The use of microbial insecticides on drying and dried fish

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Final Technical Report

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Abstract

Novel strains of the bacterium *Bacillus thuringiensis* were isolated which had larvicidal activity against some tropical species of blowfly. It was shown that this specific toxicity could be attributed to the δ-endotoxin proteins produced during sporulation.
The biochemistry, bioactivity and genetics of the production of these δ-endotoxins was characterised.

The parameters for the industrialised production of these insecticidal bacteria were deduced. A suitable pilot-scale producer was identified in Africa. An insecticidal preparation was formulated which could be applied to fish prior to sun-drying.

Toxicity tests with rats indicated no hazard resulting from exposure to very high levels of *B. thuringiensis* insecticide. Separate tests gave severe cause for concern with respect to two toxins, potentially presenting a hazard to humans, β-exotoxin and *Bacillus cereus*-type entertoxotin. Attempts to remove the former toxin from our strains of *B. thuringiensis* ultimately proved to be unsuccessful.

Due to the unacceptable risks considered to be presented by the unwanted toxins the objective of the project was altered to the use of our bio-insecticide as a bait in fish processing and drying sites. The insecticide was reformulated and tested under field conditions in Sri Lanka. The results showed that the insecticide might prove to be a useful component of an overall regime designed to reduce losses by insects to post-harvest fish. The use of low level technology to produce *B. thuringiensis* for insecticidal baits was reviewed briefly.

Contents

Chapter 1

Introduction to the Work

The importance of dried fish in the nutrition of people in developing countries is well documented (see Borgstrom, 1962, FAO Fish.Tech.Pap., 1981). It is a rich source of protein complete in the essential amino acids, and with a net protein utilisation rating close to that of eggs, the protein bio-availability is excellent (MAFF, 1978). As protein deficiency is commonplace in the developing world, increasing the quantity of dried fish could help to improve the nutritional status of large numbers of people (Borgstrom, 1972). Improving utilisation through reducing losses is an important means of increasing the production of traditionally processed fish in developing countries.

Traditionally processed fish is highly susceptible to attack by insect pests, principally blowflies (Diptera: Calliphoridae) and *Dermestes* spp. beetles (Coleoptera: Dermestidae). Damage resulting from infestation is a major cause of post-harvest losses in the traditional fish processing industry. Whilst extensive quantitative studies of insect related losses have yet to be carried out, it is generally accepted that losses of 25% are not uncommon, and, in extreme cases, losses in excess of 90% occur (Meynall, 1978, Golob *et al.* 1987, Young and Esser, 1992).

Each insect group attacks the fish at a different stage in the processing. Primarily, blowflies are attracted to the fish during the salting and sun-drying stages, whilst beetles infest the dried, stored product. There are exceptions to this with larvae of the dipteran species *Piophila casei* and *Lucilia cuprina cuprina* being reported to infest cured fish (FAO Fisheries Technical Paper 303 , Esser, 1994). There may also be seasonal variations in the predominance of pest species, with blow-flies being of most importance during the wet seasons and beetles during the warm, dry seasons (Walker, 1981) Damage through blowfly infestation results from larval feeding packs forming within the flesh of the fish. Deep burrows are created which subsequently cause the dried product to fragment, increasing losses and making transportation difficult (Meynall, 1978). During drying, the fish muscle surrounding the feeding larvae becomes putrid probably due to the combined effects of enzyme secretion onto the food initiating tissue breakdown, and pH change due to nitrogenous waste excretion in the form of ammonia. Larvae usually leave the fish to pupate, often in the earth beneath the drying racks. Thus, adults emerge within close proximity of the fish and the cycle repeats. There are two main routes of infestation: (1) larvae hatch from egg batches laid (or in viviparous species hatched larvae are deposited) on or adjacent to the fish during the early stages of processing, and; (2) larvae move onto the fish from other oviposition sites and food sources (Johnson and Esser, 1996). To be successful, control measures need to target both routes of infestation.

The beetles most commonly found infesting dried fish are *Dermestes maculatus* and *Dermestes frischii*, although *Necrobia rufipes* is also a frequent pest (Osuji, 1975a). Both adults and larvae feed on the fish causing extensive damage through tissue loss and fragmentation. Factors such as temperature, relative humidity and moisture content of the fish influence the level of infestation present. Osuji (1975b) found that daytime temperatures of around 50^{o}C cause the beetles to migrate into the centre of fish stacks where average

temperatures are 35°C. Temperatures above 40°C prevent development in the larvae of these species (Amos, 1968), whilst temperatures of 50°C for more than 15 minutes can be lethal (FAO Fish.Tech.Pap., 1981). Relative humidities of 50% or above are generally necessary for survival in all species (Howe, 1965). These factors can be manipulated for insect control purposes.
However, for optimum development and minimal mortality the moisture content of the food source should be between 10.5 and 15.9% (Scoggins and Tauber, 1951). This range coincides with that normally found in unsalted, dried fish making it an ideal food source.

Frequent and extensive losses from insect infestation cannot be sustained by processors or traders. Control measures recommended by extension workers combine the use of chemical and non-chemical methods. Of primary importance is the following of good hygiene practices. The removal of any fish waste from the vicinity of the fish processing operation will help to reduce the number of flies present at the site, as well as limiting the possibility of larval cross-infestation from other food sources. The provision of adequate physical protection to the fish during the early stages of processing can have a profound effect on reducing infestation by blowflies. Infestation during salting was prevented by simply fitting an insect proof lid to brining tanks in Indonesia (Esser, 1991). Similarly, erecting mesh screens or netting over drying racks can prevent gravid females gaining access to the fish during drying, thereby limiting infestation without markedly reducing drying rates (Esser, 1992). Salting the fish prior to sun-drying may also have a role to play in preventing blowfly infestation (Walker, 1986, Johnson and Esser, 1996).

Chemical control, through the application of insecticides to the fish during drying and storage, is a highly effective way of combating infestation by blowflies and beetles. Over 30 active ingredients have been considered for use on fish. A full review of this work can be found in Walker (1987). At present only two insecticidal compounds have recommended Maximum Residue Levels (MRL) for use on fish. These are the synergised pyrethrins and primiphos-methyl. In the former, the pyrethrins are synergised with piperonyl butoxide (ratio 1:10). Several laboratory and field studies were conducted in the UK, Canada and Norway during the 1960's. All showed that good control against blowflies could be achieved by dipping or spraying fish in emulsions of the synergised pyrethrins at concentrations of 0.3% pyrethrin or below (Olley, 1961, Somme and Gjessing, 1963, Morris and Andrews, 1968). The MRL was set at 0.1mg/kg pyrethrin and 1.0mg/kg piperonyl butoxide by the FAO/WHO Joint Meeting on Pesticide Residues (FAO/WHO, 1970), and later increased to 3mg/kg and 20mg/kg respectively (WHO, 1973). In tropical countries, early work carried out by McLellan (1963) in Uganda gave good protection to *Tilapia* spp. from the blowfly *Chrysomya marginalis* (Weid.). Data for residue levels, however, are not stated. More recent work conducted in Africa reveals that in order to gain adequate protection against insect pests, residues of synergised pyrethrins, in particular of the piperonyl butoxide component, are well in excess of the MRLs (Diouf, 1980, Walker, 1981, Walker and Donegan, 1984). Indeed Golob *et al.*(1986) found residue levels of 5.7mg/kg for pyrethrins and 225mg/kg for piperonyl butoxide six months after application to stored fish in Turkana, Kenya. Such residue levels are wholly unacceptable, and yet, despite these findings, synergised pyrethrins are still used to combat insect infestation by fish processors in developing countries (Azeza, 1986, Walker, 1987).

Pirimiphos-methyl underwent extensive trials in Malawi where it was found to provide excellent protection against blowflies and fleshflies (Walker and Donegan, 1984). Trials

conducted in The Gambia by Walker and Evans (1984) showed that control against *D. maculatus* could be achieved, although the protection declined after one month's storage. Esser (1986) found that marine catfish, *Atrius* spp., could be protected against *Chrysomya megacephala* (Fab.) during drying, and *Dermestes* spp. for ten weeks during storage, following a single dipping in a 0.03 % emulsion of pirimiphos-methyl. A MRL of 10mg/kg has been recommended for pirimiphos-methyl use on fish. Mean residues determined on experimental fish after drying have been between 4.6mg/kg and 9.7mg/kg (Esser, 1986, Walker and Evans, 1984). Residues at this level gave good control of insect pests for prolonged periods. However, liberal application can result in residues in excess of the MRL. Commercial fish sampled in Zambia had pirimiphos-methyl residues of 26mg/kg (Walker, 1987). Walker suggests that problems such as this can be overcome with good extension practices.

Whilst pirimiphos-methyl is by far the safest insecticidal preparation available, its use appears to be limited, possibly because of its restricted availability or comparative cost in some countries (Walker, 1987). Lack of training or accessible information means that many fish processors resort to the use of inappropriate, often dangerous pesticides (Walker, 1988). Products such as DDT and lindane, which are organochlorines and thus accumulate in the body, and the acutely toxic dichlorvos and phosalone, are in widespread use (Walker, 1987). Many of these products are available as household insecticides. Of additional concern is the apparent tendency to incorporate kerosene as a formulation component (Walker, 1986). Esser *et al.* (1986) reported a total absence of insects on fish treated with non-approved insecticides, suggesting that the concentration of insecticide applied to the fish was in excess of that needed to obtain efficacious blowfly control.

On consideration of the available data it is apparent that the control of insect infestation in traditionally processed fish is important for social and economic reasons. Furthermore, the willingness of processors to increase their expenditure by purchasing insecticides suggests that significant financial gains must result from controlling infestation. The lack of available food-safe insecticides has obvious grave implications to human health. Whilst a complete move towards the use of pirimiphos-methyl is to be desired for safety reasons, the likelihood of rapid pest resistance developing is great. It is therefore essential that alternative insecticides be developed for use on fish.

This project was funded with the intention of developing a microbial insecticide for use on fish. Such pesticides have several advantages over their synthetic counterparts. They have been shown to be far safer with respect to both human health and environmental considerations (Drobniewski, 1994). In addition, they are cheap and easy to produce using many locally available substrates and can therefore often be produced within the country of intended use, thus benefiting the local economy and avoiding unnecessary foreign exchange (Jones, 1988).

Several micro-organisms are currently used in pest control, the most predominant of these being *Bacillus thuringiensis*. *B. thuringiensis* is a gram positive, spore-forming bacterium which produces insecticidal crystal proteins (ICPs) at sporulation (Höfte and Whiteley, 1989). The spectrum of activity exhibited by each strain is dependant upon the class of protein or proteins produced (Höfte and Whiteley, 1989). It is an ubiquitous soil-dwelling organism which has been isolated world-wide (Martin and Travers, 1989). It has been widely used in pest control over many years (Perferoen, 1991, Feitelson *et al.* 1992) currently accounting for

92% of the biological control market (Powell, 1993). Apart from use as an agricultural pest control agent, its major success has been in vector control. *B. thuringiensis* var. *israelensis* (Bti) has been effectively used in malarial and onchocerciasis control programmes across the developing world (Walsh, 1986, Becker, 1990). Its use for these purposes is actively promoted by the World Health Organisation.

The safety record of *B. thuringiensis* is excellent (Drobniewski, 1994, Hadley *et al.* 1987, Lamanna and Jones, 1963, Siegel and Shadduck, 1990 and Siegel *et al.*, 1987). In contrast, the World Health Organisation estimates that chemical pesticides are responsible for poisoning over 1 million people each year, and 2% of these will prove fatal (Ambridge and Haines, 1987).

B. thuringiensis has commercial drawbacks being of comparatively low toxicity with high host specificity and low persistence following application (Dent, 1993). This is in marked contrast to chemical insecticides. However, these limitations offer environmental benefits which enable its use within integrated pest management programmes as well as alleviating public concerns over environmental issues. With the increase in insect resistance to chemical insecticides, *B. thuringiensis* has gained importance in the control of several important pest species. Amongst these are the diamondback moth, *Plutella xylostella*, and larvae of the blackfly, *Similium damnosum* (Jones, *et al.*, 1993). Estimates of the numbers of chemical resistant insect species were 392 in 1980 compared with 25 in 1955 (Youdeowei and Service, 1983). Assuming this apparent trend continues, *B. thuringiensis* is likely to become increasingly important in the control of major pest species. However, insect resistance is not wholly restricted to chemical insecticides and some resistance to *B. thuringiensis* has been reported (McGaughey, 1985, McGaughey and Beeman, 1988, Stone *et al.* 1989). Identification of the genes which code for the δ-endotoxin (Höfte and Whiteley, 1989) has enabled the toxin to be incorporated into hosts such as plants and other bacteria thus extending the insecticidal capability of the organism (Vaeck *et al.*, 1987). This will lead to an inevitable increase in the exposure of target insects to the toxin and so further resistance may develop.

B. thuringiensis produces a number of metabolites with insecticidal properties. Work has concentrated on two of these, the β-exotoxin and δ-endotoxin or insecticidal crystal protein (ICP). The former is structurally similar to ATP and acts by inhibiting protein synthesis through competing for binding sites thereby interfering with DNA-dependant RNA polymerase during RNA synthesis (Sebasta *et al.* , 1981). In immature insects, exposure to β-exotoxin manifests at critical development stages such as moulting or pupating. This results in prolonged development or mortality (Bond *et al.*, 1971). Adults are often infertile or have reduced fecundity (Sebasta *et al.*, 1981). Whilst the β-exotoxin is effective at killing insects its action is not specific to invertebrates (Faust, 1975). Vertebrates, including man, are susceptible to the toxin, with high levels of exposure resulting in death. Levinson *et al.* (1990) suggest that a single gene codes for the β-exotoxin, and as such genetic manipulation may allow for this toxin to be removed from the strain when its presence is undesirable.

The mode of action of the δ-endotoxin is complex (Li *et al.*, 1991, Knowles, 1994). A full review of the mechanism of action is given by Knowles and Dow (1993). It is thought to require solubilization of the protein in the midgut of the insect. The protoxin is then proteolytically cleaved to yield an active toxic fragment of 60-65 kDa. This then penetrates the peritrophic membrane and binds to specific receptors situated on the midgut epithelial wall

where it induces the formation of pores in the cell membranes (Mathavan, *et al.*, 1989). The result of this action is a net influx of ions and accompanying water (Knowles and Ellar, 1987), eventually causing the cells to swell and lyse leading to massive epithelial disruption (Endo and Nishiitsutsuji-Uwo, 1980, Reisner *et al.*, 1989). The action of the crystal alone is often sufficient to cause death. However, many authors have reported an important role for spores in enhancing the action of the toxin (Burges *et al.*, 1976, Li *et al.*, 1987, Miyasono *et al.*, 1993). Ellar *et al.* (1990) suggest that this role is one of exacerbating the onset of septicaemia. The insect pests of drying or dried fish often feed by burrowing into the flesh. As the mode of action is through ingestion of the active ingredient rather that by contact, extra care must be taken in the formulation and application to ensure that the concentration and distribution of the toxin on the target surface is sufficient to ensure adequate exposure whilst the insect is browsing on the surface.

The remit for this project was to investigate the potential role of *B. thuringiensis* in controlling the insect pests of drying and dried fish. If strains were identified with activity against these pests their application to protecting fish was to be investigated. This would entail: the safety of the strains to be assessed; the determination of fermentation parameters using substrates which are readily and cheaply available in developing countries; the development of a formulation for use on fish which would not prohibitively increase the production costs; the identification and development of a quality control method for use during production; the identification of a potential producer within the African continent. The remainder of this report details the work conducted to achieve these goals.

Chapter 2

Strain Isolation and Screening

At the onset of this project, it was not certain whether strains of *B. thuringiensis* could be identified which exhibited activity against Calliphoridae and Dermestidae. Several coleopteran active strains had been reported but few dipteran active strains, apart from the mosquitocidal strains. Hodgman *et al.* (1993) had demonstrated novel activity against larvae of the housefly in the isolate YBT-226, but, unfortunately, this strain was the property of E.I. Dupont de Nemours and was not intended for commercial release. A second report by Indrasith *et al.* (1992) showed the strain *B. thuringiensis* subsp. *kurstaki* HD-1 to be active against adult houseflies but not larvae. There had been no published reports of blowfly or fleshfly active strains. A screening programme was therefore undertaken to screen many known strains of *B. thuringiensis* for activity against species of tropical blowflies and *D. maculatus*. A toxin type was identified which exhibited activity against tropical blowfly larvae, but, despite extensive screening of known coleopteran active strains, no toxin type was found which showed activity against *D. maculatus*. Having identified the blowfly active toxin type, novel strains were isolated from soil samples collected from areas where high numbers of blowflies are found. These isolates were screened for activity against both blowfly larvae and *D. maculatus*. Several strains were identified with Calliphoridae activity, but none with Dermestidae activity. In view of the time and expenditure involved in carrying out the screening programme for Dermestidae activity, it was agreed that this aspect of the project should be curtailed. Screening of any newly available toxins continued but the isolation programme was halted.

This chapter describes the isolation and screening procedures undertaken. The LD_{50} values obtained for the active strains and their insecticidal crystal proteins (ICPs)are listed. Studies into aspects of the pathogenicity of the active strains relevant to larval infestation behaviour are described.

2.1 Materials and Method

2.1.1 Bacterial strains and growth conditions.

The following commercial strains were used in the screening:
Bacillus megaterium N.C.I.B 7581 (obtained from Dr. P.J. White).
B. thuringiensis strain 4412 (obtained from Dr. Peter Luthy)
B. thuringiensis subspecies *tenebrionis* (from DSM, Germany)
B. thuringiensis subspecies *israelensis* (donated by Dr. David Ellar)
B. thuringiensis strains HD1, HD2, and HD73 (donated by Dr. David Ellar)
B. thuringiensis strain Buibui MT44 (obtained from Mycogen)

All bacterial strains were grown on Nutrient Broth or Nutrient Agar (Oxoid) supplemented at 1ml l^{-1} with CCY salts (Stewart *et al.* 1981) to aid sporulation. This medium was termed 'BGM' medium. Liquid cultures were incubated in an orbital shaker at 30°C and 200 r.p.m. for 48 hours or until sporulation was complete. Spores and crystals were harvested by centrifugation for 60 minutes at 4,000 r.p.m. in an MSE

Mistral 3000E centrifuge. The pellet was washed twice with deionised water then resuspended in deionised water to give a final volume which represented a fifty-fold concentration with respect to the initial culture. These preparations were referred to as "fifty-fold concentrates" and were stored in small aliquots at -20^{O}C.

2.1.2 Isolation of Pure Crystals.

Pure crystals were isolated by the method of Thomas & Ellar (1983).

2.1.3 Maintenance of Insect Colonies.

Fly colonies

Breeding populations of tropical blowfly species were established from imported larvae (MAFF licence number AHZ/980E/94/46). These comprised *Chrysomya megacephala* (Fab.) from Thailand, *Lucilia cuprina* (Weid.) from Senegal and *Chrysomya albiceps* (Weid.) from Uganda. *Musca domestica* (Linn.) (WHO strain) was obtained in the UK. Colonies were maintained at 28 ^{O}C, 68 % RH with a 12:12 L:D photoperiod. Adults were given a maintenance diet of *ad-lib* sugar, milk powder and water, and were offered tinned cat-food as an oviposition medium on a twice weekly basis from the third week post-eclosion. Larvae for replacement colonies were reared on a yeast and agar based diet comprising 50g active dried yeast (DCL), 8g agar (Oxoid Bacteriological N^{O} 1), 250ml distilled water and 750ml UHT full cream milk, prepared by microwaving to boiling point.

Beetle colonies

Adults and larvae were reared in demi jars covered with filter paper and sealed with parafin wax. Whole, dried *Tilapia* spp. were placed in the jars as a food source for both adults and larvae. The insectory was maintained at 70% RH and 30^{0}C, with a series of light:dark photoperiods.

2.1.4 Bioassay Procedure.

Fly Assay

The larval rearing diet defined above was used in the bioassays. *M. domestica*, acted as an indicator species for initial screening of toxins. Those protoxins exhibiting activity against *M.domestica* were then screened against the tropical species.

The insecticidal suspension was dispensed into plastic pots (No 2 size, Ashwood Timber & Plastics Ltd.). Suspensions of pure crystals were prepared giving final concentrations of crystal protein in an exponential range from 2 to 128 $\mu g\ ml^{-1}$. The required volume of the spore-crystal fifty-fold concentrate was rediluted in distilled water, to give a final volume of 1ml. Molten diet, to give a total volume of 25mls, was added to the suspension of crystals, thoroughly mixed by agitation, then allowed to

set. The surface of the diet was scored to provide feeding crevices. 20 neonate larvae were transferred onto the diet. Pots were covered using filter paper held in place with elastic bands and placed in an incubator maintained at 27°C and 70% RH. Mortality was recorded after 48 hours. Moribund larvae were counted as living. The following controls were used for all bioassays unless otherwise specified: *B. thuringiensis* subsp. *tenebrionis* was used as a negative control at a dose equal to the highest dose used in the test range; the strain 4412 was used as a positive control at a dose of 20μgml^{-1}. All doses were replicated 5 times, and assays were repeated at least twice.

