also indicated the presence of a number of enzymes in the crude culture supernatants.

SECTION 4: DISCUSSION

The aim of the fermentation studies (3.1) was to optimize the fermentation of B. thuringiensis with respect to insecticidal activity production, cost, and fermentation time.

Preliminary studies on the effect of aeration and casein on growth and sporulation of B. thuringiensis indicated that the latter was inhibited by high levels of casein and sub optimal aeration, when the organism was cultured in the Drake and Smythe medium in flasks. When casein was omitted from this medium and aeration increased by decreasing the medium volume to flask capacity ratio, high levels of sporulation were observed without significantly affecting the levels of viable counts recorded. Similar results were obtained during flask culture experiments using the Megna medium. Using this medium, only the medium volume to flask capacity ratio was varied and it was found that low aeration rates significantly delayed the onset of sporlation without markedly affecting the viable counts. The results of this type of investigation had not been previously reported, but, nonetheless, provided important information on some of the factors likely to affect biomass and bioactivity yields in the fermenters.

Initial investigations into the development of an inoculum production process involved a comparision of the use of vegetative cells and spores as the inoculating cell types. The results of these studies revealed that in both a soluble, non commercial medium and in an industrial medium, the use of vegetative cells as the inoculating cell type enabled logarithmic growth in the culture to begin at least two hours earlier than was observed when spores were used. In addition, the use of vegetative cells gave higher sporulation efficiencies at the end of the production stage fermentation. For these reasons, it was decided that when developing an inoculum medium, one which gave a high level of biomass in which sporulation was prevented should be

selected.

A range of inoculum media with an imbalance in one nutrient source was tested and the casein inoculum medium chosen as the most suitable of those examined. A study of the growth of the organism in flasks, and later in fermenters, showed that high yields of biomass were obtained using this medium. In addition, sporulation was inhibited for at least 24 hours due to its high casein content. The optimum inoculum size used to seed the production stage media was investigated, and found to be 5% of the production medium volume, similar to inoculum levels described by other workers (Dulmage. 1970 b; Goldberg et al., 1980; Margalait et al., 1983). Growth of B. thuringiensis in this inoculum medium was found to be highly reproductible and well suited to the production of seed cultures. Furthermore, it was found during the course of the work that casein inoculum medium culture ages of 14-24 hours could be satisfactorily used in the fermentation process. This was of considerable importance in the context of an industrial fermentation where a degree of flexibility is desirable.

In order to develop a suitable medium for the production stage fermentation, a number of literature-reported media were investigated with respect to biomass production and sporulation characteristics of the organism when grown in these media. Following this,a range of variant media were studied with respect to the same parameters; in addition, considerations of cost and cycle time were taken into account during the variant media investigations, 3.1.3. The final step in this section was a study of the production of insect toxicity by B. thuringiensis in a number of media examined,3.1.4. The result of this work was the development of the SM medium which had been originally derived from the Dulmage medium, Table 3.1.6.

The SM medium represented a considerable improvement in the Dulmage medium and was also more economically attractive than all other media examined. In only one other medium formulation were similar levels of viable counts observed (Obeta and Okafor, 1984), but this was in a cycle time of 72 hours using raw materials locally available in the author's country, Nigeria. The media described by Smith (1982), and the media in Table 3.1.6 were, in all cases, inferior in cost and performance to the SM medium. As has been mentioned before, direct comparison of bioactivity levels reported in various media was not possible for a variety of reasons, 3.1.4.3. However, on the basis of analysis of other workers processes done during this study, it was possible to conclude that the process described here is at least as commercially useful, if not, considerably more so, as those previously reported.

During the course of the investigation described above a number of other points of importance to the B. thuringiensis fermentation process arose. Firstly, with respect to suitable carbon sources for the growth of B. thuringiensis, glucose and starch proved to be preferable to sucrose, Table 3.1.9. However, the combined use of starch and cane molasses (whose primary sugar component is sucrose) allowed as good growth and sporulation of the organism as a combination of starch and glucose, Table 3.1.8. However an excessive level of glucose (20 g/l) caused considerable acid production during the early fermentation and resulted in an inhibition of sporulation which has been previously reported for B. thuringiensis (Dulmage, 1981) and for other bacilli (Freese, 1981). In contrast to glucose, sucrose at levels as high as 45 g/l allowed growth and sporulation of the organism to occur in a familiar pattern, Table 3.1.7. The basis of this relative insensitivity of the organism to sucrose is unknown, but it underlies its unsuitability as a carbon source for the

fermentation of B. thuringiensis.