Beetle Assay

Toxins were screened individually. Assays were conducted in triplicate using 5 first instar larvae for each pot. 1ml of the fifty-fold spore crystal concentrate was mixed into 5g of fish meal placed in 20ml plastic pots. Filter paper was secured over the pots to allow ventilation but prevent moisture build-up which would cause the fish meal to become too moist for the beetles to survive on. Pots were placed in an incubator maintained at 30°C and 70% RH. Mortality was recorded after 48 hours. A negative control was provided by using known non-toxic strains. No positive control was available.

2.1.5 Isolation procedures

About 0.25 g (dry weight) of environmental sample were placed in test-tubes containing 2 ml of a Nutrient Broth (Oxoid). The tubes were heat-shocked at 70°C for 10min in a water-bath. The contents of each tube were poured into 250ml flasks containing 50 ml of Nutrient Broth (Oxoid) supplemented with CCY salts (Stewart, 1981), 1m l^{-1} to aid sporulation, and 20 International Units ml^{-1} of penicillin G (Sigma Chemicals).The flasks were incubated at 30°C at 200 r.p.m. until sporulation was complete. The particulate matter was recovered by centrifugation for 60min at 3,600 rpm in an MSE Mistral 3000E. The pellets were resuspended in 2ml aliquots of liquid growth medium and put through a second cycle of the procedure above. The pelleted matter from the second centrifugation step was resuspended in 2ml of broth. Serial dilutions of these suspensions were plated out onto Nutrient Agar (Oxoid) containing CCY salts and supplemented with 20 International Units ml^{-1} of penicillin G. After incubation at 30°C until sporulation was complete individual beige colonies with a matt texture were examined microscopically for the characteristic spores and crystals of *B. thuringiensis* (Bulla *et al.* 1980).

2.1.6 Identification of Isolates with Activity Against Blowfly Larvae.

Individual isolates were grown and harvested as previously described. Pooled samples comprising 300 μl of the fifty-fold concentrate from each of three isolates were initially bioassayed. Where larvicidal activity was exhibited, each isolate was individually screened to identify the toxic strain(s).

2.1.7 Electrophoresis of Crystal Proteins.

SDS-PAGE was carried out by the method of Laemmli (1970).

2.1.8 Protein Determination.

Protein concentrations were determined by the method of Lowry *et al.* (1951), using bovine serum albumin (Sigma Chemicals) as a standard.

2.1.9 Investigation into the Pathogenicity of the Novel Isolate 13B.

2.1.9 *Effect of Replacing Spores with Vegetative Cells.*

Vegetative cells, grown overnight in liquid media, were harvested and washed twice in distilled water, then kept on ice until the population density of the viable cells could be assessed by plate counts. Cells, at a level of colony forming units equivalent to the number of spores normally associated with an LD_{50} dose of crystals, were added to pure crystal preparations constituting an LD_{50} dose, then dispersed into the larval diet.

2.1.9.2 *Investigation into the Potential for Survival Following Limited Exposure to a Lethal Dose of B. thuringiensis.*

Large numbers of neonate larvae were placed onto diet containing a lethal dose of spores and crystals. Twenty larvae were transferred in triplicate to untreated diet at the following time intervals: 1 hour, 2 hours, 4 hours, 12 hours and 24 hours after the initial exposure. Pots containing recovered larvae were placed in an incubator at 27°C and 67% R.H. and monitored until all animals had either died or completed their life cycles. The experiment was repeated four times.

2.1.9.3 *Lethality of the Isolate 13B to Adult Flies.*

Toxicity of the ICP was assessed against adults of both *M. domestica* and *L. cuprina.* Four mesh cages covered with netting were set up to house the adult flies. Water and carbohydrate were provided respectively in the form of damp cotton wool pads and 250mg finely ground sucrose contained in an open dish. Ten, two day old flies were placed in each cage. Assays were set up as follows: Test cage 1: 50mg of 13B pure crystals were thoroughly mixed in with the sugar; Test cage 2: 50mg of 13B pure crystals were thoroughly mixed in with the sugar and 100mg of crystals were evenly spread onto the water pad; Control cage 1: 50mg of pure crystals of *B. thuringiensis* subsp. *israelensis* were thoroughly mixed in with the sugar; Control cage 2: an additional 50mg of sucrose was added to the sugar. Mortality rates were recorded daily for each cage. Water and sugar / sugar crystal mixes were replaced as necessary. The trial was considered complete when 100% mortality was attained in the test cages. To ensure that the lethality observed was a result of exposure to the *B. thuringiensis* and not a factor associated with the rearing conditions, controls remaining alive at the end of the experiment were maintained under the same conditions for a further 2 weeks.

2.2 Results

2.2.1 Strain assessments.

Of the known strains of *B. thuringiensis* that were bioassayed against fly larvae, only strains HD-2 and 4412 were found to be active. Isolation of novel strains from soil samples revealed six isolates exhibiting larvicidal activity out of 37 assayed. Three of these were classed as high activity (13B, 1B and 3B) and three as moderate activity (10B, 3A and AB). LD_{50} values were calculated using probit analysis of the bioassay data. Values are detailed in Table 2.1. The lethal dose range for the fifty-fold concentrate of spores and crystals of each strain assessed, against the larvae of a given species of fly, is given in Table 2.2. Values obtained from the protein analysis showed the protein production to be comparable in all strains.

Pure crystals of five of these isolates were analysed by SDS-PAGE (Fig 2.1). Four of the isolates produce very similar profiles indicating the presence of two protoxins in the 130-140 kDa range and one in the 60-70 kDa range. One of the isolates however, strain 1B, had only one protoxin type in both size categories. In spite of the similar electrophoretic pattern of the protoxins in the four strains, their bioactivity against fly larvae was markedly different, indicating that they are not the same isolate.

None of the strains screened for activity against *D. maculatus* were found to be toxic

Table 2.1
LC_{50} values, calculated by logit transformation, for the two commercial strains, 4412 and HD-2, and the novel isolate 13B, against neonate larvae of *M. domestica* and two tropical blowfly species, *L. cuprina* and *C. albiceps*.

Species	Strain	LC_{50} (μg ml^{-1})	Standard Error	95% Confidence Interval
M. domestica	13B	77.6	1.0	(72.5, 83.0)
	4412	79.4	1.0	(76.7, 82.3)
	HD-2	81.3	1.0	(78.5, 84.2)
L. cuprina	13B	150		(107, 193)
	4412	303		(151, 454)
	HD-2	200		(148, 252)
C. albiceps	13B	607	141	(300, 914)
	4412	807	1320	(118, 1495)
	HD-2	1,390	316	(0, 4270)[a]

[a] Concentration required to effect an LC_{50} response is in excess of the maximum feasible concentration used thus resulting in a very poor logit fit for these data.

Table 2.2.

The minimum concentration of the fifty-fold concentrated spore/ crystal preparation required to effect an LD_{100} response.

Strain	Fly Species		
	Musca domestica	*Lucilia cuprina*	*Chrysomya megacephala*
4412	10	50	200
HD-2	40	300	450
HD-1	NA	NA	NA
HD-12	NA	NA	NA
B. thuringiensis ssp. *israelensis*	NA	NA	NA
B. thuringiensis ssp. *tenebrionis*	NA	NA	NA
Isolate 13B	25	100	200
Isolate 1B	30	150	250
Isolate 3B	30	100	300
Isolate AB	150	170	500
Isolate 10B	100	500	1000
Isolate 3A	200	200	1000

NA not active

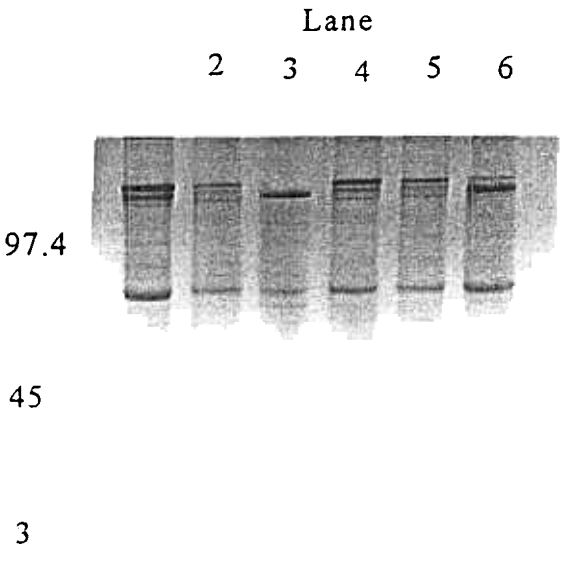


Fig. 2.1. Polypeptide profile of δ-endotoxins for the *B. thuringiensis* novel isolates on an SDS/10% polacrylamide gel (Coomassie Blue-stained). Lane 1, M_r marker; lane 2, 13B; lane 3, 1B; lane 4, 10B; lane 5, AB; lane 6, 3A

2.2.2 Replacing Spores with Vegetative Cells.

The addition of vegetative cells to the crystal preparations had a potentiating effect on the toxicity of the crystals ($p<0.001$) This effect was found to be equivalent to that obtained following the addition of the corresponding number of spores (Fig 2.2).The addition of spores did, however, increase the rate of mortality (data not shown).

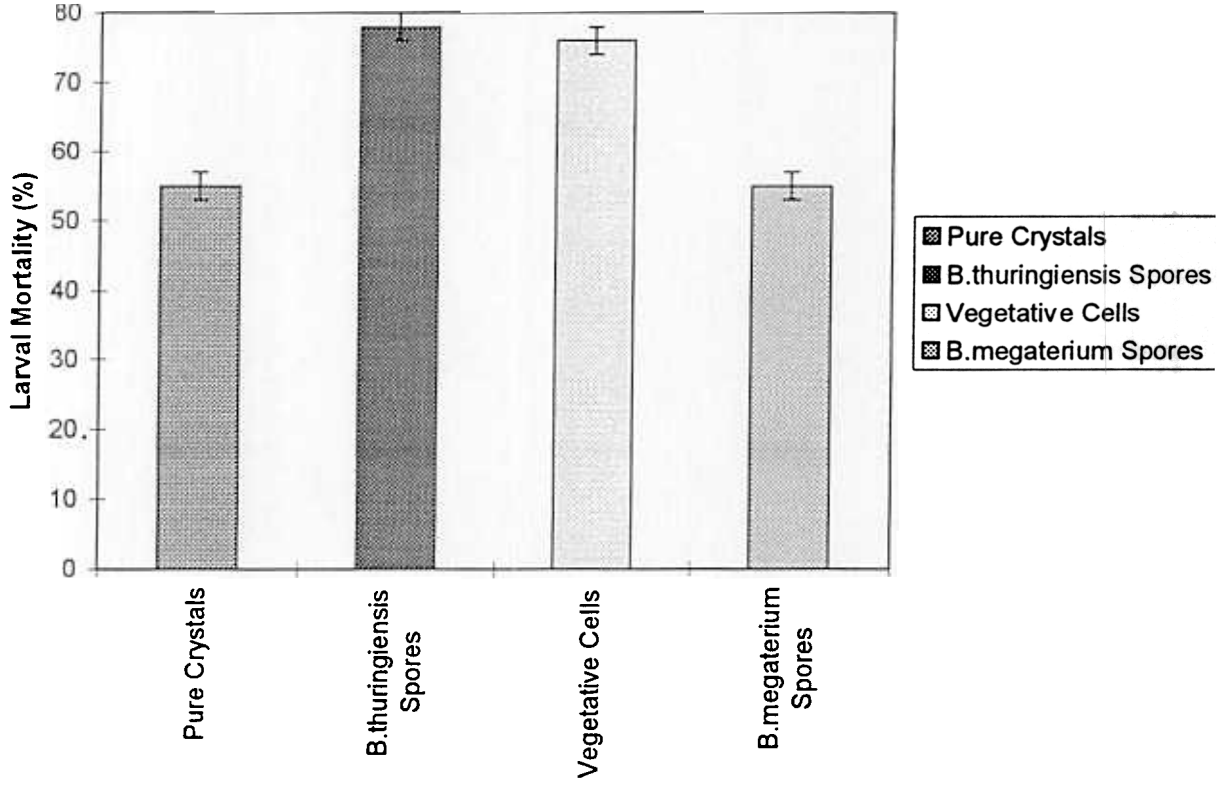


Fig 2.2: Comparative effects on mortality in *Musca domestica* larvae, of the addition of spores of *B. megaterium* and spores or vegetative cells of *B. thuringiensis* to a standard crystal preparation constituting an LD_{50} dose.

2.2.3 Larval Survival Following Limited Exposure to a Lethal Dose of B. thuringiensis.

Of the 1,200 larvae transferred from the insecticide treated to untreated diet across all four trials, only 20 larvae (1.7%) survived to pupation. Of these, only six (0.5% of total) successfully completed their development to adult flies. Mortality in the remaining larvae occurred within 72 hours of their transfer. It can be seen, therefore, that in almost all cases a lethal dose was consumed by the larvae within one hour of their exposure to the insecticide. No larvae survived when transferred after 12 hours exposure. The trend in survival both within a trial and between trials was non-uniform,

with the survival rate following each period of exposure being sporadic. Surviving larvae exhibited severe lethargy, and a reluctance or inability to move, but continued to feed within a confined area as measured by their weight gain and instar changes. This perhaps suggests that the observed lethargy is not attributable to paralysis, unless it is partial in effect. The variability in survival may be a result of natural variations in the population.

2.3 Discussion

These results extend the findings of Hodgman *et al.* (1993) in showing that blowflies as well as the house fly are susceptible to ICPs of *B. thuringiensis.* The latter report is, in fact, erroneous in that strain YBT was claimed not to be a producer of β-exotoxin Hodgman *et al.* (1993). This strain has subsequently proven to be a strong producer of this toxin (Yu Ziniu, pers. comm.). The bioassay data cited are therefore wrong. Further work has shown that the ICPs of strain YBT are toxic to house fly larvae, in addition to the toxic effect of the β-exotoxin. Our work shows that it is exclusively the CryIB protoxin that is responsible for this toxicity. This has not been shown before. No other protoxin type had this activity. This makes the work of Indrasith *et al.* (1992) even more suspect in that they claimed that a CryIA toxin resulted in the death of adult and larval flies. We could not find any toxic effect of our Cry IB crystals on adults.

The variation in LC_{50} required for different strains of *B. thuringiensis* is typical of the species variation seen with this organism when applied to, say, lepidopteran larva. There is also an interesting variability between strains in that the most effective one against one larval species is not necessarily the most potent against another. Unfortunately the blowfly larvae are the most resistant to the ICPs,

Chapter 3

Strain Development and Safety

The expected end use of the insecticidal product is for application to fish intended for human consumption. The safety status of the strain to be used is therefore of primary importance. Hazardous metabolites potentially produced by *B. thuringiensis* include β-exotoxin and *B.cereus*-type enterotoxin and emetic toxin. A strain suitable for the purpose of this project must be unable to produce any of these products. Autoclaving β-exotoxin at pH 3 destroys its toxic activity; it is stable in neutral or alkaline solutions (Levinson *et al.*, 1993).

3.1. β-Exotoxin Assessment

3.1.1. Materials and Methods

a) Bacterial strains and growth conditions
Bacterial strains and growth conditions were as detailed in section 2.1.1. with the following variations: following centrifugation the supernatant liquid, which will contain the water soluble, heat-stable β-exotoxin if present, was retained and autoclaved for 15 minutes at 121^{0}C to destroy any non-heat stable insecticidal metabolites.A second assessment was made using triplicate samples with their pH adjusted to 3, 7 and 12 respectively, prior to autoclaving. The sterilised culture fluids were then adjusted to pH 7.

b) Maintenance of insect colonies
Insect colonies were maintained as in section 2 .3

c) Bioassay Procedure
Bioassays were carried out as described in section 2.1.4., with the following variations. In place of the spore/ crystal concentrate, 3mls of the autoclaved supernatant was dispersed in 22 mls of molten diet. Positive and negative controls were provided by using the autoclaved culture fluids from *B. thuringiensis* strain HD-2 and *B. thuringiensis* ssp. *tenebrionis* respectively. For assays involving the supernatant liquids whose pH values had been adjusted further controls were provided which incorporated a volume of acid or alkali, equivalent to that which was required to adjust the pH value, into the molten diet. Larvae were reared on the assay diets until they either died or completed their life-cycles. Death at any instar change, or failure to successfully pupate, strongly indicated the presence of β-exotoxin. Successful pupation and adult emergence confirmed the negative β-exotoxin status of the strain.

3.1.2. Results

Bioassay results showed that all six of the novel isolates were positive for β-exotoxin as was *B. thuringiensis* strain 4412. Adjusting the pH prior to autoclaving further confirmed these results. Growth and mortality rates in larvae exposed to the

supernatant at pH 7 and pH 12 were comparable to one another and equivalent to the positive control, whilst those exposed to the supernatant at pH 3 were equivalent to the negative control (see table 3.1.1). No attempt was made to quantify the level of β-exotoxin production as this was not considered to be relevant to the project purpose (any strain producing β-exotoxin will be unacceptable).

Table 3.1.1: β-Exotoxin assessment of strains using autoclaved supernatant fluids

Strain	Exotoxin assessment
13B	positive
1B	positive
3B	positive
3A	positive
AB	positive
10B	positive
4412	positive
B.thuringiensis subsp. *israelensis*	negative
Controls[1]	negative

[1] Culture fluid of strain 13B autoclaved at pH 3 and then neutralised.

3.1.3. Discussion

These results show that all blowfly toxic strains in our possession are β-exotoxin producers. In view of the non-specific action of this type of toxin, the strains as they are cannot be used for their intended purpose of application to a food product. Several commercially available strains are β-exotoxin producers, but, more recently, agrochemical companies will only market β-exotoxin minus strains.

Levinson *et al.*(1990) found that β-exotoxin production was carried on a single plasmid. It may therefore be possible to remove this plasmid either by conjugation or through plasmid curing (growing the organism under conditions which will make it lose some or all of its extrachromasomal DNA). The success of this, however, may be dependent upon the plasmid encoding for the β-exotoxin not also encoding for the δ-endotoxin. In their investigation, Levinson *et al.* (1990) found that in the five strains of *B. thuringiensis* producing β-exotoxin they assessed, the β-exotoxin plasmid also encoded one or more insecticidal crystal protein.

3.2 Strain Development to produce β-exotoxin minus status

3.2.1 Introduction

The finding that all of the fly-toxic strains in our possession produce β-exotoxin lead to the decision to try to remove this trait. Theoretically the simplest way to achieve this would have been to clone the gene encoding the active δ-endotoxin and express it in an

acceptable vector such as *B. thuringiensis* subspecies *israelensis*. The problem with this approach is that it is currently highly unlikely to obtain permission in any country to liberate any micro-organism which has been genetically manipulated. This term covers techniques which alter the genetic material of an organism in a way that could not occur naturally. Fortunately bacteria are adept at transferring DNA between themselves and also seem to lose DNA, particularly extrachromosomal DNA quite easily. Two strategies, involving naturally-occurring processess, designed to arrive at a strain of *B. thuringiensis* incapable of producing β-exotoxin were adopted. These were plasmid curing and conjugation.

3.2.1(i) Plasmid curing.

The development of a strain lacking the ability to synthesise exotoxin by the conjugational approaches described below is a time-consuming task and the frequency of transfer of plasmids carrying the *cry* genes may be low. In addition, there is no guarantee that these genes will be transcribed and then translated into crystal proteins which retain the same high activity as they exhibited in the donor strain.

Alternatively, plasmid curing could be used to achieve the same end result as exotoxin is thought to be plasmid encoded. This would provide a rapid, simple means of obtaining a strain unable to synthesise exotoxin, but synthesising a highly active δ-endotoxin as the 'donor' strain could be rid of exotoxin directly. Such an approach was validated by the fact that a gene for a 32kDa protein (not a toxin) was found to be lost preferentially during heat curing of plasmids (Aileen Van Nguyen, PhD thesis). Plasmid cured colonies were first examined by phase-contrast microscopy to make sure crystal producing ability was retained and then tested for exotoxin production by bioassay.

3.2.1(ii) Conjugation

This approach was used to transfer DNA from a donor strain of *B. thuringiensis* synthesising a highly active toxin to an exotoxin negative, recipient strain of *B. thuringiensis* in such a way as would occur in Nature. Any resultant strain would therefore stand a good chance of being approved for release into theenvironment by the appropriate regulatory bodies. The problem with such an approach proved to be a quick, reliable way in which to distinguish donor from recipient and transcipient colonies following conjugation without the use of plasmid antibiotic markers.Two possible means by which this could be achieved were devised and tested:

(a) *Antibiotic Selection on the basis of minimal inhibitory concentrations (MICs).*

Many different bacterial species are inherently resistant to certain inhibitory compounds.It is well known that some species will synthesise compounds toxic to susceptible, or less resistant species. This puts such species at a competitive advantage in terms of survival, especially in situations where essential nutrients are limiting i.e. in a contained, finite system such as an agar plate or an insect larva. It would be highly desirable if the recipient strain was resistant to an antibiotic and the donor strain was

not. Following conjugation, the cells could be plated out onto agar plates containing the antibiotic and only recipient and transcipients would be able to grow. As the donor and recipient strains are of the same species and as such, highly homologous, this would be unlikely to occur naturally. All strains of *B. thuringiensis* are penicillin resistant but it would be feasible that the degree of resistance could differ between strains. This would be reflected by differences in the minimal inhibitory concentrations of penicillin between strains. Another possibility was that these strains could have differing degrees of resistance to other antibiotics such as chloramphenicol and streptomycin. Strains 4412 and 13B were selected as potential 'donors' as had been previously shown to synthesise a highly active crystal protein. Strain 10B was selected as the 'recipient' as it was shown to be exotoxin negative and originally thought also to be enterotoxin negative. In order to determine whether or not it was possible to transfer crystal-producing ability from a donor to recipient strain, an acrystalliferous recipient strain was required. This was achieved by 'plasmid curing'.