With regard to the nitrogen sources used during this work, a considerable number of different materials were found to be suitable. As with the carbon sources, an imbalance in the levels of nitrogenous materials severely affected the sporulation of the organism thus a high level of casein was used to ensure that cells in the inoculum medium were in a vegetative state, Table 3.1.3.4. High concentrations of yeast extract and corn steep solids were also found to give altered sporulation patterns, Table 3.1.3,4.

A variety of soya preparations were investigated with respect to their usefulness for the production of B. thuringiensis, Table 3.1.8. Of these, soya bean meal was found to give the best growth and sporulation of the organism. Soya peptone was the least useful of the soya materials in that it gave poor viable counts and delayed the onset of sporulation, Table 3.1.8. The reasons for the unsuitability of this nutrient source were unknown, but may, possibly have been due to the presence of amino acids which had a sporulation-inhibition effect and which were released during the proteolysis used to manufacture the peptone. In this regard cysteine, in the presence of glucose, has been reported to inhibit sporulation of B. thuringiensis (Rajalkashmi and Shetna, 1977).

The drop in pH during vigourous vegetative growth of B. thuringiensis described here, Figures 3.1.1, 2, 3, 4, has been reported previously (Tyrell et al., 1981; Yousten and Rogoff, 1969).

During their study on the metabolism of B. thuringiensis in relation to spore and crystal formation Yousten and Rogoff (1969) reported that this drop in pH was due to the production of acetic acid from glucose during exponential growth. These authors also stated that this acid

was metabolized during early sporulation in a tricarboxylic acid cycle - dependent process, which demonstrated the requirement for oxygen during sporulation as well as during the exponential phase. Thus it would appear that the changing pH pattern during the fermentation of B. thuringiensis reflects the physiological state of the culture and therefore represents another parameter by which to judge the progress of the fermentation.

It was shown during the investigation into the relationship between viable count and bioactivity that the former was not an absolute indicator of the levels of the latter, 3.1.4.4. However, it was noted that, in general, the media which produced the highest amounts of biomass also tended to be the best producers of insect toxin. This relationship was used, to a certain extent, to judge which media were likely to be most useful, and would obviously be an important parameter to monitor in future studies on further improving the process.

Notwithstanding the lack of absolute agreement between viable counts and levels of bioactivity in the fermentation broth, it was firmly established that the time at which the highest bioactivity was observed was that at which maximal levels of sporangial lysis occurred, Figures 3.1.5 - 12. This correlation was extremely important as it allowed a rapid, reliable method for the determination of the time of maximum bioactivity to be used. This is important to large scale fermentation as it enables the time at which to harvest the culture to be easily established.

The occurance of strain deterioration with storage and/or subculturing provided an important warning as to the inherent genetic instability of the organism when maintained under unfavourable conditions. This

conclusion is supported by the frequent reports describing spontaneous mutation of B. thuringiensis in the laboratory, where these mutations led to the isolation of acrystalliferous strains (Gonzalez et al., 1981; Faust et al., 1983; Kamdar and Jayaraman, 1983; Gonzalez and Carlton 1984). Thus, for continued maintenance of this organism in the laboratory, frequent and rigourous checks must be made to ensure that the strain under use retains its original phenotype.

During the scale-up investigations, B. thuringiensis results obtained in flasks were successfully transferred to fermenters for growth of both the inoculum and production stages, 3.1.5. The classsical scale up problems of inoculum production, medium sterilization, and culture mixing/aeration (Banks, 1979, Lilly, 1983) did not not impose limitations on the fermentation at the large scale level. This represented another major attraction of the process in that this step in its development was easily achieved where other processes have proved difficult to develop during the same stage.