(b) *Selection against donor strain achieved by growth in non-permissive growth medium.*

It was discovered that the novel isolates varied in their ability to sporulate on a particular medium (BFB). All, however, could produce good vegetative growth. This could be taken advantage of if the donor strain was unable to sporulate and the recipient strain (a *cry-* mutant) sporulated well in a particular medium. It would mean that if conjugation was carried out in such a medium and the resultant cells allowed to sporulate, only recipient and transcipient cells should have the ability to produce heat - resistant spores. In this way, after heat shock, there would be no donor cells present and transcipients (of Cry^+ phenotype) could be subsequently identified by phase contrast microscopy. These would be recipient cells which had gained the ability to produce ICP from the donor i.e. they would have gained the blowfly-toxic gene.

3.2.2 Materials and Method

a) β-exotoxin Assessment

The protocol followed was as described in section 3.1. Individual colonies from 44 plates grown at 42^0C were bioassayed for exotoxin activity.

b) Conjugation

(Based on method of Jarrett *et al.*, 1990). 'BFB' medium is composed of:
Blood, fish and bone-meal (B&Q) 18g/ l
Chickpea flour (Holland and Barrett) 18g/ l
Molasses (Holland and Barrett) 5ml/ l
To make solid media Technical Agar (Oxoid) was added at 1.5%(w/v).

Donor and recipient colonies were grown separately in boiling tubes containing BGM or BFB growth media (5ml), respectively. These were incubated overnight for approximately 18h at 30^0C. Aliquots of donor and recipient cultures 100 µl) were

added to fresh medium (5ml). Aliquots of each (100 µl) were added separately to fresh medium as a control. These were incubated at 30°C, 40 r.p.m for 24hrs, then at 200 r.p.m until sporulation was complete. Samples of culture (1ml) were transferred to Eppendorfs tubes (1.5ml) and heat-shocked at 60°C for 10 minutes. Serial dilutions were made and these plated out onto BGM or BFB agar plates then incubated at 30°C overnight.

c) Minimal Inhibitory Concentration (MIC) determination -liquid medium

B. thuringiensis strains AB, 4412 and 13B- were grown on BGM + CCY plates at 30°C overnight. A single colony was then resuspended in sterile BGM medium and an OD_{600} measured, then adjusted to a value of 0.6. An inoculum (10µl) was added to BGM + CCY test tubes (5ml) containing different volumes of a filter sterilised solution of penicillin G (10mg/ml). Penicillin concentrations tested ranged from 0 - 500 International units/ml. The tubes were then incubated at 30°C overnight and growth examined the next day.

-antibiotic plates.

Lawns of novel strains 10B and AB (medium activity isolates - potential recipient strains), 4412 and 13B (high activity isolates - potential donor strains) were spread over BGM agar plates containing either a 1/50 concentration or a 1/100 concentration of a penicillin/streptomycin (5,000 I.U./ ml and 5mg/ ml, respectively) mixture (Sigma) or chloramphenicol (CAP) at 5µg/ ml. Control lawns were also spread onto BGM plates with no antibiotic present.

(d) 'Total' plasmid curing to remove the ability to produce ICPs

Strain AB, which was to be the recipient strain, was streaked out to single colonies on BGM + CCY agar plates, incubated at 42°C until sporulation was complete (as determined by phase contrast microscopy) and then restreaked and incubated at 30°C, again until sporulation. Colonies were then microscopically examined for the absence of crystalline inclusions in the spore mother cell (mutants with the *spo+ cry-* genotype). Sometimes, several rounds of curing were required to result in the disappearance of crystals. A better method involved incubating at 42°C for 24h and then transferring the plates to 30°C directly. In this way it was possible to determine the phenotype immediately.

(e) δ-Endotoxin assessment of cured strains

Bioassays were carried out as detailed in Chapter 2.

(f) Partial plasmid curing to lose the ability to produce β-exotoxin

Strains were streaked out onto nutrient agar plates supplemented with CCY salts (Stewart *et al.* 1981). Plates were incubated at 42°C until sporulation was complete

Individual colonies were then examined microscopically for the presence of crystal inclusions. Those colonies retaining crystal production were restreaked onto Nutrient Agar and grown at 30^0C, then re-examined for the presence of crystal inclusions.

(g) Conjugation using antibiotic selection

1.Three boiling tubes containing 5ml of BGM + CCY were inoculated with either donor strain 4412 pBC16 (Tet^R) , 4412 pC194 (Cap^R) or recipient strain 10B *cry-*. These were incubated overnight at 30^0C.
2. The OD_{600} of each tube was measured and, where necessary, made equivalent.
3. A sample (100µl) of donor and recipient was added to fresh BGM + CCY medium (5ml) and incubated at 30^0C with very gentle shaking (40 r.p.m) for 24h. A sample (100µl) of donor and recipient cultures were also added to fresh BGM + CCY (5ml) and incubated separately as controls.
4. A dilution series was performed for each tube (recipient strain 10B cry-, donor strains 4412 pBC16 and 4412 pC194 alone ; strain 10B *cry-* + strain 4412 pBC16 ; strain10B *cry-* + strain 4412 pC194) and appropriate dilution(s) plated out onto BGM + CCY and/ or BGM + CCY + CAP/TET plates. These were incubated at 30^0C overnight and examined the next day.

(h) Electroporation

From Bone & Ellar (1989), modified by S. Gash (pers. comm.).
1. A recipient lawn of isolate 4412 was grown on BGM, overnight at room temperature.
2. Sterile water (2ml) was added to the lawn and the bacteria scraped off into an eppendorf tube.
3. The tube was centrifuged for 10 secs. and the resulting pellet resuspended in electroporation buffer (272mM sucrose ; 8mM HEPES pH 7.4 ; 15% glycerol).
4. Centrifugation was repeated followed by resuspension in electroporation buffer (120µl per intended electroporation). The electroporator (Bio-Rad, with Pulse controller) was set at 400W ; 25mF ; 1.8 kV)
5. Plasmid DNA solution(1ml) was added to 120µl of 4412 recipient cell suspension in 0.4 cm gap electroporation cuvettes (Bio-Rad).
6. Electroporation was carried out then cuvette contents transferred to a boiling tube containing 1ml of LB and incubated at 30^0C, 200 rpm for 40 mins.
7. Chloramphenicol (CAP) was added to the tube at a concentration of 5µg/ml, and the incubation continued for a further 20 min.
8. Dilutions were plated out onto LB + CAP (5mg/ml) or LB + TET (25µg/ml) plates and these incubated overnight at 30^0C.
NB. Recipient lawns incubated overnight were left for no longer than 17 h before electroporation was carried out.

3.3 RESULTS

3.3.1 (a) Conjugation: selection against donor by growth on a medium inconducive to sporulation

Initial observations of the variablity of sporulation and ICP formation in BFB medium are shown in the tables, below. All strains were able to produce excellent vegetative growth in this medium. Cultures exposed to a heat shock (after an incubation period adequate for sporulation to be completed) will only give rise to colonies if spores have, indeed, been formed. Thus strains with a decreased capacity for sporulation in this medium will give rise to fewer colonies than those able to sporulate successfully.

Table 3.2.1 Microscopic analysis of strains of *B.thuringiensis* grown in BFB medium with and without CCY salts.

Strain	Liquid medium	
	BFB medium minus CCY salts	BFB medium plus CCY salts
Isolate 13B	A few spores and crystals	Many spores and crystals
Strain 4412	Crystals, no spores	Crystals, v. few spores
Isolate 10B cry-	A few spores	Many spores and crystals
Strain	**Solid medium**	
Isolate 13B	Spores and crystals	Many spores and crystals
Strain 4412	v.few spores and crystals	Few spores and crystals
Isolate 10B cry-	many spores, few crystals	Spores and crystals

Table 3.3.2 Plate counts (10^{-6} dilution) of strains of *B.thuringiensis* grown on BFB medium with and without CCY salts.

Strain	BFB medium - CCY salts	BFB medium+ CCY salts
Isolate 13B	156	170
Isolate 4412	38	52
Isolate 10B	276	>300

The results obtained from the preliminary experiments strongly suggest that when incubated in BFB medium strain 4412 sporulates very poorly. Isolates 13B and 10B are able to sporulate more efficiently, with the latter yielding the most spores. This is true in either the presence or absence of CCY salts, although all three strains fare better when CCY salts are included in the growth medium.

3.3.1 (b) Conjugation and selection of *B. thuringiensis* transcipients grown in BFB medium.

Experiments involving the conjugational transfer of plasmid DNA were subsequently carried out using isolate 4412 as donor and the plasmid-cured acrystalliferous mutant strain, 10B *cry*-, as recipient. The conjugation method described in 3.2.2 (b) was used. As a direct consequence of the results obtained from the preliminary experiments, a method was devised to separate the donor from recipient strain following conjugation. It was so envisaged that only recipient and transcipient colonies would ultimately survive and these could be distinguished by phase-contrast microscopy. 4412 was chosen as donor in preference to 13B as it contains only one type of *cry* gene, *cry* IB(a), whereas 13B contains additional *cry* genes (inferred from SDS PAGE analysis, see previous chapter). In addition, strain 4412 produces characteristic bipyramidal crystals. It was hoped that in transcipient cells the crystals would retain this conformation, facilitating the screening of large numbers of colonies.

It was discovered that donor colonies still had the ability to grow on the final plates following the heat- shocking treatment i.e. strain 4412 was sporulating in the 'non-permissive' growth medium. In an attempt to eliminate the donor strain, a more intense heat-shock was applied (70^0C for 30 min.) but this made no significant difference. The experiment was then again repeated, but higher dilutions plated out as it was assumed that even though the donor strain was sporulating, the level at which it was doing so was likely to be much less than that of the recipient/transcipient strain. This dilution idea, however, also proved unsuccessful and it was concluded that the donor strain could not be successfully 'weeded out' using this approach.

There was one useful observation resulting from these experiments in that 4412 donor and isolate 10B *cry*- recipient colonies were of differing morphology when grown on either BFB or BGM plates: the latter strain produced colonies with ragged edges, those of the former strain were very crisply defined. This clear difference in colony morphology was thought to be a potential method for distinguishing donor from recipient colonies (as opposed to the traditional method of exclusively preventing the growth of the donor colonies). In such a way, it would be possible to then identify colonies with isolate 10B type morphology and screen these for the presence of crystals i.e. identify the transcipients.

A possibility creating cause for concern was the fear that there was no DNA transfer (or a very small, undetectable amount) occurring at all. An initial approach was consequently devised to (i) demonstrate DNA transfer was in fact taking place, (ii)separate out donor colonies on the basis of differing morphology and (iii) show that crystal producing ability was specifically being transferred to the recipient strain. Were the results of this approach encouraging the antibiotic selection could be omitted so that the desired transcipient could be sought using methods not requiring genetic manipulation. In order to demonstrate DNA transfer was occurring, plasmids pBC16 (Tet^R) and pC194 (Cap^R) were first isolated from P. Jarrett's strain of *B. thuringiensis* subsp. *aizawai* and *B. subtilis* 168, respectively, using the alkaline lysis method (section 4.2.2). These were then separately electroporated into donor strain 4412.

Colonies to which either antibiotic resistant plasmid had been transferred (those growing on the antibiotic plates) were subsequently conjugated with the acrystalliferous recipient strain 10B using using the antibiotic selection method as described in 3.2.2 (g). Selection for tetracycline resistance was made at 25μgml^{-1}.

It was discovered that when grown separately (i.e. in the control tubes), an equivalent number of donor 4412 and recipient 10B *cry-* colonies resulted. The strains could be seen to differ in morphology as strain 4412 produced small, well-defined round colonies whereas strain 10B colonies were larger with ill-defined, ragged edges. When grown together in order to allow conjugation to take place, however, no colonies of isolate 10B type morphology resulted on BGM + CCY + TET/CAP plates. This could have meant that the antibiotic resistant plasmids were not being transferred from donor to recipient ie. no DNA transfer had taken place. Colonies of isolate 10B type morphology did, however, grow on the BGM + CCY plates, but in a number significantly less than that of 4412 colonies.

From this, it was concluded that perhaps strain 4412 had some kind of competitive advantage over isolate 10B since when cultures of identical optical density were incubated together, 4412 colonies greatly outnumbered 10B colonies. It would be reasonable to suggest that this could possibly be due to the fact that strain 4412 is exotoxin positive while strain 10B exotoxin negative. It has been previously shown that β-exotoxin inhibits the polymerase activities of strains of both *Escherichia coli* and *B.thuringiensis* (Johnson, 1978). It could well be imagined that this toxin could effect the replication of a non-producer, such as strain 10B, thus giving the producing organism, strain 4412, a competitive advantage.

Following this assumption, the conjugation method previously described was modified slightly. The 4412 donor cultures were diluted 5 × and 25 ×, before 100µl of each was added to 100µl of recipient strain 10B *cry-* and conjugation allowed to take place. Strains were again distinguished on the basis of differing colony morphology, the results obtained were as shown below.

Table 3.3.3. Controls of electroporation experiment

Ratio	4412 pBC16 (dil. 10^{-4})		4412 pC194 (dil. 10^{-4})		10B *cry-*(dil. 10^{-4})
	BGM+CCY	BGM/CCY/ TET	BGM+CCY	BGM/CCY/ CAP	BGM+CCY
	>300	273	>300	>300	>300
5:1	>300	218	>300	>300	>300
25	>300	191	>300	>300	>300

It was observed that a greater number of colonies were present when the antibiotic resistant strains of 4412 were plated out onto BGM + CCY as opposed to BGM + CCY + TET/CAP. This was put down to the fact that as the strains were incubated in

would be no selective pressure or advantage in exhibiting antibiotic resistance. When conjugation cultures of strain 4412 pBC16 + 10B *cry-* were plated onto BGM + CCY + TET no growth of either strain on any of the BGM + CCY + TET plates was observed.

Table 3.3.4. Conjugation culture plated onto BGM + CCY

Ratio	4412 pBC16 + 10B *cry-* (dil. 10^{-4})		4412 pC194 + 10B *cry-* (dil. 10^{-4})	
	10B morphology	*4412 morphology*	*10B morphology*	*4412 morphology*
	22	188	56	200
5:1	>350	0	292	65
25:1	>350	0	360	10

Table 3.3.5. Conjugation culture plated onto BGM + CCY + CAP

4412 pC194 + 10B *cry-* (dil. 10^{-4}) colonies					
1:1 ratio		5:1 ratio		25:1 ratio	
Strain 10B morphology	*Strain 4412 morphology*	*Strain 10B morphology*	*Strain 4412 morphology*	*Strain 10B morphology*	*Strain 4412 morphology*
0	218	2	78	ND	ND
0	238	2	71		
0	280	0	65		
0	230	4	75		
0	292		83		
0	280	1	145		
0	222		116		
0	224	10	70		
0	300	0	68		
0	380	3	87		
0	218		87		
0	252	0	104		
1	70	16	50		
	44	4	52		
0	200	4	70		
2	103		67		
	300	0	83		
0	289	0	70		

Determination of Transformation Efficiency:
Total number of strain 10B *cry-* (BGM+CCY plates) = 292 colonies/ plate
(This represents 10B *cry-* CAP^R + 10B *cry-* CAP^S)

Number of strain 10B *cry-* (BGM + CCY + CAP plates) = 50 colonies/ 18 plates
= 2.78 / plate

Therefore the percentage 10B *cry-* CAP^R = 2.78/ 292 × 100 = 0.95%

The results show that plasmid pC194 (Cap^R) has been transferred from the 4412 donor to isolate 10B *cry-* recipient as colonies of 10B - type morphology were present on the BGM + CCY + CAP plates. DNA transfer has taken place. Subsequently, these colonies were examined by phase contrast microscopy for the presence of crystals in order to ascertain whether or not the plasmid(s) for crystal-producing ability had been transferred along with that (those) for antibiotic resistance. None of the isolate 10B *cry-* colonies appeared to contain convincing parasporal bodies. In view of the very large size of the plasmids of *B. thuringiensis* (Gonzalez *et al.* 1981) one would expect that transformation by conjugation of ICP-encoding plasmids would be a very rare event. There can be little doubt that it is possible, nevertheless, as the profusion of strains of *B. thuringiensis* carrying similar protoxin genes would seem to testify.

3.4 Plasmid curing to lose the ability to produce β-exotoxin

The development of a strain lacking the ability to synthesise exotoxin by the conjugational approaches previously described is a time-consuming task and the frequency of transfer of plasmids carrying the *cry* genes may be low. In addition, there is no guarantee that these genes will be transcribed and then translated into crystal proteins which retain the same high activity as they exhibited in the donor strain. Alternatively, plasmid curing could be used to achieve the same end result as β-exotoxin is thought to be plasmid encoded (Levinson *et al.*, 1990). This would provide a rapid and simple means of obtaining a strain unable to synthesise exotoxin, but synthesising a highly active δ-endotoxin. Such an approach was validated by the fact that a gene for a 32kDa protein (not a toxin) was found to be lost preferentially during heat curing of plasmids over plasmids encoding *cry* genes (Aileen Van Nguyen, PhD thesis). Plasmid cured colonies were first examined by phase-contrast microscopy to make sure crystal producing ability was retained and then tested for exotoxin production by bioassay.

Two separate attempts were made to 'cure' strains of the ability to produce β-exotoxin: the inital attempt gave rise to several mutants which were shown conclusively by bioassay to have lost the ability to produce β-exotoxin. These mutants retained the ability to produce ICPs and were, in fact, used in the studies on fermentation media (Chapter 5). During a programme of sub-culturing, designed to check the stability of these mutants and which took place over a period of about 9 months these strains slowly regained the ability to produce β-exotoxin. An attempt to explain this very unexpected occurrence is made later in this chapter.

During the re-emergence of the production of β-exotoxin in the mutants obtained in the first round of curing a second attempt was made. The mutants derived from this attempt were studied more closely, the results are tabulated in Table 3.4.1. Of the 44

colonies assayed 9 appeared to have lost the ability to produce β-exotoxin, as determined by larval survival through to adult emergence. Colonies which were deemed β-exotoxin minus were restreaked to assess strain stability. These were then re-assessed by bioassay. Only three of the cured strains remained β-exotoxin minus (A1, S3 and S9). Larvae exposed to A1 and S9, however, grew more slowly and pupated at significantly lower body weights than those exposed to S3 or the control. This perhaps suggests that a reduced level of β-exotoxin production had resumed in these two cured strains.

The δ-endotoxin activity of these cured strains was then assessed (see Table 3.2.2). δ-Endotoxin activity equal to that of the parent strain was present in strains A1 and S9. δ-Endotoxin activity was absent in S3.

Repeated β-exotoxin assessments using bioassays for the plasmid cured strains indicated that β-exotoxin production increased with each subsequent generation in strains A1 and S9, but remained absent in strain S3 (see Table 3.4.1). The δ-endotoxin activity in strains A1 and S9 was also found to increase with the increase in β-exotoxin production (see Table 3.4.2). As mentioned above β-exotoxin production was also found to resume in colonies from plates which represented earlier generations originally found to be β-exotoxin minus (data not shown).

Table 3.4.1: β-Exotoxin Assessment of Plasmid Cured Strains

Cured Strain	Plate 1		Plate 2 (restreak)		Plate 3 (restreak)		Plate 4 (restreak)	
	Exo. Status	[a]Larval Weight (mg)	Exo. Status	[a]Larval Weight (mg)	Exo. Status	[a]Larval Weight (mg)	Exo. Status	[a]Larval Weight (mg)
A1	-	4.1	-	3.7	+	3.0	++	2.5
D1	-		+++	1.9	+++	1.9	+++	1.8
D3	-		+++	2.1	+++	2.0	+++	2.1
D5	-		+++	1.6	+++	1.6	+++	1.7
D6	-		+++	2.0	+++	1.9	+++	2.0
E2	-		+++	2.1	+++	2.1	+++	2.1
E3	-		+++	1.6	+++	1.6	+++	1.6
S3	-	4.4	-	4.1	-	4.4	-	4.3
S9	-	4.5	-	3.8	++	2.6	+++	1.9
Controls								
Btt	-	4.4	-	4.1	-	4.3	-	4.4
4412	+++	1.8	+++	1.8	+++	1.9	+++	1.8

Larval weight represents the mean larval weight calculated from the gross weight of 20 larvae recorded at 48 hours.
β-Exotoxin production absent : determined by normal larval growth and development through to adulthood.

+ Some β-exotoxin production likely : determined by reduced larval growth and failure to pupate.

++ Increased β-exotoxin production : determined by further reduction in larval growth and failure to pupate.

+++ Confirmed β-exotoxin producer : determined by severe effect on larval growth and death at instar change.

Table 3.4.2: δ-Endotoxin Activity of Plasmid Cured Strains A1, S3 and S9.

Cured Strain	Plate 2 (restreak)		Plate 3 (restreak)		Plate 4 (restreak)	
	Endotoxin Status	[a]Larval Weight (mg)	Endotoxin Status	[a]Larval Weight (mg)	Endotoxi nStatus	[a]Larval Weight (mg)
A1	+	3.3	++	2.6	++	2.0
S3	-	4.3	-	4.4	-	4.3
S9	+	3.3	++	2.5	+++	All dead
Btt	-	4.5	-	4.4	-	4.4
4412	+++	All dead	+++	All dead	+++	All dead

Dose rate used was that which would result in 100% mortality with the parent strain.