Direct comparison of the cost effectiveness of this process with that of other manufacturers is, for obvious reasons, impossible. However, a number of generalizations and assumptions can be made in order to draw some conclusions. Teknar, a flowable formulation marketed by Sandoz Inc., U.S.A. costs approximately $6 per litre and contains 6 x 10^8 ITU in the same volume, and therefore sells at $600 per 10^{10} ITU. Table 3.1.14 show that the SM medium produces 10^{10} ITU at a cost of approximately $0.50 (assuming parity between the American dollar and Irish pound). Assuming that medium costs account for about 50% of the total manufacturing costs (Stowell and Bateson, 1983) this would mean that the overall fermentation price is about $1 per 10^{10} ITU. Additional expenses in the production of B. thuringiensis include those associated with formulation and packaging, and the substantial

contribution of plant, labour and quality control overheads. Thus, outside these expenses, the SM medium and the process described during this work would seem to be commerically viable.

Given the wide variation in the potential of the media tested during this work for the production of bioactivity, it is unlikely that the medium and process described here cannot be improved by further investigation. Likely methods for the improvement of toxic unit yields include the study of increased aeration during vegetative growth and early sporulation where dissolved oxygen studies have shown that these stages have the highest aeration requirements of the production fermentation. The use of high, balanced nutrient levels combined with sparging oxygen gas as well as compressed air into the fermenter may allow the production of higher levels of viable counts and bioactivities than have been reported here. In addition, the use of alternative nutrient sources, such as cottonseed materials, may enable yield improvements to be attained.

During the course of the project a system was set up at the Institute for the biological assay of the insecticidal materials. This work involved the maintenance of adult mosquito colonies, guinea pig colonies, production and storage of mosquito eggs, as well as larval production for use as the bioassay insect target. Results obtained with the conventional bioassay system compared favourably, in terms of reproducibility, with data of previous workers (de Barjac and Larget 1979; Nugud and White 1982b). Furthermore, investigations into the novel bioassay showed that this system had considerable potential for the evaluation of the bioactivity of fermenter broths more rapidly than was possible with the standard international method of Rishikesh and Quelennec (1983).

Since organism-free formulations are of considerable interest to the

manufacturers of biological insecticides, studies were carried out on the purification of the parasporal crystals, which was easily done using an aqueous biphasic system, 3.3. The protocol used during this work was not economically viable, but other biphasic separation procedures have been described requiring cheaper raw materials (Sacks and Alderton, 1961). Further investigations using this cheaper system may prove attractive if crystal yields similar to those obtained during these investigations could be achieved.

During the course of this work, evidence was obtained for the presence of a number of proteases in the culture broths of B. thuringiensis. Analysis of gel filtration fractions using the azocasein digestion assay revealed the presence of three enzymes of molecular weight 16,100, 22,900 and 29,500. The 29,500 dalton protease had a pH optimum of 8.0 while the 16,150 dalton enzyme was maximally active at pH 10.0. The optimum pH of the third protease was not determined, although the peak at its elution volume was only detected at pH 10.0. Inhibitor and effect of divalent metal ions studies suggested that the casein digestion and leunase activities were catalysed by different enzymes. Using a crude culture supernatant, the casein hydrolysis was maximally active at pH 8.0 and was inhibited by PMSF and EDTA, but not by inhibitors of sulphydryl proteinases. By contrast, the leunase activity in the same crude culture supernatant was maximally active at pH 10.25 and was inhibited by EDTA, pCMB, pHMB, but not by PMSF. In the case of both activities, inhibition by EDTA was found to be irreversible. This similarity in pH optima of the two different activities with peaks in the elution pattern of the gel filtered protease sample suggested that the 29,500 dalton protease described above may be inhibited by PMSF and EDTA, and would thus be very similar to the intracellular serine protease isolated from B. subtilis

(Strongin et al., 1978, 1979). The leunase activity may therefore be catalysed by one, or both of the lower molecular weight proteinases, but in either case is inhibited by EDTA, and sulphydryl proteinase inhibitors.