[a] Larval weight represents the mean larval weight calculated from the gross weight of 20 larvae recorded at 48 hours.

+ Low activity : some reduction in growth in comparison to the negative control. Larvae pupated but adults failed to emerge.

++ Medium activity: Further reduction in growth. Some larvae pupated, many died prior to pupation.

+++ High activity: lethality equivalent to that of the parent strain at the same dose rate.

3.4.3. Discussion

The plasmid which encodes for β-exotoxin production can be removed by plasmid curing. From our results, however, elimination of the capability to produce β-exotoxin, in some cases, also detrimentally affected the δ-endotoxin activity. This is in partial agreement with the findings of Levinson *et al.* (1990). These authors, however, found that in the cases they studed all β-exotoxin minus mutants were also devoid of ICPs (*'cry-' genotype*). Prolonged exposure to elevated temperatures will, as this report, for example, shows, cause all plasmids to be lost. In this instance we used limited exposure to elevated temperature in the hope of obtaining partial plasmid loss. In the case of S3, crystal proteins were still produced at sporulation but activity towards our target insects was no longer present. It may be that only the CryIB toxin is absent and that other toxins remain complete. If this is so, then it may be possible to remove the β-exotoxin plasmid and retain the CryIB activity using plasmid curing. Some of the initial

β-exotoxin minus mutants that we isolated retained comparable activity in their ICPs with the parent strains. These, along with the other such mutants that we isolated regained the ability to produce β-exotoxin with successive sub-cultures. This observation is hard to explain. One explanation could be that the initial heat-cured population was not entirely homogenous: a small proportion of parental-type, β-exotoxin producers may have remained. If the ability to produce β-exotoxin confers a selective growth advantage over non-producers, as is hypothesized above, 3.3.1.(b), the former type will, upon sub-culture, become more prevalent in the population. This would explain the apparent re-appearance, over time, of the ability to produce β-exotoxin in a population which was thought to have lost this ability. Alternatively, the observed initial absence of β-exotoxin production in the cured strains A1 and S9 is difficult to explain. It is possible that the β-exotoxin production is switched off when the organism is under extreme stress as occurs during growth at elevated temperatures.

Attempts to transfer the plasmid carrying the δ-endotoxin gene of strain 4412 to a strain incapable of producing β-exotoxin and enterotoxin were unsuccessful. The initial stumbling block in the conjugation process was the effective removal of the donor cells from the population after any genetic transfer had taken place. The approaches that we devised to circumvent the inability to use any sort of antibiotic selection produced by genetic manipulation were unsuccessful. Control experiments showed that the conditions that we used were suitable to facilitate conjugation. It must be borne in mind, however, that the antibiotic resistance plasmids that were transfered are small. In contrast the plasmids of *B. thuringiensis* that carry the δ-endotoxin genes of *B. thuringiensis* are very large (Gonzalez *et al.*, 1981; Carlton and Gonzalez 1985). Since ease of transfer is inversely related to plasmid size one might have to be rather optimistic (or have a very good selection method) to be able to isolate transconjugants of *B. thuringiensis* plasmids with ease.

3.5. Mammalian toxicity assessment

The mammalian toxicity assessment was carried out under Home Office Licence number PPL 30/1130. Whilst the toxicological safety of *B. thuringinesis* has been well studied (see Drobniewski, 1994), its previous applications have been for non-food use[1]. In view of this it was considered necessary, ethically, to establish the toxicity status of our strain.

3.5.1. Materials and Methods

a) Toxicological Study

A total of fifty female rats were used for the study. Exposure was via the oral or sub-cutaneous routes. The oral dose comprised 10^{12} spores administered daily for 21 days. The sub-cutaneous dose involved a single injection of 10^{6} spores, administered on Day 1 of the treatments. Controls were provided by equivalent doses of autoclaved preparations. Ten rats were used in each treatment group. A further ten untreated rats

[1]Plants intended for human consumption are not classified as food until after harvesting.

were used as a control against the handling involved in administering the treatments. Rats were kept for seven days prior to commencing the treatments to ensure all animals were healthy.

Treatments were carried out by trained personnel in accordance with Home Office legislation. Rats exposed via the oral route were orally intubated and the contents of one mini-centrifuge tube administered by injection into the stomach via the tube. Rats exposed via the sub-cutaneous route were injected into the fat pad situated dorsally at the base of the neck.

All rats were maintained for 23 days from the commencement of the treatments

b) Preparation of inoculum

Strain 13B was grown up and harvested as detailed in section 2.1.1. The concentrated spore/crystal mix was held on ice at 4^0C until the population density of viable cells could be assessed by plate counts. The spore/crystal concentrate was then resuspended in sterile distilled water to give a final concentration of 10^{12} spores ml^{-1}. Half of this stock was autoclaved to provide a control. A total of 210 aliquots (1ml) of each stock, representing a single oral dose, were then dispensed into labelled, sterile mini-centrifuge tubes (Eppendorf). Tubes were batched in tens (a single day's treatment per batch of rats) placed in plastic bags and immediately placed in a freezer at -20°C until needed. Both stocks were continuously stirred on magnetic stirrers whilst the aliquots were being dispensed.

The remaining stocks were further diluted with sterile distilled water to give a final concentration of 10^6 spores ml^{-1}. A further 10 aliquots(1 ml), representing a single sub-cutaneous injection dose, of each stock were dispensed into labelled, sterile mini-centrifuge tubes (Eppendorf). These were again batched and placed in a freezer at -20°C until needed.

c) Post-treatment analysis

On completion of the treatments rats were weighed on a top pan balance in groups of five, each group having undergone the same treatment. They were then sacrificed by cervical dislocation.

Full post-mortem examinations were conducted by a veterinary surgeon experienced in pathology. Blood samples were collected from the heart using sterile needles with heparinised vacutainer tubes. The following organs were removed for culturing for the presence of *B. thuringiensis*: liver, heart, spleen, kidneys, lungs, section of small intestine, and in those animals exposed via the sub-cutaneous route the fat pad was also removed. All organs were incised after removal and examined for pathological abnormalities. Organs were pooled according to exposure and mean weights were recorded using a top-pan balance. Organs were held on ice at 4^0C until results from cultures were obtained.

With the exception of the viscera, organs were homogenised in sterile Ringers solution (Oxoid) and plate counts from the homogenate carried out using nutrient agar plates. Blood samples were mixed by inverting several times then 1 ml samples spread on nutrient agar plates. Viscera were incised using a sterile blade and the contents emptied into 10 ml aliquots of sterile distilled water. Plate counts from serial dilutions were carried out. These dilutions were then heat shocked at 80^0C for 10 minutes to eliminate any vegetative cells as well as the non-spore forming bacteria naturally present in the gut. Comparative plate counts were carried out on the heat shocked dilutions.

3.5.2. Results

Table 3.5.1 details the mean weights recorded for total body weights and organs pooled by treatment. Total body weight for control rats was lower than that recorded for the other groups. In contrast, all weights recorded for organ types were heavier than for any of the other groups. Whilst this may suggest that organ development was retarded by the treatments carried out this does not explain the lower total body weight recorded. It is possible that organs were not removed with the same degree of presision between treatment groups - a reduction in tissue recovered resulting in a reduction in weight recorded. Alternatively, the difference may be explained by biological variations between animals. If the weight difference was directly related to the exposure to *B. thuringiensis*, one would expect to see enlargement of the spleen and thymus with a concurrent weight increase. As this was not the case, it appears unlikely that the weight differences observed are a factor of the treatments carried out.

All animals were in excellent body condition. This, coupled with total body weights above those of the controls, indicates that exposure to the organism did not (a) affect feeding or the general health and well-being of the animals, or (b) result in large energy expenditure in clearing the organism from the animal's system. The increased weights recorded for treated animals over the untreated control animals suggests that no adverse effects resulted from handling the animals or administering the treatments.

Post-mortem examination did not reveal any gross abnormalities. There were no visible tissue changes, oedema or localised inflammation in any of the organs from any of the rats. The following points are of particular relevance with relation to the route of exposure: (i) in those animals exposed via the oral route, the gut contents were normal and there was no evidence of gut inflammation or luminal fluid accumulation; this indicates that the organism did not cause infection or a local reaction during its passage through the intestinal tract, nor did it appear to affect the gut transit time of faecal matter, and (ii) in animals exposed by sub-cutaneous injection there was no localised reaction around the injection site nor any swelling in the lymph nodes responsible for draining the area. This indicates that the organism did not appear to cause either a local or more general infection and was rapidly cleared from the site of exposure.

B. thuringiensis was not isolated from any of the tissue samples taken, with the exception of the visceral contents of orally treated animals (see Table 3.5.2). This confirms that the organism was rapidly cleared from the body of those animals exposed by sub-cutaneous injection, and there was no evidence to suggest that the organism crossed the intestinal wall following oral treatment.

The number of colony forming units isolated from the visceral contents following heat shocking was lower than was found prior to heat shocking, but there was no significant difference between those rats given oral doses of active *B. thuringiensis* and the controls. This suggests that the colonies destroyed by heat shocking were non-spore forming gut flora and not vegetative cells of *B. thuringiensis*.

Table 3.5.1: Table of mean weights of total body and of organs for rats following each of the procedures.

Dose Type	Route	Mean Weight (g)						
		Total body	Heart	Lungs	Liver	Kidney	Spleen	Thymus
Active	Oral	181.5	1.28	2.30	8.67	2.31	1.07	0.89
Autoclaved	Oral	182.7	1.52	2.22	7.91	2.07	1.35	1.13
Active	Sub-cut.	179.3	1.33	1.82	8.13	2.35	1.19	1.12
Autoclaved	Sub-cut.	181.6	1.13	2.31	8.56	2.08	1.20	1.23
None	None	175.4	1.82	2.78	8.84	2.84	1.92	1.86

Weights are means of ten pooled samples.

Table 3.5.2: Isolation of *B. thuringiensis* from tissue samples

	Dose Type				
	Active	Autoclaved	Active	Autoclaved	None
Route	Oral	Oral	Sub-cut	Sub-cut	None
Heart	x	x	x	x	x
Lungs	x	x	x	x	x
Liver	x	x	x	x	x
Kidney	x	x	x	x	x
Spleen	x	x	x	x	x
Thymus	x	x	x	x	x
Blood	x	x	x	x	x
Fat Pad	ND	ND	x	x	x
Visceral Contents	+	x	x	x	x

x = No *B.t.* isolated + = *B.t.* isolated ND = Not Done

3.5.3. Discussion

Our findings are in keeping with the other studies have been published concerning the toxicity to mammals of entomopathogenic bacilli administered by various routes. Not

surprisingly toxicity can be demonstrated by intraperitoneal (Lamanna and Jones 1963 Siegel and Shadduck 1990) and intracranial injection (Siegel *et al.* 1987). These are obviously not realistic routes of entry from normal exposure of bioinsectides. Clearance of bacteria from such challenging sites of infection seems to be slower if both vegetative and spore forms are present (Lamanna and Jones 1963; Siegel and Shadduck 1990). Clear evidence for multiplication of bacteria is debatable, however.

No reports exist of fatality following oral nor subcutaneous administration of *B. thuringiensis* although the latter route of exposure was lethal when spores of the closely related *B. anthracis* were injected (Lamanna and Jones 1963). Oral dosage (4 x 10^7 spores) of *B. thuringiensis* subsp. *israelenesis* to mice gave rise to no illness in the subsequent seven days (Siegel *et al.* 1987). The feeding of a commercial preparation of *B. thuringiensis* (DiPel) to sheep over five months gave rise to insignificant physiological changes in comparison to controls: no change was seen with respect to body weight nor food consumption (Hadley *et al.* 1987). Considering the differences in digestive system the relevance of this finding to humans should, perhaps, not be over-emphasised. The fact that no ill effects were observed from sub-cutaneous injection of rats would seem to be an observation which could be extrapolated to human safety. Exposure of humans to spores of *B. thuringiensis* through cuts or abrasions in the skin appears to be without undue risk.

These results would indicate very strongly that there was no risk to humans from the ingestion of large numbers of spores of *B. thuringiensis*. The oral dose given dailyto the rats (10^{12} spores) represents a very significant challenge. Furthermore, in terms of dosage/ kg body weight this would be massively above any dosage which would conceivably be consumed by a human. The strain used (13B) has been shown to be a producer of β-exotoxin. It is also a producer of *B. cereus*-type enterotoxin, see below. The complete absence of deleterious effects to rats seems hard to reconcile with the production of these toxins. The only plausible explanation is that spores of *B. thuringiensis* do not germinate in the gut of the rat and are thus precluded from producing these toxins. The use of rats as models for the safety assessment of oral dosage of *B. thuringiensis*to humans must, therefore, be seriously questioned.

Few reports exist of human trials although Fisher and Rosner (1959) exposed humans to doses of a commercial preparation of *B. thuringiensis* strain HD-1 orally and by inhalation over five days. There were no adverse clinical indications in the subjects nor in laboratory tests on them. This strain was indicated in both of the tests that we used to be an enterotoxin producer. Conceivably the level of toxin produced is too low to cause symptoms of food poisoning or else other factors such as diet are important. In contrast most of the volunteers eating food contaminated with a different strain, *B. thuringiensis* subsp. *galleriae*, showed signs typical of food poisoning following an incubation time of eight hours (Ray 1991). The possibility that the symptoms were due to b-exotoxin were not ruled out. This toxin is not produced by strain HD-1. It is, however, produced by the strain used in the toxicity tests in this report. The fact that we found no evidence of germination of these spores in rats would negate any ability to produce toxins which only occur in the vegetative form.

3.6. Enterotoxin Assessments

3.6.1. Materials and Methods

a) Bacterial Strains and Growth Conditions

The following strains were cultured for enterotoxin assessment:
six novel isolates (13B, 1B, 3B, AB, 3A, 10B)
two plasmid cured mutants (13BA1, 4412E)
five commercial strains (4412, HD-1, HD-2, *B. thuringiensis* var. *tenebrionis*, *B. thuringiensis* var. *israelensis*).
Strains were grown up in Brain Heart Infusion Broth (Oxoid) supplemented with 10g/l of glucose, at 30^0C for 12 hours at 200 rpm. Cultures were centrifuged at 3,400 r.p.m. for 10 minutes at 4^0C in an MSE Mistral 3000E centrifuge. The supernatants were filtered through 0.22 μm Millex-GV filters (Millipore SA. France).

b) Assay

Enterotoxin status was initially assessed using a commercially available reversed passive latex agglutination (RPLA) assay (Unipath BCET-RPLA TD950). The protocol followed was that supplied by the manufacturers.

A selection of these strains were submitted to the Central Public Health Laboratory Service, Collingdale, for independant verification of results. These comprised: all commercial strains with the exception of HD-2; the novel isolates 13B and AB; two of the plasmid cured mutants 13BA1 and 4412E. The assays used by the CPHLS are the RPLA kit (Unipath BCET-RPLA TD950) and the diarrhoeal enterotoxin visual immunoassay (Tecra). Analysis for emetic toxin was also carried out by the CPHLS.

3.6.2. Results

All strains assessed were found to be positive for enterotoxin production but negative for emetic toxin production. Results obtained in the laboratory mirrored those obtained by the CPHLS. There was good correlation between results obtained by the CPHLS using the two different test methods. All results are tabulated in Table 3.6.1. The commercial strains *B. thuringiensis* var. *israelensis* and *B. thuringiensis* var. *tenebrionis* seem to be poorer producers than do the other strains assessed. The mutant strains appear to have increased their enterotoxin production, although how this may be genetically or biochemically possible is uncertain.

Table 3.6.1: Assessment of enterotoxin status in six novel isolates and five commercially available strains.

Strain	Laboratory	CPHLS	
	RPLA test	RPLA test (titre)	Tecra kit
13B	positive	positive (128)	
1B	ND	ND	
3B	ND	ND	
3A	ND	ND	
AB	ND	positive (2048)	
10B	positive	ND	
13BA1	positive	positive (1024)	
4412E	positive	positive (512)	
4412	positive	positive (512)	
HD-1	positive	positive (512)	
HD-2	positive	ND	
B.t. var. *tenebrionis*	positive	positive (16)	
B.t. var. *israelensis*	positive	positive (32)	

3.4.4. Discussion

Controversy exists about the reliablity of the two kits used in this study (Buchanan and Schultz 1994). These authors favoured the Tecra ELISA kit in comparison to the BCET-RPLA kit. The latter was recommended as a simple and reliable method for detecting enterotoxigenic strains of *B. cereus*, however, by Granum *et al.* (1993). False positive and negative results have been reported with the RPLA method (Day *et al.* 1994; Lund and Granum 1996). Variation between the two products was also noted by Rusul and Yaacob (1995). In this study we used both methods to determine the ability of strains of *B. thuringiensis* to produce diarrhoeal enterotoxin and found complete agreement in the limited number of strains tested. The use of an *in vitro* cell assay using transformed human colonic cells was unsuccessful (data not shown): lysis of these cells could not be correlated with the addition of preparations of enterotoxin. The only reliable method of assessing enterotoxin production is the use of ileal loops of live rabbits (Duncan and Strong, 1969), a practice now regarded as unethical.

Rusul and Yaacob (1995) assessed the prevalence of *B. cereus* in various foods. Most of the cooked and all of the dried foods that they tested contained *B. cereus*, *B. thuringiensis* or *B. mycoides*. Over 80% of the isolates tested were deemed to be enterotoxigenic. These authors suggested that more than 10^5 cfu g^{-1} food are required to cause food poisoning in humans. Granum (1993) suggested that levels of enterotoxigenic bacilli in foods above 10^3 to 10^4 cfu g^{-1} could pose a health risk. Interestingly he suggested that only strains capable of producing high levels of enterotoxin should be of concern and that their prevalence is normally quite low. The rats in this study were, however, given a dosage of about 5 x10^{10} spores/ d for three weeks. This represents a much higher dose of enterotoxigenic organism and, on a dose

per body weight basis, an even larger, relative challenge. Despite high doses of spores to rodents no reports exist of signs of food-poisoning. Our results support these findings in spite of the evidence that the strain of *B. thuringiensis* used was capable of producing the enterotoxin.

These results strongly indicate that all strains assessed are enterotoxin producers. This includes those strains which are commercially available and extensively used in agriculture and horticulture, as well as *B. thuringiensis* var. *israelensis* which is incorporated into drinking water in some temperate and many tropical countries to combat the vector borne diseases malaria and onchocerciasis. Strain HD-1 is also available for home use as a bio-pesticide for organic produce. It would seem probable that large quantities of these products are consumed on vegetable and grain produce, or in drinking water, on a regular basis without any apparent detrimental effects. Their safety must therefore be dependant upon the food source to which they are applied being unsuitable for the production of the enterotoxin *in situ*. Fish however, being rich in protein would readily support enterotoxin production. As such the possibility of utilising these strains as insecticidal preparations for application to fish can no longer be considered.

These results potentially have relevance to the traditional application of *B. thuringiensis*. With current trends for increasing popularity of organically grown foods and decreasing cooking times for vegetables a potential food-poisoning risk exists with the use of *B. thuringiensis* as a biopesticide. Some strains of *B. thuringiensis* can, obviously, produce *B. cereus*-type enterotoxin; extrapolation of safety tests using rodents to risk assessments for humans appears to be unreliable . A further problem in the use of this biopesticide on foods is that if an enterotoxigenic strain of *B. cereus* grew in, say, a dairy product to such a level that there was a risk of food poisoning the food would become unacceptable for consumption. Spraying pre-cultured spores of *B. thuringiensis* on leaf crops will not produce a comparable decline in the organoleptic qualities of the food and so it would still be considered suitable for consumption. The residual spore load that can remain on leaves shows that a potentially significant number of spores can be ingested after normal food preparation practices. Should washed salad leaves, for example, be supplemented with carbohydrate- or protein-rich foods and kept unrefrigerated the potential for replication, from an already high inoculum, is obviously great. Harvesting leaves the day after spraying was intended to show as a high a residual spore load as possible; instructions concerning a period after spraying during which the crop may not be consumed are, however, not generally given with insecticides of this type.

As public health laboratories become more conversant with the identification of *B. thuringiensis*, as distinct from *B. cereus*, it is possible that the instances of food poisoning being attributed to the former organism will increase. To ensure that biopesticides containing members of the genus *Bacillus* continue to enjoy the excellent safety record that they have to date it would seem to be advisable to ensure that existing and newly registered products are reliably tested for their potential as food poisoning agents.

With respect to this project even if a pesticide is produced for use in a bait it will have to be enterotoxin-negative. This is obvious from a point of view of the people producing it and from the possible cross contamination risk to the drying fish (perhaps from adult flies). No attempts were made to develop an enterotoxin-negative strain from a positive one as was attempted with the β-exotoxin. This was partly as a result of (a) the experience with the latter toxin, (b) the lack of a reliable assay and (c) the complex nature of the polypeptides required to produce toxicity (Lund and Granum, 1996).

Chapter 4

Genetic analysis

At the outset of the project the identity of the δ-endotoxin type found to be toxic to larvae of *M. domestica* (Hodgman *et al.*,1993) was unknown. From the established strains of *B. thuringiensis* found to be active against larvae of blowflies we deduced, by a process of elimination and using a knowledge of the δ-endotoxin types that these strains produced, that toxins of the CryIB family were involved. Since this toxin type (nor any other) had not previously been associated with this spectrum of toxicity we decided to characterise the relevant genes. A further incentive to this work was that the CryIB(a) protoxin produced by *B. thuringiensis* strain HD-12 was completely inactive, even against *M. domestica*. This was regardless of whether the native crystal or crystal which had previously solubilised or activated *in vitro* were used. The specificity of this activity was, therefore, very precise. As a corollary of this it would not be unexpected if the spectrum and potency of toxicity varied with the exact DNA sequence within the sub-family of CryIB found to be responsible. To try to ensure that we were working with the strains of *B. thuringiensis* that had the highest potency and most appropriate spectrum of activity we carried out a selective DNA sequencing programme.

As the project progressed it became known that a former colleague had devised pairs of polymerase chain reaction (PCR) primers which were specific for almost all known δ-endotoxin gene sub-families (pers. comm. Dr S. Gash, University of Cambridge). Having been given access to this information and permission to use it our task of obtaining DNA sequences and of characterising our strains was greatly facilitated.

4.1 Materials & Methods

4.1.1 Chemicals used and their abbreviations (unless stated otherwise the supplier was Sigma Aldrich)

Agarose (Type 1A Low EEO)
Agarose (NBL Gene Sciences Ltd.)
Ammonium acetate
Ammonium persulphate (AMPS)
5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal)
Chloramphenicol (CAP)
Chloroform
Dithiothreitol (DTT)
Ethylenediamine tetraacetic acid (EDTA)
Ethanol (EtOH)
Ethidium bromide
Hydrochloric acid (HCl)
Isopropyl β - D - thiogalactopyranoside (IPTG)
Magnesium chloride ($MgCl_2$)
N,N,N,'N' tetramethylethylenediamine (TEMED)
Orthophosphoric acid

Penicillin G (pen G)
Phenylmethylsulphonylfluoride (PMSF)
Phenol (Fisons)
Potassium acetate
Sodium acetate
Sodium chloride
Sodium dodoecyl sulphate (SDS)
Sodium hydroxide
Tetracycline (TET)
Trichloroacetic acid (TCA)
Tris (hydroxymethyl) - methylamine ('Tris buffer')

4.1.2 Buffers

SMM	0.5M sucrose 0.02M maleic acid 0.02M $MgCl_2$ adjusted to pH 6.5 with conc. NaOH
TBE 5× conc	Tris buffer 54.0g/l Boric acid 27.5g/l EDTA (pH8.0) 20ml/l
TE (Tris.EDTA) EDTA	Tris.Cl (pH 8.0) 10mM (pH8.0) 1mM
SDS PAGE: Gel running buffer	Tris buffer 6.0g/l Glycine 28.8g/l SDS 0.1% w/v
Gel sample buffer	SDS 0.1% w/v EDTA.Na_2 1mM (inhibits metallo-proteases) PMSF 2mM (inhibits serine-proteases) Glycerol 10% w/v Tris.Cl (pH7.5) 50mM Bromophenol blue 0.5% v/v of a 0.5% w/v soln. Fresh DTT added at 25mM (3.8mg/ml) just before use

4.1.3 Solutions prepared

SDS PAGE	30% acrylamide stock: acrylamide 300g/l bis-acrylamide 20g/l 1.5M Tris.Cl (pH8.8) 0.5M Tris.Cl (pH6.8) 10% w/v ammonium persulphate

	10% w/v sodium dodoecyl sulphate TEMED
Gel stain	Coomassie blue R 0.1% w/v AR quality methanol 50% Acetic acid 10%
Gel destain	AR quality methanol 10% Acetic acid 10%

4.1.4 DNA sequencing reagents

Annealing buffer (5x conc.)	200mM Tris.Cl (pH7.5) 100mM $MgCl_2$ 250mM NaCl
Dithiothreitol (DTT)	0.1M

Labelling nucleotide mixture (use with radiolabelled dATP):
1.5μM dGTP, 1.5μM dCTP and 1.5μM dTTP

Termination nucleotide mixtures (one for each dideoxy nucleotide)

Each mixture contains 80μM dGTP, 80μM dATP, 80μM dTTP, 80μM dCTP and 50mM NaCl.

In addition, the 'G' mixture contains 8μM dideoxy-dGTP; the 'A' mix, 8μM ddATP and the same for the 'T' and 'C' mixes.

Stop solution	95% formamide 20mM EDTA 0.05% bromophenol blue 0.05% xylene cyanol FF

Labelled dATP (Amersham):
[α-^{35} S] dATP specific activity 1000-1500 Ci/mMol

DNA SEQUENCING GEL

10x TBE	Tris base 108.0g/l Boric acid 55.0g/l EDTA.Na_2 9.3g/l
40% acrylamide stock:	acrylamide 380.0g/l bis-acrylamide 20.0g/l

0.5x TBE 6% gel mix	40% acylamide stock 15ml/0.1l 10x TBE 5mL/0.1l Ultrapure urea 46g/0.1l

25% ammonium persulphate solution (AMPS)
35% TEMED

4.1.5 Media

LB	10g/l Bacto-tryptone 10g/l NaCl 5g/l Bacto-yeast extract
BGM (Basic growth medium)	See section 2.1.1
BHI (Brain Heart Infusion)	Oxoid

4.1.6 Enzymes (Sigma)

Lysozyme
Mutanolysin
Proteinase K
RNAse A
Trypsin

4.1.7 Kits for DNA manipulation

DNA Sequencing - Sequenase® Version 2.0 (Promega)
pGEM Cloning - pGEM® - T Vector System (Promega)

4.1.8 Bacterial strains and plasmids

Bacillus cereus strain FM-1 (obtained from Prof. P.E. Granum
Bacillus thuringiensis (novel isolates: 13B, 3B, 1B, 10B, 3A, AB)
Bacillus thuringiensis subsp. *israelensis* (Bti)
Bacillus thuringiensis subsp. *tenebrionis* (Btt)
Bacillus thuringiensis strains HD-1, HD-2, HD-12, YBT, 4412.
Escherichia coli inv α F' } purchased from
Escherichia coli JM109 } Promega

Plasmids:
pBC16 Tet^R }obtained from
pC194 Cap^R } P. Jarrett
pSV5 (cloned *cry*IB(b) gene from HD12 in Bti cry- strain IPS78/11)
pSV4 (C terminally truncated version of SV5 full protein product)

4.2 Methods

4.2.1 Total DNA preparation

(a) Method from personal communication, Dr E. Bone.

LB medium was inoculated with a strain of *B. thuringiensis.*

2. A sample of culture (20ml) was removed, centrifuged at 6 000 r.p.m for approximately 20 minutes in an MSE HS18 centrifuge, the supernatant discarded and the pellet resuspended in 1ml of TE buffer (pH 8.0).
3. Addition of lysozyme (2mg), 240µl 0.5M EDTA (pH 8.0), 26µl proteinase K (10mg/mL) and 140µl of 10% SDS, was followed by incubation at 37°C for 1-2 h.
4. The DNA was precipitated with 2 x vol. EtOH (95%), and left for 5 min at 37°C.
5. The DNA was spooled into 500µl TE using a sealed Pasteur pipette.
6. Steps 4 & 5 were repeated.
7. The DNA was dissolved into TE at 37°C for 1-2 h.
8. Extraction with 1:1 phenol:chloroform was carried out.
9. of The aqueous phase)about 400µl) was removed, 133µl of 7.5 M ammonium acetate and 1ml of EtOH (95%) added. The tube was inverted and left to stand at room temperature for 2mins.
10. The tube was microcentrifuged at 13 000 r.p.m for 10 mins.
    The EtOH was aspirated off and the pellet washed with EtOH (70%). This was microcentrifuged for 2 mins, the supernatant removed and the pellet aspirated then desiccated for 30-60 mins.
12. The pellet was dissolved in 200 µl TE (pH 8.0) and approximately 2 µl were run on a 0.6 or 0.8 % agarose gel at 50mA for approximately 1h.

(b) Adams *et al.*, (1989)

LB medium (2ml) was inoculated with a colony of *B. thuringiensis* and incubated at 37°C overnight no shaking).

2. This was transferred to a 2l flask containing 250ml LB and grown at 37°C, 200 r.p.m, until an OD_{600} = 0.4 was reached.
   The flask contents were harvested at 8,000 r.p.m for 45 min. using a Sorvall G.S.A rotor.
4. The cell pellet was resuspended in 20ml of TE (pH 8.0), lysozyme(20mg) added, the tube inverted to mix and then incubated at 37°C for 1-2 h.
5. The addition of 1ml of 0.5M EDTA (pH8.0) was followed by 0.5ml of 2M Tris.Cl (pH 7.9) and the tube was inverted to mix followed by incubation at 37°C for 15 min.
6. RNAse A (10mg/ml) solution (200µl) was added and the tube incubated at 37°C for a further 15 min.
   Addition of of 10% SDS (2.3ml) resulted in considerable clearing of the cell suspension due to cell lysis.
8. Incubation for 2 h at 50°C with 2mg of proteinase K (or trypsin) was carried out and the suspension then split into 2 tubes.
9. Two phenol extractions were followed by two with phenol : chloroform (1:1)

10. Total DNA was precipitated with 0.1 vol. 3M sodium acetate and 2.5 vol. EtOH (95%).
11 After mini-entrifugation at maximum speed for 5 min. the pellet was washed in EtOH (70%), re-centrifuged and then aspirated and desiccated.
12 The pellet was resuspended in 10 ml TE and dissolved by heating at 60^0C.
13. Samples were run on a 0.6% agarose gel at 50mA for approximately 1h.

4.2.2 Plasmid preparation

This method was developed and initially follows the procedure of Adams *et al.*(1989) followed by the alkaline lysis protocol (Maniatis 1989).

B. thuringiensis was streaked out onto nutrient agar + 5μg/ml CAP and incubated overnight at 37^0C.
2. A single colony was used to inoculate 2ml LB medium + 5μg/ml CAP and incubated overnight at 37^0C.
3 This was transferred to LB medium + 5μg/mL CAP (250ml) and grown at 37^0C, 200 r.p.m until an OD_{600} = 0.4 - 0.6 was reached.
4. Flask contents were harvested for 45 min at 8000 r.p.m in a Sorvall G.S.A rotor.
5. The pellet was resuspended in 20ml TE and lysozyme (20mg), inverted to mix and incubated for 1 - 2 h at 37^0C.
6. Addition of 0.5M EDTA(1ml) + 0.5ml of 2.0M Tris.Cl (pH 8.0) was followed by mixing by inversion and incubation for 15 mins. at 37^0C.
7. A sample (2.5ml) of the suspension was removed (the remainder frozen) and 1.25ml of 0.8M NaOH/ 4% SDS added then mixed by inversion and put on ice for 5 min.
8. Ice cold potassium acetate (pH 4.8), 1.875ml was added, the tubes inverted to mix and put on ice for a further 5 min.
9. The suspension was separated into Eppendorf tubes and mini-centrifuged at 13000 r.p.m for 5 mins.
10. The supernatant layer was transferred to new tubes and an equal volume of phenol:chloroform (1:1) added, the tubes were inverted and minifuged at 13000 r.p.m (max. speed) for 2 min.
The supernatant layer was again transferred to clean tubes and ethanol precipitated with 2 vol 95% EtOH. The tubes were then mixed by inversion and left to stand at room temperature for 2 min.
12. The tubes were minifuged (max. speed), the supernatant layer removed by aspiration and the remaining pellet washed in 70% EtOH, minifuged then the pellet aspirated and desiccated.
13 The pellet was dissolved in approximately 200μl of TE (pH8.0), RNase treated (20μg/ml) and incubated at 37^0C for 30 mins.
4. Samples were run on a 0.6% agarose gel at 45mA for about 1.5 h

4.2.3 Polymerase chain reaction (PCR) amplification of the *cry*IB gene.

The method and primers were developed by Dr S. Gash, Univ. of Cambridge (pers. comm.).

Bacterial lawns of high activity (13B) and medium activity (3A, AB) isolates, together with strains YBT, HD2 and HD12 (positive controls for *cry* IB(a) and *cry* IB(b) genes, respectively) were grown up from single colonies by being incubated at 30°C overnight.

2. Resulting growth was scraped off into 0.5ml sdH_2O, whirlymixed briefly, frozen at -20°C for approximately 15 min, thawed, then boiled for 10 min and the process repeated.
3 The suspension with microcentrifuged for 20 - 30 s and the tube placed on ice until the supernatant was required as the template in the PCR reaction mixture.
4. All constituents were added to autoclaved Eppendorf tubes (0.75ml), the *Taq* polymerase last of all, and a 'hot start' PCR method used. This involved incubating the reaction mixture at 85°C for 1 min, pausing whilst dNTPs, prewarmed to 60°C were added, then continuing to incubate at 85°C for a further minute.

PCR reaction mixtures:

Constituent	Volume (µl)
NH_4 reaction buffer	10.0
$MgCl_2$ (23mM)	39.3
sdH_2O	18.5
specific primer, forward (0.25µM)	2.5
specific primer, reverse (0.25µM)	2.5
'red hot' DNA polymerase (Adv. Biotech Ltd.)	0.2
dNTPs (2.5mM)	12.0
template	15.0

cry IB(a) specific primer sequences:

1 Ba (f3):	ATGCAAGAACGAGAAGTGTTCTTTATACC	position 425 - 453 in the coding sequence
1 Ba (r1):	CCTATTGGCCGAGATTGAATCGTGTGC	position 1079 - 1053 in the coding sequence

PCR thermal profile using a Techne 3A thermal cycler:

Programme 35

	85°C	2 min

Programme 36

	94°C	1 min
2.	54°C	1 min
3.	72°C	1 min

24 cycles

Programme 37

	94°C	1 min
2.	54°C	1 min
3.	72°C	7 mins
4.	4°C	24 h

4.2.4 Agarose gel electrophoresis

Agarose gels (0.4/ 0.5/ 0.6 and 1.2%) were prepared by dissolving the appropriate amount of agarose in 0.5 x TBE buffer(50 ml). This was done by placing the mixture in a 650w microwave oven at medium heat for approximately 2 min. The agarose solution was allowed to cool, 0.5µl of ethidium bromide dissolved in ethanol (10mg/ml) added and the gel mix poured into minigel apparatus (NBL).
The gels were run under the appropriate conditions (current and voltage settings) dependent upon the DNA being analysed.

4.2.5 pGEM cloning protcol

4.2.5. *Ligation of the p-GEM®-T Vector and PCR product*

The PCR product was purified from the reaction mix by RNAse A treatment, followed by ethanol precipitation, centrifugation, aspiration and desiccation. The resulting pellet was resuspended in 25µl TE (pH 8.0).

2. The DNA was quantified by measuring its OD_{260}.

DNA Quantification

Isolate	OD_{260} (dil.x 250)	DNA(µg/ml)
13B	0.008	100.0
3A	0.021	262.5

Calculation

The DNA was diluted 1/ 250 then its OD_{260} measured using a Lamda 3 spectrophotometer.
The amount of DNA, in μg/ml = Absorbance (260nm) × 50 × 250
(since OD_{260} = 1 ≡ 50μg/ml DNA)
The amount of DNA was also quantified by visualising it on a gel when run against a DNA standard (a molecular weight marker, ϕX174, Hae III digest, NBL) of known concentration. In this way, an approximate determination was made as to how much DNA was present in the 13B and 3A products by comparing the intensity of the band with those of the standard.

3 The pGEM®-T Vector and pGEM®- T Vector control tubes were centrifuged to collect contents at the bottom of the tube.

4. Ligation reactions were set up as follows:

Constituent	Sample Reaction	Control Reaction
T4 DNA Ligase 10× Buffer	1μl	1μl
pGEM®-T Vector (50ng/μl)	1μl	1μl
PCR product (or pGEM®-T Vector control)	xμl	1μl
T4 DNA Ligase (Weiss unit/ml)	1μl	1μl
	xμl	5μl
final volume	10μl	10μl

For an optimal ligation, a 1:1 molar ratio of vector : insert (PCR product) was recommended.The following equation was used to calculate the appropriate amount of PCR product:

$$\frac{\text{ng vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert : vector molar ratio} = \text{ng of insert}$$

T- vector supplied at 50ng/μL
T- vector ≈ 3kb
Insert (PCR product) ≈ 655bp

5. Ligation mixtures were incubated at 15°C for approximately 24 h. and a sample taken after overnight incubation at 15°C and kept at 4°C for 6 h.

6. A 2μl aliquot of each sample was used for the transformation step.

7 Transformation into *E. coli* strain INVαF'/ *E. coli* JM109 (Promega) $CaCl_2$ competent cells.

4.2.5.2 *Preparation of competent cells of Escherichia coli*

Competent cells were prepared by the calcium chloride method (Mandel & Higa 1970) Centrifugation steps were done at 5500 rpm at 4^0C with a Sorvall G.S.A rotor.

A sample of 3ml from an overnight culture (5ml) was used to inoculate 47 ml of fresh LB broth.

2. This was incubated at 37^0C, 225 rpm until an OD_{650} of 0.4 reached, the culture centrifuged and the supernatant layer discarded.
3. The cells were resuspended in 25ml of ice cold 100mM $CaCl_2$, held on ice for 10 mins, re- centrifuged and the supernatant liquid discarded.
4. Ice cold 100mM $CaCl_2$ (5ml) was added and the cells gently resuspended in this solution then held on ice for a further 10 mins.
5 The cells remain competent for 24h at 4^0C.

4.2.5.3. *Transformation of Ligated PCR: pGEM®- T Vector*

A single LB/pen G/X-gal plate was prepared for each ligation reaction. The plates were equilibriated to room temperature prior to plating (step 7).

2 The tube containing the ligated PCR product: pGEM®-T Vector reaction was centrifuged to collect contents at the bottom of the tube. A sample (2μl) of the ligated PCR product: pGEM®-T Vector was transferred to a sterile 1.5ml microcentrifuge tube on ice.

3 Freshly prepared *E. coli* strain INVαF'/ *E. coli* JM109 $CaCl_2$ competent cells (100μl) were carefully aliquoted into each tube prepared in step 2.

4 The tubes were gently flicked to mix and placed on ice for 20 min.

5. The cells were heat shocked for 90 seconds at exactly 42^0C, then the tubes returned to ice for 2 min.

6. LB broth (1ml) was added to the tubes, gently inverted to mix and incubated at 37^0C for approximately 1 h.

7. Serial dilutions of the transformation culture (100μl) was spread onto LB/ penG/ X-gal plates.

8 Plates were incubated overnight at 37^0C and colonies examined and counted the next day. Plates were then stored at 4^0C after a proportion of each white colony had been removed using a sterile needle. These colonies were suspended in sterile glycerol (10%) and stored at -20^0C.

4.2.6 Isolation of single stranded phagemid (pGEM) DNA

From Maniatis (1989).

1. A bacterial colony containing the pGEM T vector with ligated PCR product (a white colony) was inoculated into of 2 ×YT (2ml) medium containing penicillin G (100μg/ml).
2. This was incubated at 37°C with strong agitation (300 rpm) until the culture reached saturation (about 5h).
3. 20 μl of the saturated culture was diluted into 2 × YT medium (2ml) containing M13KO7 (helper phage) at a concentration of 2×10^8 - 4×10^8 pfu/ ml. M13KO7was selected because it will result in the preferential secretion of single-stranded p-GEM phagemid DNA as opposed to its own. This is because it preferentially recognises the pGEM origin of replication (*ori*) over its own. 'Wild type' M13 cannot be used with the pGEM system as it will replicate its own *ori* at a higher level. This means the single stranded DNA secreted will simply be that of the bacteriophage and not the recombinant phagemid DNA (pGEM vector and PCR product insert) which one wishes to sequence.
4. The bacteria + phages were incubated for 1 hr at 37°C with strong agitation.
5. Kanamycin (25mg/ml in H_2O) was added to a final concentration of 70μg/ml. The incubation was continued for a further 14 to 18 h at 37°C.
6. Single-stranded bacteriophage M13 DNA was prepared as described below.

4.2.7 Small-scale preparation of M13 bacteriophage DNA

From Maniatis (1989)

1. A sample (1.0-1.5 ml) of the infected culture was transferred to a microcentifuge tube and centrifuged at 13,000 r.p.m for 5 min at 4°C in an MSE minifuge.
2. Some (1.2-1.3ml) of the supernatant was transferred to a fresh microfuge tube and 200μl of a solution of 20% polyethylene glycol (PEG 8000) in 2.5M NaCl (filter sterilised) were added. The tube was inverted several times to mix, followed by gentle vortexing. The tube was left to stand for 15 min at room temperature.
3. Precipitated bacteriophage particles were recovered by centrifugation at 13,000 r.p.m for 5 min at 4°C in a microfuge.
4. a All of the supernatant was carefully removed using a disposable pipette tip attached to vacuum line. The tube was re-centrifuged for 30 s and any residual supernatant removed. A tiny pellet of precipitated bacteriophage particles was visible at the bottom of the tube.
5. The pellet was resuspended in 100μl TE (pH8.0) by vigorous vortexing.
6. Phenol equilibriated with Tris.Cl (pH8.0), 50μl, was added and mixed well by vortexing for 30 s. The sample was allowed to stand for 1 min at room temperature, and then vortexed for another 30 s.
7. The sample was centrifuged at 13,000 r.p.m for 1 min at room temperature in a minifuge. Phenol extraction was then repeated and a chloroform extraction also carried out. The upper, aqueous layer was transferred to a clean tube containing 300μl of a 25:1 mixture of absolute ethanol: 3M sodium acetate (pH5.2). This was vortexed briefly and stored for 15 min at room temperature.