These findings are in agreement with those of Chilcott et al. (1983) who found peaks in the protease activity associated with crystals from B. thuringiensis var israelensis at pH 8.0 and pH 10.0. However, these activities were not further characterized. Lecadet et al. (1977) isolated a protease of molecular weight 23,000 which was maximally active at pH 8.0, and was inhibited by both PMSF and EDTA. This may have been the same enzyme as that of 22,900 daltons described here by the gel filtration data,and whose pH optimum and inhibitor response pattern were unkown. A 29,000 dalton protease, maximally active at pH 8.0 and inhibited by PMSF has also been previously described (Epremyan et al., 1981; Stepanov et al., 1981). However, this enzyme was claimed to be only weakly inhibited by EDTA and was strongly inhibited by pCMB. These workers also claimed this enzyme to be extracellular, although it was isolated from a culture in which sporulation had occurred, but in which levels of lysis were not reported.

Therefore, the results presented here substantiate previous studies which have examined protease activities in B. thuringiensis culture broths. However, no evidence was obtained to support the claim of Stepanov et al. (1981), who suggested the recognition of a new subfamily of serine proteinases which are inhibited by sulphydryl reagents. Neither was there any evidence found for a netural protease of 37,500 molecular weight as described by Li and Yousten (1975). Indeed, this report is the only one known describing such an enzyme from B. thuringiensis. However, growth and protease production in the

medium of Li and Yousten was poor in comparison to that observed in the SM medium. It is possible that neutral protease production in the SM medium was masked by levels of other proteinases, and that in the Li and Yousten medium, a proportionately greater amount of neutral protease was produced. A more detailed examination of the proteases produced by B. thuringiensis in this medium would resolve this question. It should, however, be borne in mind that the protease type elaborated may not only be dependent on the medium used, but also on the age of the culture, and possibly also on the strain of B. thuringiensis studied.

Further investigation into the nature of the proteases produced by B. thuringiensis could be conducted along a number of lines. As was mentioned above, it would be interesting to study the types and relative amounts of proteases produced by the various serotypes of the organism. If it was found that proteinase production patterns varied from variety to variety, this may be used as a simple taxonomic tool for the classification of the organism.

A more detailed study into the types, cellular locations, and times of production of the proteases of B. thuringiensis, and a comparison of these enzymes with those of other bacilli would enable a clear picture of the importance of these enzymes to be established. The appearance of particular protease types at given stages during the growth of the organism (as was suggested during section 3.5.4.2.) may cast some light on the role of these enzymes, if any, in the complex differentiation process of bacterial sporulation (Doi, 1972; Maurizi and Switzer, 1980).

During the course of this project, studies were carried out on the development and characterization of fermentation conditions for the production of B. thuringiensis var israelensis. Methods for analysis of the biological activity of tne insecticide were established and further developed. A protocol for the purification of the parasporal crystals was quantified with respect to its crystal purification efficiency. Finally, proteases produced by the organism were partially purified and characterized. On the basis of these results, it is hoped that this dissertation will contribute to the knowledge, both basic and applied, of B. thuringiensis. Continued research on this organism has proven in the past to be beneficial to man, and will, almost certainly, be so in the future.

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APPENDIX 1

Calculation of insecticidal potencies of culture broths

1. Crystal Protein Concentration

LC_{50} pure crystal suspension = a ng/ml

LC_{50} culture broth = b X 10^{-6}

Therefore, crystal protein of culture broth = a/(b X 10^{-6}) ng/ml

= a/b mg/ml

2. Insecticidal Potency

Culture broth dry weight = a mg/ml

Culture broth $LDil_{50}$ = b X 10^{-6}

Therefore, culture broth LC_{50} = a X b X 10^{-6} mg/ml

= a X b ng/ml

Potency IPS.82 = 15,000 International Toxic Units/mg

Potency of culture broth = $\frac{LC_{50}\ IPS.82}{LC_{50}\ Culture\ broth}$ X 15,000 ITU/mg

But, dry weight of culture broth = a mg/ml

Therefore, potency per ml culture broth =

$\frac{LC_{50}\ IPS.82}{LC_{50}\ Culture\ broth}$ X 15,000 X a ITU/ml

DECLARATION

It is hereby declared that the work described in this thesis is that of the candidate, Dermot Pearson, as performed at the School of Biological Sciences, National Institute for Higher Education, Dublin, under the supervision of Dr Owen Ward, Head of the School of Biological Sciences.

Dermot Pearson

DERMOT PEARSON

Owen P Ward.

DR OWEN WARD

JUNE 1985