8 The precipitated single-stranded bacteriophage DNA was recovered by centrifugation at 13,000 r.p.m for 10 min at 4^0C in a microfuge. The supernatant layer was removed by gentle aspiration, and care taken not to disturb the pellet. The tube was re-centrifuged for 15 s and any residual supernatant liquid removed.

9 70% ethanol (200µl)was added at room temperature, vortexed briefly and centrifuged. The supernatant layer was immediately removed by gentle aspiration.

10. The open tube was left to stand on the bench for 10 min to allow any residual ethanol to evaporate. The pellet was dissolved in 30µl TE (pH 8.0). The single-stranded DNA was stored at -20^0C.

4.2.8 DNA sequencing

This was done using a Sequenase® Version 2.0 kit by following the manufacturer's instructions.

4.2.9 Photography and autoradiography

Photographs were taken with a Polaroid MP.4 land camera using Polaroid print film type 55 (4" x 5") or with a Polaroid mini camera using Polaroid print film type 665 (3.25" x 4.25").

Autoradiography was done as detailed below:

1. S^{35} paper was removed from the gel in the dark . This was placed in diluted developer [(1:9 Ilford Ilfosol developer] for 5 min then rinsed in water.
2. The paper was then transferred to dilute fixer (1:9)[Ilford Hypam fixer] for a further 5 min then rinsed again before being visualised in the light.

4.3 Results

4.3.1 Specific PCR with *cry*IB(a) primers

High and medium activity isolates were characterised in terms of the DNA encoding the crystalline protein(s) synthesised. The Cry IB(a) toxin was identified to be the important toxin when considering blowfly toxicity by a process of elimination as strains producing only Cry 1B(a) protoxins (HD-2 and 4YBT, not HD-12) gave a positive result upon bioassay. Strain HD-12 contains a *cry* IB(b) gene. Consequently genes of the *cry* IB(a) sub-family were the focus of DNA analysis. Firstly, PCR using *cry* IB(a), *cry* IB(b) and *cry* IB(c) specific primers (Dr S. Gash, pers. comm.) was carried out to determine which *cry*IB gene the isolates contained. All the novel, blowfly-toxic isolates were found to contain a *cry* IB(a) gene. Similarly *B. thuringiensis* strains 4412 and HD-2 also possessed this gene. The former strain is significant since it only produces one type of protoxin (Hofte *et al.*, 1988). Since strain 4412 was one of the most active tested we could assume that no other toxin type was necessary for the activity that we sought.

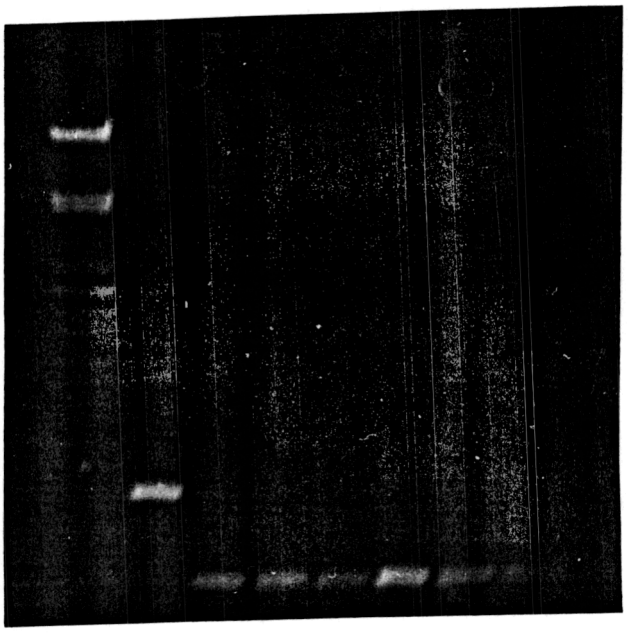
PCR products from the reactions described previously were run on a 1.2% agarose gel alongside *Pst* I digested λ DNA marker (NBL Gene Sciences Limited) (Fig. 4.1). All of the strains of bacteria which have been shown by larval bioassay to be toxic to flies have produced amplification products of approximately 655 base-pairs in length from the *cry*IB(a) primers.

Having tested all of the other gene families we have found that only δ-endotoxins of this sub-family are active.

Figure 4.1 PCR amplification using primers specific for the *cry*IB(a) and *cry*IB(b) genes from various isolates of *B. thuringiensis*

Lane

1 2 3 4 5 6 7 8



655 bp *cry*IB(a) amplification product

Lane

1. *Pst* I λ marker
2. HD12 (*cry*IB(b) positive control)
3. YBT (*cry*IB(a) positive control)
4. HD2 (*cry*IB(a) positive conttrol)
5. 13B-
6. 3A
7. AB
8. 4412

4.3.2 Cloning and expression of the *cry*IB(a) gene in *E. coli*

PCR products from the amplification of DNA from Bt strains 13B and 3A were cloned into the pGEM® vector. The transfection efficiencies are shown in Table 4.1.

Table 4.1 Colony forming units (c.f.u.s) of transformed *E. coli* on LB/ penG/ X-gal plates

Origin of DNA	Colour of colony	Ligation temperature 15°C	15°C/4°C
pGEM® T- vector control	Blue	9	4, 2*
	White	14	32
Strain 3A	Blue	7, 3*	10, 1*
	White	2	?
Strain 13B	Blue	7, 2*	9
	White	13	10

* pale blue colonies

White colonies (those which presumably contained the *cry*IB(a) gene insert) were scraped off the plates and grown up as previously described. Partial DNA sequence from the novel strains 13B and 3A is shown in Fig. 4.2

Fig. 4.2 DNA sequence comparison of the *cry*IB(a) crystal protein genes from *B. thuringiensis* strain HD2 and the novel strains 13B and 3A.

(i) ATGCAAGAACTATAAGTGTTCTTTATACCCAATATATAGCTTTAGA
(ii) ATGCAAGAACGAGAAGTGTTCTTTATACCCAATATATAGCTTTAGA
(iii) ATGCAAGAACGAGAAGTGTTCTTTATACTCAATATATAGCTTTAGA

(i) ACTTGATTTTCTTAATGCGATGCCGCTTTTCGCAATTAGAAACCAA
(ii) ACTTGATTTTCTTAATGCGATGCCGCTTTTCGCAATTAGAAACCAA
(iii) ACTTGATTTTCTTAATGCGATGCCGCTTTTCGCAATTAGAAACCAA

(i) GAAGTTCCATTATTGATGGTATATGCTCAAGCTGCAAATTTACACC
(ii) GAAGTTCCATTATTGATGGTATATGCTCAAGCTGCAAATTTACACC
(iii) GAAGTTCCATTATTGATGGTATATGCTCAAGCTGCAAATTTACACC

(i) TATTATTATTGAGAGATGCCTCTCTTTTTGGTAGTGAATTTGGGCTT
(ii) TATTATTATTGAAATGCTCTCTTTGTGATATT.....
(iii) TATTATTATTGAAATGCTCTCTTTGTGATATT.....

(i) *cry* IB(a) gene Bt subsp. *thuringiensis* strain HD2 (Brizzard & Whiteley 1988).
(ii) *cry* 1B(a) gene isolate 13B (high activity)
(iii) *cry* 1B(a) gene isolate 3A (medium activity).

Discussion

It has been clearly demonstrated that the genes amplified and cloned from the novel isolates of *B. thuringiensis* are *cry* IB(a) genes with considerable homology to the holotype, reference gene of *B. thuringiensis* strain HD2, previously known as gene *cry* A4 (Brizzard and Whiteley, 1984). In addition, the *Cry* IB(a) genes from the novel isolates differ by one nucleotide (C/ T) of the 170 nucleotides for which the sequence is shown (Fig.4.2). As such, it can be concluded that these are unique genes.

It is not, however, possible to make assumptions as to the likelihood that different *cry* IB(a) gene sequences of the novel *B. thuringiensis* isolates are directly responsible for the differing levels and/ or spectra of insecticidal activity that they exhibit. This is due to the fact that the nucleotide sequences shown are positioned within Domain1 of the crystal protein. It would be more likely that differences in Domain 2 could be responsible for the different activities exhibited as it is this domain which appears important in determining specificity (Li *et al.*, 1991). It must, nevertheless, be assumed that differences in the structure of the two toxins are responsibe for these differences in activity since, as previously stated (section 2.2.1), the levels of ICP production is very similar.

Chapter 5

Fermentation Studies

One of the initial goals of the project was to investigate the parameters for the eventual industrial production of our most effective strain(s) of *B. thuringiensis.* To this end a variety of media, suitable for large scale fermentation, were tested with several of our strains. The objective was to find the medium and strain giving the highest level of ICP production. Initial experiments in shake flasks lead to more detailed studies in a 10 litre constantly stirred tank reactor (CSTR). The latter experiments give a better approximation of the yields, conditions and thus costs pertaining to industrial fermentation. This, together with ICP toxicity data from the field can give an estimation of the cost-effectiveness and feasibilty of the use of microbial control for the intended application.

Quality assurance of the insecticide produced was identified as an important aspect of production in view of the potential risks of applying a batch of fermentation product contaminated with another organism directly to a food. For this reason a scheme for checking that no other organism was present in the fermentation broth was developed. To prove the feasiblity of this DNA finger-printing of the novel strains was carried out using random PCR primers.

Towards the end of the project its objectives were changed in the light of safety considerations. Instead of producing an insecticide to be applied to fish an insecticide to be incorporated into a bait became the agreed goal. For this reason a very brief analysis of the feasibility of producing *B. thuringiensis* by simple, semi-solid fermentation was carried out.

Materials and Methods

5.1 Protein Determination

The method Lowry *et al.*, (1951) was used

5.2 Hexose assay

Total hexoses were assayed by the 'anthrone' method of Herbert (1971).

5.3 Phosphate assay

Phosphate was assayed using molybdic acid reagent (Vogel, 1961)

5.4 Nitrogen assay

Total nitrogen was assayed by the method of Kjeldahl (Vogel, 1961)

5.5 Growth media and conditions

(a) Media

'BGM' - based on Nutrient Broth see Chapter 2.

Medium 1 - Molasses-based commercial medium (Lisanski *et al.* 1993)

Molasses (50% solids)	14g/ l
Yeast extract	3g/ l
Ammonium sulphate	1g/ l
Corn steep liquor	1g/ l
Calcium carbonate	1g/ l

adjusted to pH 6.8 after sterilisation

Medium 2 - Starch-based commercial medium (Lisanski *et al.* 1993)

Starch	13g/ l
Yeast extract	10g/ l
Sodium hypophosphate	4g/ l
Corn steep liquor	2g/ l
Calcium carbonate	8g/ l

adjusted to pH 6.8 after sterilisation

Medium 3 - Glucose-based commercial medium (Lisanski *et al.* 1993)

Glucose	40 g/ l
Yeast extract	12g/ l
Ammonium sulphate	4g/ l
Corn steep liquor	4g/ l
$MgSO_4.7H_2O$	0.60g/ l
$MnSO_4.H_2O$	0.10g/ l
$CaCl_2$	0.16g/ l
$ZnSO_4.7H_2O$	0.01g/ l
$CaSO_4.5H_2O$	0.01g/ l
$FeSO_4.7H_2O$	0.001g/ l

adjusted to pH 7.0 after sterilisation

Medium 4 - B_4 medium of Dulmage (1971

(b) Shake flask experiments

Conical flasks (250ml) containing medium (50ml) were inoculated with spores of different strains of *B. thuringiensis* and incubated at 30°C, 200 rpm for up to 48h, or until sporulation was complete. Five replicates of each flask were inoculated. These were pooled at the end of incubation to minimise experimental variability.

(c) Fermentation studies using a constantly stirred tank reactor (CSTR).

Seed cultures of 500ml of medium under test in 2l conical flask were inoculated with the strain being analysed and incubated at 30°C with shaking (200 r.p.m.) When the cultures had reached the mid-logarithmic phase the culture was added to 7.5l of the same medium in the fermenter. Polypropylene glycol 2000 (Sigma) was added (1ml/ l) to each medium to minimise foaming. During growth the medium was maintained automatically at pH 7.0 (+/-0.2) by the addition of sterile sodium hydroxide solution (5M) or sterile sulphuric acid (5M). The dissolved oxygen concentration was maintained above 20% of the saturated level by pumping in compressed, filter-sterilised air. The two impellers were rotated at 190 r.p.m. and baffles fitted to the tank to improve gaseous exchange in the culture. The temperature was maintained at 28°C (+/-2°C) by heating and cooling coils. The above parameters were constantly monitored with pH, temperature and oxygen probes.

(d) Analysis of cultures

Samples (1ml for analysis; 10ml for sucrose gradient purification) were transferred to Eppendorf tubes (1.5ml). These were centrifuged at 13,000 r.p.m for 10 minutes in an MSE Microcentaur minifuge. The supernatant fluids were used for chemical analysis. The pellets were resuspended in distilled water and washed twice. The pellets were then loaded onto sucrose density gradients (Thomas and Ellar, 1983) and the fraction(s) containing spores and crystals retained. This procedure was used to minimise the carry over of proteinaceous medium ingredients since the recovered material was to be analysed for protein: contamination from insoluble medium components would greatly distort the protein readings. This method was very time-consuming and suffered from errors such as loss of bacterial material during isolation but there was no alternative.

For assesments of spore yields serial dilutions were made of the pooled culture fluids and aliquots (100µl) of each were spread and counted in those dilutions containing between 30 and 300 colonies per plate. An average was made from two, duplicate plates for each experiment. A drop of suspension from the flasks was also examined microscopically for the presence or absence of spores and/ or crystals.

5.6 DNA finger-printing using random primer PCR (RAPD)

The method for template preparation was adapted from Brousseau *et al.* (1993)

A single colony of *B. thuringiensis* was streaked out and incubated overnight (approximately 18h) at 30°C.

2 A loopfull (preferably of a single colony) was transferred to a 1.5ml Eppendorf tube containing 250µl sdH_2O and this was vortexed for 20 s.

3. The suspension was boiled continuously for 10 min and then centrifuged for 30 s.

4. The tube was stored on ice until the supernatant layer was required as template in the PCR reaction mix.

5. PCR reaction mixes were set up using 13 different random primers. The sequences of the four found to be most useful are given below.

PCR Reactions

Constituent	**Volume (µl)**
template	15.000
sdH_2O	6.425
NH_4 reaction buffer	2.500
$MgCl_2$ (250mM)	0.375
dNTPs (25mM)	0.250
random primer	0.250
Taq polymerase	0.200

PCR Reaction profile:

Programme 31
1. 95°C 5mins.
2. 30°C 2mins.†
3. 72°C 2mins.

Programme 32
1. 95°C 1min.
2. 35°C 1min *
3. 72°C 2 mins.
45 cycles

Programme 33
1. 72°C 5 mins.
2. 4°C ∞

†A ramp rate of 30 was used between the annealing and extension steps (2 & 3).

* The annealing temperature was varied, values of 45°C, 47°C and 50°C being used also. The temperature was altered in an attempt to increase the stringency of the conditions and so give fewer, easily interpretable bands.

Random primer sequences:

	5' 3'
	GGAAGGCTGT
	CTCTCGTGCG
OPC 05	GATGACCGCC
DT 37	CGATTCATGG

5.7 Results

Protein assays for strains of *B. thuringiensis* grown on different media.

Nine strains were assayed for their optimum protein production using five different media. The strains were the wild types of strains 13B and 4412. In addition some of the plasmid cured, '*exo*" derivatives (section 3.4) were assessed. The subsequent protein assays of the pelleted material (Tables and Figures 5.1 - 5.5, below) produced some striking findings. These may be summarised as follows:

i) there was large variation in the protein yields given by the diffferent media. Medium 1 produced the highest overall yields of ICP and, significantly, has one of the cheaper feedstock formulations.

ii) no single strain remains the most productive in all media. The strain yielding the highest amount of ICP for each medium was: 13B Exo$^-$ E (BGM); 4412 Exo$^-$ E (Medium 1); 13B wild type (Medium 2); 13B Exo$^-$ 20(Medium 3); 13B wild type (Medium 4).

iii) in the same media some of the mutants produced more than twice as much protein as their parents. In terms of making a more cost effective product this would be an avenue worthy of further exploration.

Table 5.1 Results of protein assay for strains grown in BGM.

Strain	Protein µg/ml
13B wild-type	144
4412 wild-type	156
4412 exo- 'E'	310
13B exo-E	388
13B exo-11	64
13B exo- 12	268
13B exo-18	264
13b exo- 20	152
13B exo- 21	204

Fig. 5.1 Graph of protein production for strains grown in BGM.

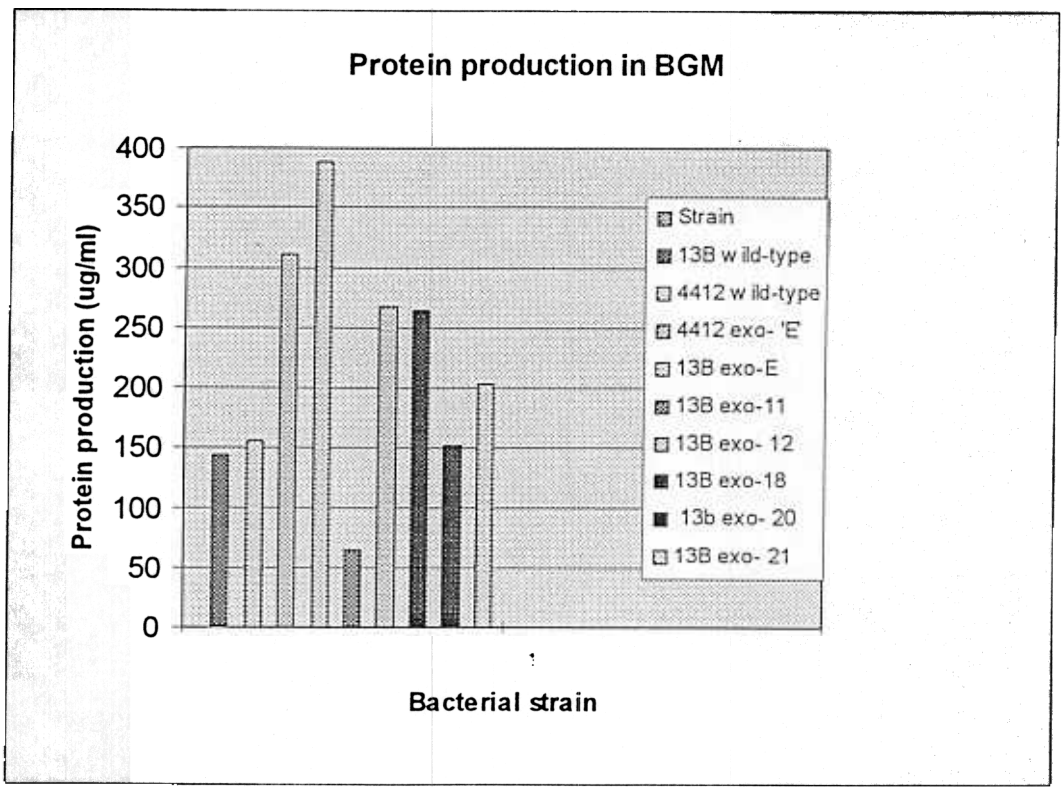


Table 5.2 Results of protein assay for strains grown on Medium 1.

Strain	Protein µg/ml
13B wild-type	252
4412 wild-type	212
4412 exo- 'E'	472
13B exo-E	378
13B exo- 12	368
13B exo-18	436
13B exo- 20	296
13B exo- 21	320

Fig. 5.2 Graph of protein production for strains grown in Medium 1

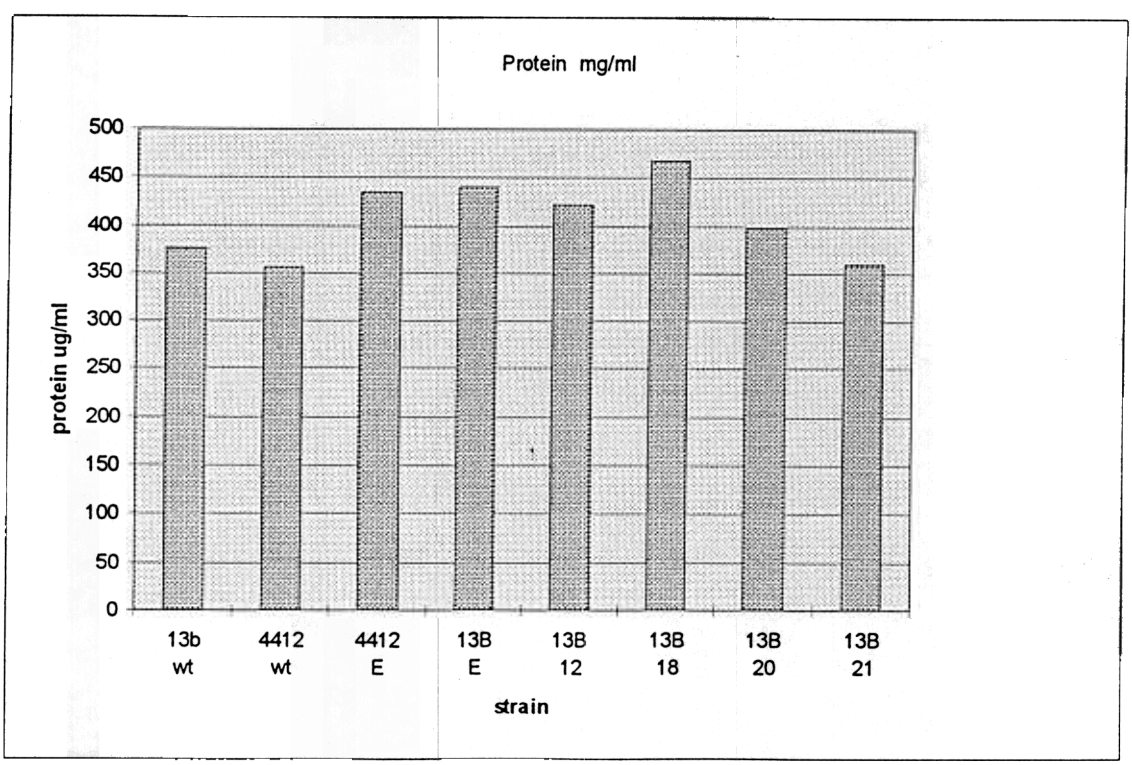


Table 5.3 Results of protein assay for strains grown with Medium 2.

Strain	Protein
	µg/ml
13B wild-type	292
4412 wild-type	258
4412 exo- 'E'	216
13B exo-E	162
13B exo-11	212
13B exo- 12	208
13B exo-18	242
13B exo- 20	224
13B exo- 21	224

Fig. 5.3 Results of protein assay for strains grown on Medium 2.

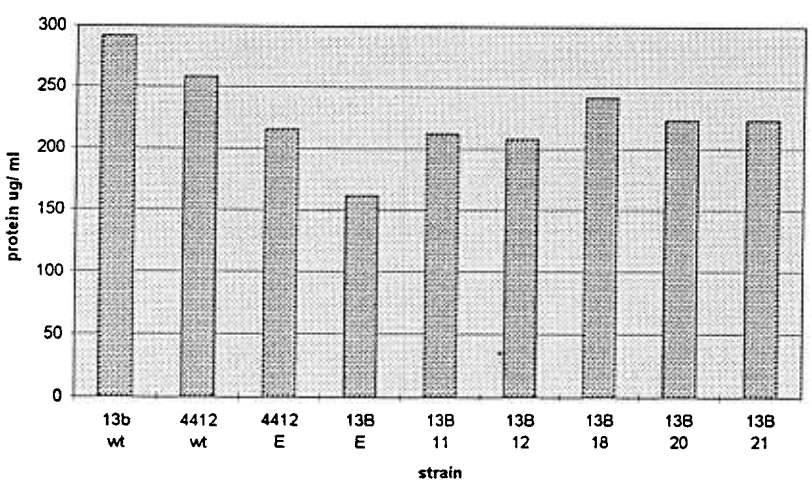


Table 5.4 Results of protein assay for strains grown on Medium 3

Strain	Protein µg/ml
13B wild-type	246
4412 wild-type	238
4412 exo- 'E'	

Fig. 5.4 Results of protein assay for strains grown with Medium 3.

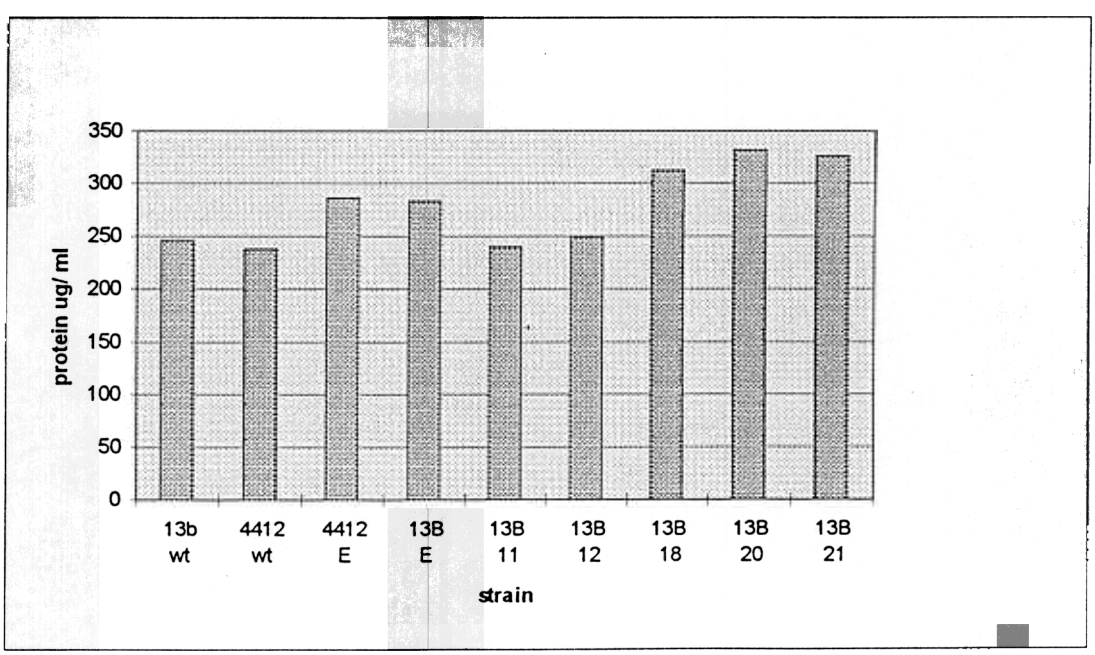
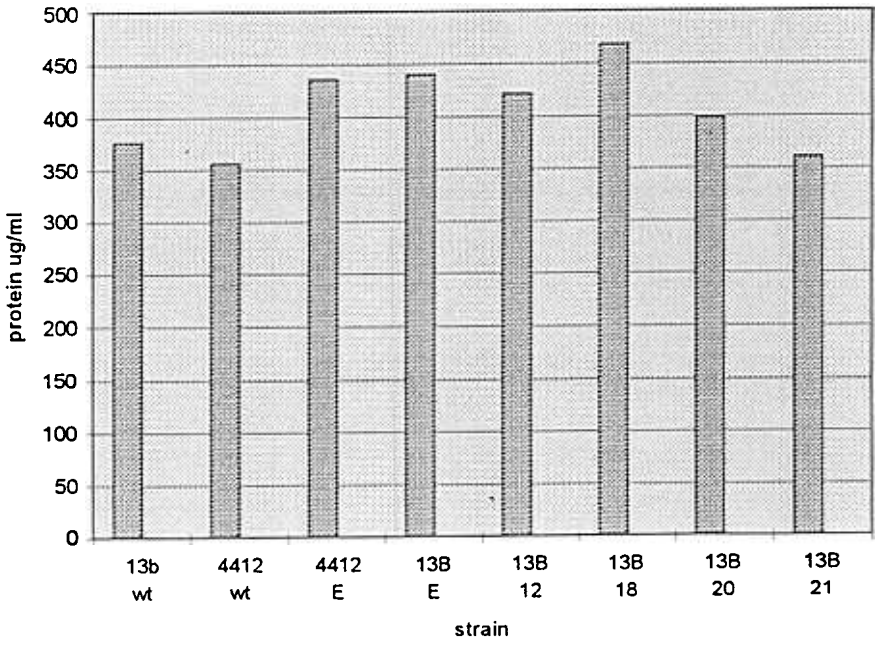


Table 5.5 Results of protein assay for strains grown on Medium 4.

Strain	Protein µg/ml
13B wild-type	234
4412 wild-type	194
4412 exo- 'E'	
13B exo-E	

Fig. 5.5 Results of protein assay for strains grown on Medium 4.



5.7.2 Analysis of varying the concentration of molasses added to Medium

The results above indicate that, of the media tested, Medium 1 gave the highest overall yield of protein. This medium was, therefore, manipulated to study the effect of varying the major ingredient and carbon source (molasses) with a view to finding the optimum level for its addition. The parent (wild-type) strains of 13B and 4412 were used.

Table 5.6 Results of anthrone (total hexose) assay of culture supernatants before and after strain 13B was grown in Medium 1 with varying amounts of molasses.

Molasses conc. g/l	0	2	6	10	14	18	22	26	30	34
Sucrose µmol/ml originally	0	3	8.9	15	21	27	33	39	45	51
Sucrose µmol/ml remaining	.05	0.5	2.8	2	3.3	2.7	3	6.9	21	2
Sucrose µmol/ml utilized	0	2.5	6.1	13.0	17.7	24.3	30	32.1	24	39

Fig. 5.6. Graph of total hexose assay of culture supernatants before and after strain 13B was grown in Medium 1 with varying amounts of molasses.

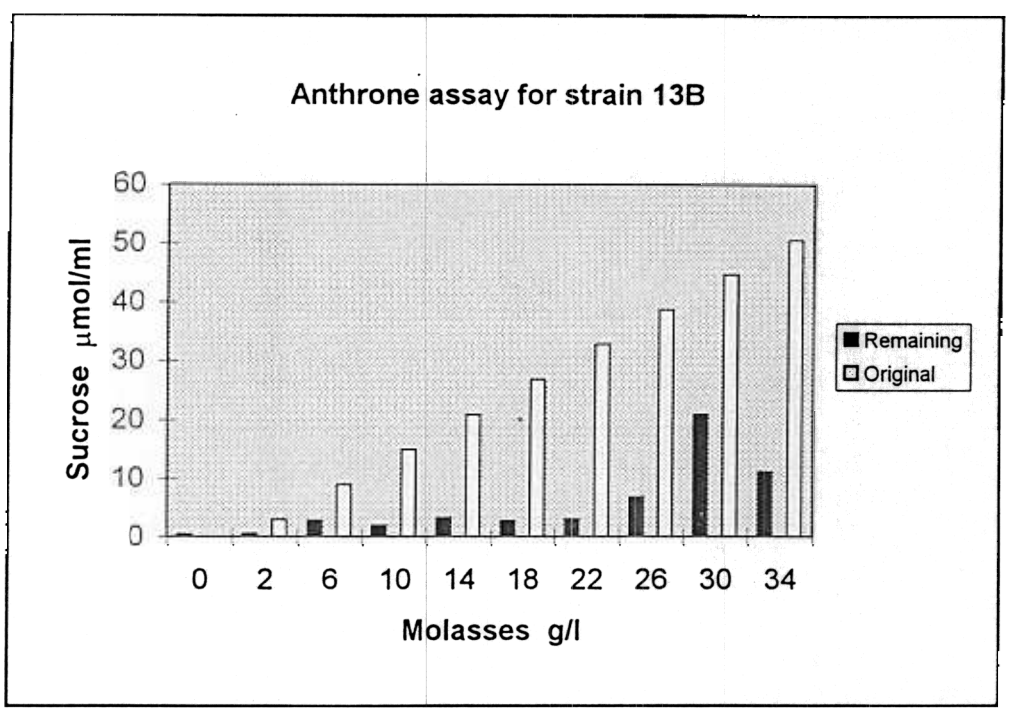
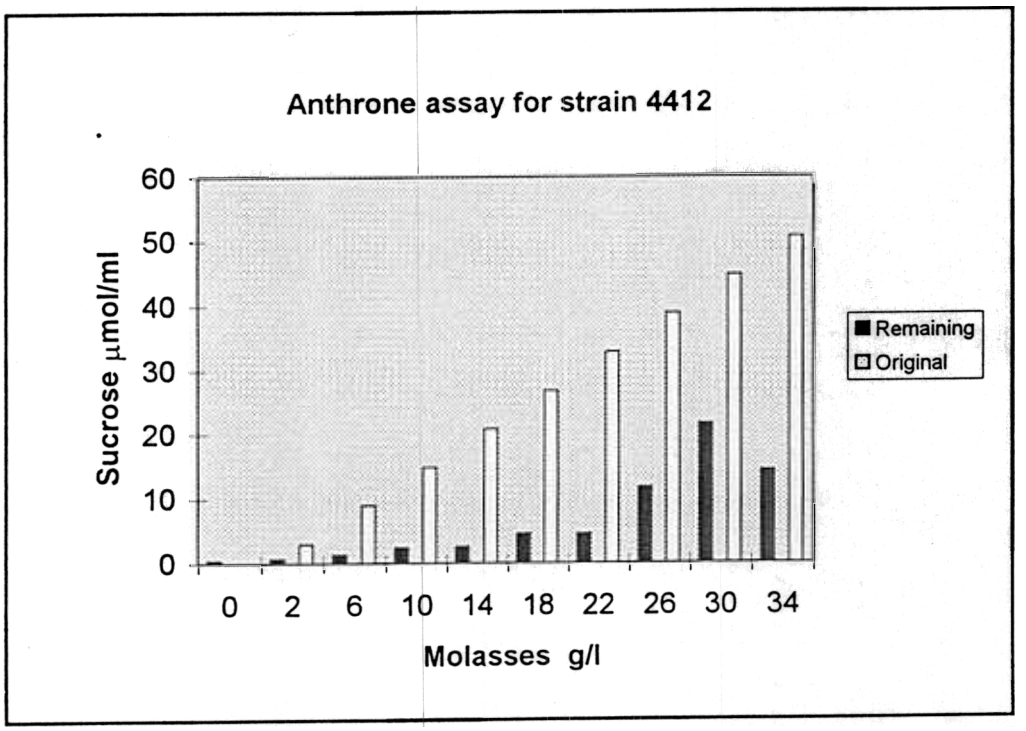


Table 5.7 Results of anthrone (total hexose) assay of culture supernatants before and after strain 4412 was grown in Medium 1 with varying amounts of molasses.

Molasses conc. g/l	0	2	6	10	14	18	22	26	30	34
Sucrose µmol/ml originally	0	3	8.9	15	21	27	33	39	45	51
Sucrose µmol/ml remaining	0.0	0.7	1.4	2.5	2.6	4.5	4.6	11.7	21.5	14.3
Sucrose µmol/ml utilized	0	0.3	7.5	12.5	18.4	22.5	28.4	27.3	23.5	36.7

Fig. 5.7 Graph of total hexose assay of culture supernatants before and after strain 13B was grown in Medium 1 with varying amounts of molasses.



The data for both strains are remarkably similar in that the addition of molasses up to about 22 g/ l results in its almost total utilisation by both strains. At an addition rate of 30 g/ l there is a peak in the amount of molassses remaining but, instead of this fraction increasing when the molasses concentration is increassed to 34 g/ l, the proportion remaining actually drops. At the highest concentration, therefore, both strains consumed about 75% of the hexose addded while at 30 g/ l they only consumed about 50% of the hexose added. The reason for this finding is unclear.

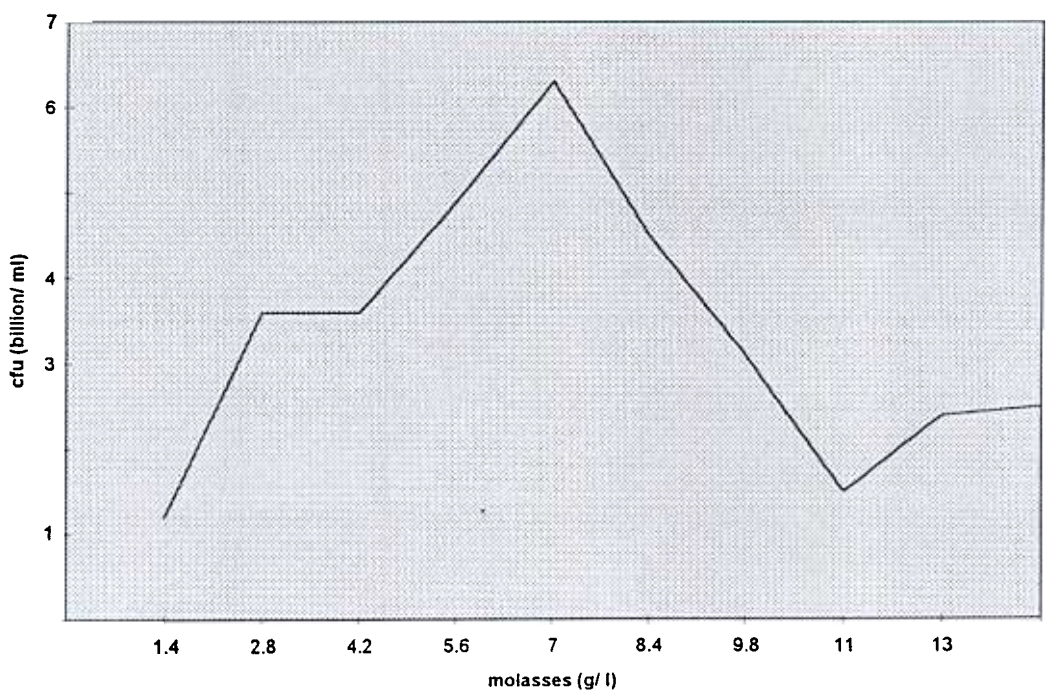
The results indicate that the region of interest should be where molasses is added at rates below about 22 g/ l since here the added hexose was largely consumed: residual

media components are wasted input. The concentration of molasses was, therefore, varied around the concentration stipulated in the original formulation and the resulting spore density of strain 13B assessed in terms of c.f.u./ ml.

Table 5.8 Density of spores of 13B (c.f.u./ ml) produced from varying concentrations of molasses added to Medium 1

Molasses g/l	1.4	2.8	4.2	5.6	7	8.4	9.8	11	13	14
Spore count cfu/ml 10^9	1.2	3.6	3.6	4.9	6.3	4.5	3.1	1.5	2.4	2.5

Figure 5.8 Graph of density of spores of 13B (c.f.u./ ml) produced from varying concentrations of molasses added to Medium 1



This experiment clearly shows that the optimum concentration of molasses to be added to this medium (for spore production by this organism) is 7 g/ l: the original

concentration used. The reason for the sub-optimal density achieved at the lower addition rates of molasses could simply be explained by lower levels of carbon source resulting in a lower density being achievable. Why the density decreases with increasing molasses beyond the optimum concentration is less obvious. It is possible that the vegetative cells are experiencing unbalanced growth and are producing detrimental by-products which diminish the ability to produce spores.

Having generated data in shake flasks to suggest that this medium was the best of those initially selected growth of 13B was further studied in a 10 litre CSTR. The information gained would be of use in determining the conditions suitable for the large-scale industrial production of the insecticide.

5.7.3 Constantly Stirred Tank Reactor (CSTR) studies

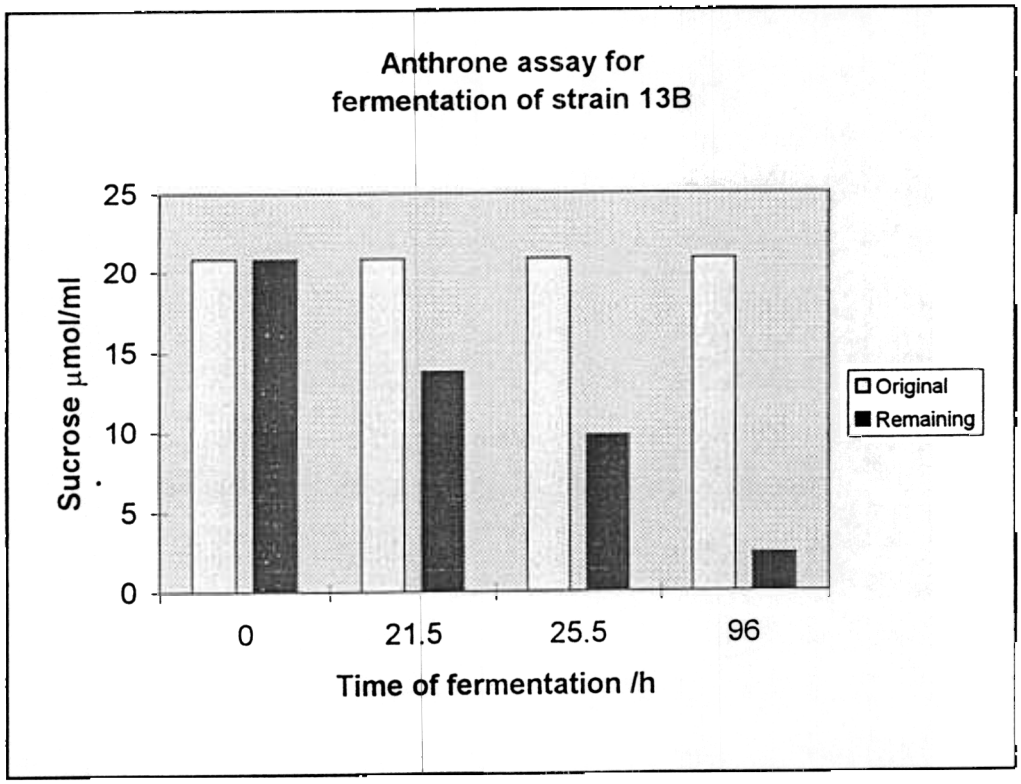
The conditions for operating the fermenter were derived empirically and are as described in section 5.5(c). The temperature (fairly uniform for *B. thuringiensis*) and pH value (specified by the medium composition) did not require investigation. The main consideration is oxygenation, *B. thuringiensis* being 'oxygen-hungry' if it is to realise its maximum sporulation and ICP production levels. Supplying oxygen on an industrial scale is expensive and the figure quoted in section 5.5(c)of 20% DO_2 may be regarded as a comfortable minimum. Increasing gas-liquid mass transfer is improved by increasing agitation (increasing rotor speed and/ or the presence of baffles in the fermenter vessel). Factors operating against this include the power requirement (increasingly important as the process is scaled up) and foaming. The conditions and equipment used produced high yields of spores (~ 4 x 10^{10}/ ml), approximately ten-fold greater than achieved in shake flasks. Microscopic examination of the cultures showed very good rates of sporulation (> 95%) with each spore mother cells invariably containing large ICPs. Morris *et al.* (1996) recently studied factors affecting the yield of ICPs in different media using a single strain of *B. thuringiensis.* Although their study was limited to the use of shake flasks they also found that oxygenation was one of the most important factors.

Using optimal conditions the progress of one fermentation experiment was followed. Samples were taken initially, at the end and at points leading up to sporulation.

Table 5. 9 Time course of anthrone (total hexose) assays for strain 13B growing in Medium 1 (7 g/ l molasses) in a CSTR.

Fermentation Time (h)	Original sucrose conc. (μmol/ml)	Remaining sucrose conc. (μmol/ml)	Sucrose utilized (μmol/ml)
0	20.9	20.9	0
21.5	20.9	13.8	7
25.5	20.9	9.8	
96	20.9	2.4	18.5

Fig. 5.9 Graph of time course of anthrone (total hexose) assays for strain 3B growing in Medium 1 (7 g/ l molasses) in a CSTR.



The results above show that there is still excess anthrone-positive material in the medium at the end of sporulation. This assay will detect hexoses even if they are present in polysaccharides and so could, theoretically, be showing sugars which are unavailable to the bacteria. In view of the composition of the medium, however, this is not the case in this instance. The remaining sugars (probably sucrose) are testament to the efficiency of the growth in the fermenter. Sporulation occurs very rapidly (within 36 hours) and so, presumably, less energy is expended in, say, maintenance energy requirement than in a shake flask.

Assays for total nitrogen and phosphate (two vital nutrients) were also made at the same time. These were also shown to be in excess throughout the fermentation. Data for phosphate only are shown in Table 5.10.

Table 5.10 Changes in the concentration of phosphate duration fermentation of strain 13B growing in Medium 1 (7 g/ l molasses) in a CSTR.

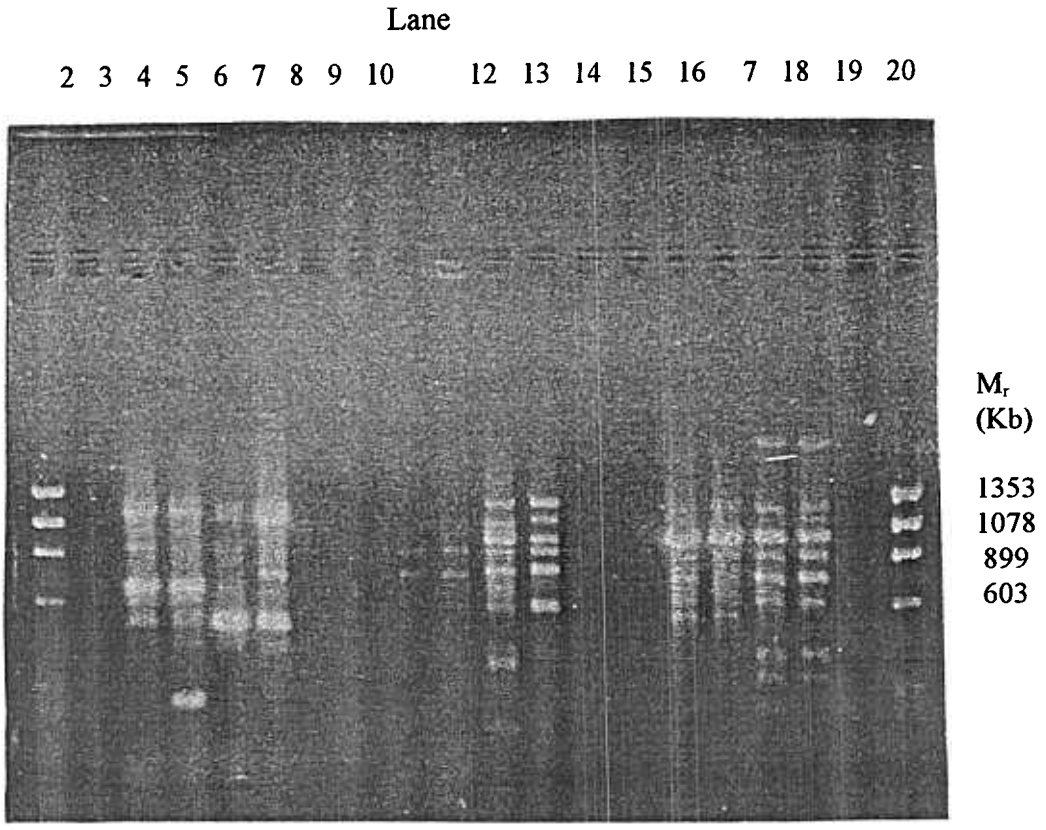
Sample	Phosphate (pmol/ ml)	% remaining of initial
Initial medium	71	100
Culture supernatant after 21h	44	61
Culture supernatant after 25.5h	25.5	35
Culture supernatant after 96h	24.5	34

5.7.4 Quality Assurance

Random amplification was carried out with template DNA of strains 4412, HD-2, 13B, 3A, AB and 10B.Nine of the primers initially selected gave amplification bands with few of these DNA samples. Of the four remaining primers three (OPC-05, 403 and 405) produced banding patterns but not in any way that clearly allowed the strains to be distinguished from one another. The final primer (DT 37), used with an annealing temperature of 50°C, produced a clear amplification band of about 2kb only with DNA from strain 13B (Fig. 5.10). Decreasing the annealing temperature to 45°C or 47°C resulted in the loss of this specific band.

Using both random and specific PCR techniques it thus seems feasible to develop a quality assurance scheme to ensure that the insecticide produced during fermentation is not contaminated (Fig. 5.11)

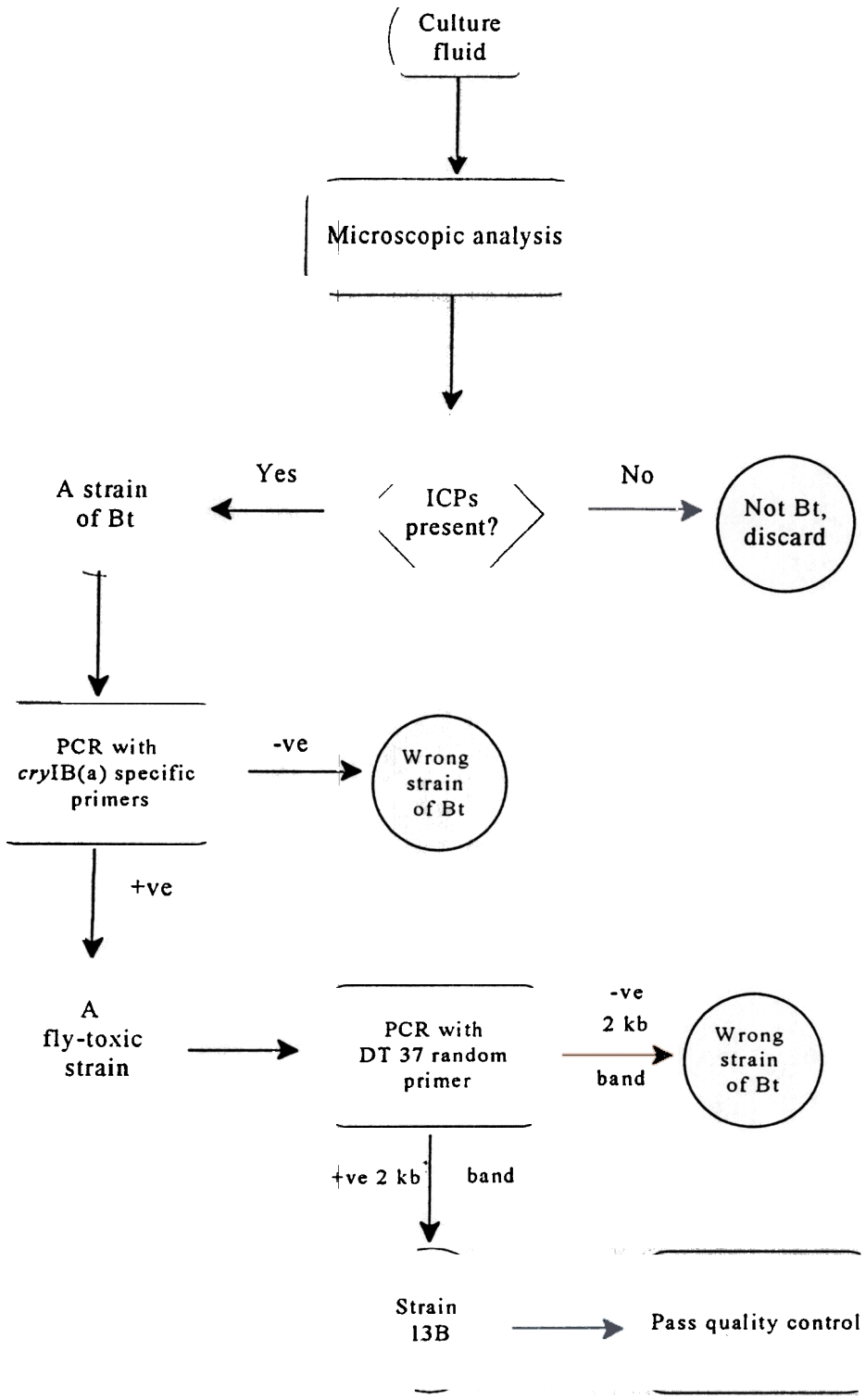
Fig. 5.10 Random PCR products from DNA derived from six of the novel strains of *B. thuringiensis* (Bt).



Lane

1. Hae III ØX174 marker
2. Negative control
3. Bt strain 4412
4.
5. Bt strain HD-2
6. Bt strain HD-2
7. /
8. /
9. Bt strain 3A
10. " Bt strain AB
12. Bt strain AB
13. /
15. Bt strain 10B
16. Bt strain 10B
17. Bt strain13B
18. Bt strain13B
19. /
20. Hae III ØX174 marker

Fig. 5.11 Quality assurance scheme for the production of *B. thuringiensis*



5.8 'Low technology' fermentation using semi-sold media.

With the change in project goal from using *B. thuringiensis* as a food-grade insecticide applied to fish to employing it in a bait the intended manner of its production also changed. As an insecticide applied to food the control of quality was paramount. In becoming incorporated with fish wastes as a bait the necessity to control contamination during production of the insecticide diminished. At the same time the importance of the cost of producing an effective product increased. The necessity therefore arose, late in the life-span of the project, to evaluate means of producing *B. thuringiensis* using low technological inputs.

The production of *B. thurigiensis* has been taking place successfully in less developed countries for decades. The equipment used is necessarily less sophisticated than the CSTRs used in the industrialised nations. The specific toxicity of the products is consequently lower than those from carefully controlled fermenters but is, nevertheless, able to compete with chemical insecticides. The production of *B. thurigiensis* by simple means has been well studied and is reviewed, for example, by Salama and Morris (1993).

Attempts were made to grow strain 13B using semi-solid media. An example of this was the mixing of wheat bran with liquid media, e.g. BGM or Medium 1 in a ratio by weight of about 5:1. Following incubation at 30°C for 48 h, while moistening the bran with fresh, liquid medium, spore counts were carried out. The increase in yield was minimal and consisted largely of vegetative cells. Due to the nature of the medium protein assays, to gauge ICP production, were impossible but one would imagine the yield to be negligible. Adding large amounts of fermenter-grown spores and crystals to similar media or those containing scraps of fish was found to be futile: the spores germinated and the vegetative cells only perhaps doubled in number. The use of such expensive inocula for such a poor return is not practical in any situation.

Larvae of *M. domestica* readily ate such preparations and were, as a consequence, killed. The toxicity was probably due in no small part to the production of β-exotoxin by the vegetative cells of 13B. Even though the insecticide is not to be added to fish for human consumption the use of any strain producing β-exotoxin cannot be sanctioned.

It is undoubtedly possible to produce *B. thurigiensis* for the incorporation into insecticidal baits using simple technology. The limited air flow in the incubator used probably had a detrimental effect on microbial growth and the vessels used were probably not ideal. Application of published methodologies would, doubtless, produce an effective bait.

5.9 Discussion

The data presented in this section high-light the variability of ICP production with growth media and bacterial strain. The production of insecticidal baits using 'village level technology' should be successful and cost-effective assuming that the inter-relationship between medium, bacterial strain and fermentation method are analysed

and optimised. From field data (Chapter 6) gained from the use of fermenter-grown *B. thuringiensis* in baits it is possible to derive a *very rough* estimation of costs. Based upon current industrial fermentation costs in the U.K. the production of 100ml of bait would cost about £0.30. This would seem to be uneconomic. Local production costs are currently difficult to predict. The results (e.g. Table 5.2) presented here, however, show that judicious selection of production strain can more than double the specific yield of ICP.

It should be pointed out that yield of ICP might not the most useful parameter for deciding the choice of medium. It has been reported, for example, that the use of leguminous seeds in media produces ICPs with lower specific toxicity than those grown in other media (Salama *et al.* 1983). Similarly Morris *et al.* (1996) report that the presence of significant levels of L-cystine in culture media can have a detrimental effect on the growth of *B. thuringiensis.*

The most useful measure might be International Toxicity Units/ ml (ITU/ ml) of culture medium with respect to a target insect. This is obviously a time-consuming procedure but an essential one before the final choice of strain and medium could be made. Should the insecticide be produced in different regions one might have to re-evaluate the most cost effective medium (depending on locally available feedstocks) and hence strain (depending upon ITU/ ml in that medium and also upon varying efficacy of strains against the endemic species of blowfly, see Table 2.2).

Another medium using cheap feedstock was studied. This was 'BFB' medium, see section 3.2.2 (b), which was designed to replicate the type of medium which could be produced from fish wastes which might be cheaply available in areas where the insecticide was being used. The nitrogen source for this medium was blood, fish and bonemeal. Data are not presented here because of the impossibility of removing components of the medium from the bacterial products at the end of the fermentation. Protein assays would, therefore, be meaningless. From microscopic analysis of cultures where sporulation did take place (see Table 3.2.1) excellent crystal production and a high density of spores was observed. This observation underlines the suggestion that attempts to improve the cost-effectiveness of any insecticide produced will be dependent upon using the highest yielding strain in the most cost-effective medium.

Chapter Six

Screening of Novel Isolates of *Bacillus thuringiensis* for Strains free from β-Exotoxin and *Bacillus cereus*-type Enterotoxin

The studies on the safety of *B. thuringiensis* (Chapter 3) high-lighted two areas of concern: the production of β-exotoxin and of *B. cereus*-type enterotoxin by our novel isolates. The ability to produce either was deemed unacceptable in any strain to be used other than for research purposes. All of the strains that we found to produce high activity ICPs against blowflies also produced both of these unwanted toxins. Our attempts to remove the ability to produce β-exotoxin from our strains were ultimately unsuccessful (Chapter 3). No attempts were made to remove the ability to produce enterotoxin.

During the course of this project we had developed and/ or applied techniques useful for:

i) efficient isolation of novel strains of *B. thuringiensis* (Johnson and Bishop, 1996);

ii) the identification of strains with the genetic potential to produce ICPs toxic to fly larvae (section 4.2.3)

iii) bioassaying larvae with δ-endotoxin and β-exotoxin (sections 2.1.4 and 3 1.(c), respectively), and,

iv) the identification of *B. cereus*-type enterotoxin by using antibody-dependent diagnostic kits (this chapter).

In view of the instability of our β-exotoxin mutants and the perceived difficulty of removing the gene(s) for enterotoxin production (Chapter 3) it was decided to try to find isolates of *B. thuringiensis* which naturally had the desired genotype (i.e. *cry*IB(a)$^{+}$; *exo-; entero-*). In other words they would be toxic to fly larvae but unable to produce either of the undesired toxins. The expertise that we had gained during this project, outlined above, made this objective a feasible undertaking. The percentage of natural isolates of *B. thuringiensis* which are able to synthesize β-exotoxin is, so far, unreported; it has been suggested (Prof. Granum, pers. comm.) that 25% of isolates of *B. cereus* are unable to produce enterotoxin. Even if, in this initial survey, we were unable to isolate a strain with all of the desired characteristics one would be able to predict the sample size required to achieve this goal thereby making possible an assessment of its feasibility.

6.1 Methods and Materials

6.1.1 Isolation of novel strains of *B. thuringiensis*

The method involving penicillin (Johnson and Bishop, 1996) was used. In addition, strains were also selected after heat shock (60°C for 30min) on the basis of their ability to produce ICPs visible under light microscopy (adapted from Meadows *et a.*,. 1992). The samples from which bacteria were isolated were collected from soil adjacent to an intensive livestock unit in Warickshire, U.K.

6.1.2 Bioassay for β-exotoxin production

The method described in section 3. .(c) was used.

6.1.3 Polymerase chain reaction (PCR) using primers specific *cry*IB(a) genes

The methods described in section 4.2.3 were used.

6.1.4 Identification of strains capable of producing *B. cereus*-type enterotoxin

The latex agglutination kit produced by Oxoid (BCET-RPLA) and the enzyme-linked immunosorbent assay (ELISA) kit produced by Tecra (VIA) were both used according to the manufacturers' instructions.

6.2 Results

6.2.1 Isolation of novel strains

From the agar plates containing soil samples that had just been heat shocked 202 colonies were selected as being of *B. thuringiensis*-type appearance (beige with matt texture). Of these 42 possessed one or more visible ICPs within the spore mother cell and were, therefore, classified as strains of *B. thuringiensis.* This represents a frequency of 21% true strains in the presumptive *B. thuringiensis* population. Similarly for those selected by the method involving penicillin 48 isolates were identified as being *B. thuringiensis* out of 101 presumptive positives. This represents an isolation frequency of 48%. A description of the crystals produced by a number of these strains is given in Tables 6.1 and 6.2.

All of the isolates of *B. thuringiensis* were analysed by PCR amplification. All of those which were positive (and some negatives) were screened for the production of β-exotoxin and of *B. cereus*-type enterotoxin. The results are summarised in Tables 6.3 and 6.4.

Table 6.1 Microscopic appearance of ICPs produced by representative strains of *B. thuringiensis* isolated by heat shock and plating out alone.)

Colony number	Microscopic appearance
1A	several small, irregular crystals
3A	several small, irregular crystals
4A	1 large crystal
5A	several small crystals
6A	1 large bipyramidal crystal
7A	several, irregular small crystals
9A	1 large, irregular crystal
10A	1 large, irregular crystal
11A	1 large, irregular crystal
12A	several small, irregular crystals
13A	1 large, bipyramidal crystal
14A	1/2 large crystal
15A	1 large crystal
19A	several small crystals
20A	several small crystals
21A	several small crystals
23A	several small crystals
24A	1 / 2 large crystals
25A	1 large, bipyramidal crystal
26A	1 large, irregular crystal
34A	several small, irregular crystals
36A	several small, irregular crystals
37A	several small, irregular crystals
43A	1 small crystal

Table 6.2 Microscopic appearance of ICPs produced by representative strains of *B. thuringiensis* isolated by penicillin selection.

Colony	Description
3	1 irregular crystal
4	several irregular crystals
6	several irregular crystals
13	1 irregular crystal
14	1 irregular crystal
15	several irregular crystals
17	several irregular crystals
18	several irregular crystals
23	several small irregular crystals
27	several irregular crystals

Table 6.2 (cont.) Microscopic appearance of ICPs produced by representative strains of *B. thuringiensis* isolated by penicillin selection.

Colony	Description
32	1 irregular crystal
38	1 irregular crystal
39	several irregular crystals
40	1 irregular crystal
41	several irregular crystals
46	1 irregular crystal
47	1 irregular crystal

There was no correlation in the appearance of the ICPs and the presence of the cryIB(a) gene, or any character studied (Tables 6.1- 6.4).

Table 6.3 Assays on isolates of *B. thuringiensis* derived from penicillin selection to identify the presence of the *cry*IB(a) gene; B. *cereus*-type enterotoxin and β-exotoxin.

Isolate number	cryIB(a) gene	enterotoxin ELISA	enterotoxin RPLA	β-exotoxin
1A	+	+		+
3A	+	+		+
4A	+		-	+
5A	-		-	+
6A	+	+	+	-
7A	+	+		+
9A	+		+	-
10A	+		+	+
11A	+		+	+
12A	+		+	-
13A	+		+	+
14A	+		+	-
15A	-		-	+
19A	+	+		+
20A	+	+		-
21A	-		-	-
23A	+	+	+	-
24A	+	+		+
25A	+	+	+	-
26A	-		-	-
34A	+	+	+	+
36A	-		-	-
37A	+	+	+	+
43A	+	+		+
3A	+		+	+
4A	+	+	+	-
6A	+	+	+	+
13A	+	+	+	+
14A	+	+		+
15A	+		+	+
17A	+		+	+
18A	+		+	+
23A	+	+		-
27A	+	+		+
29A	+	+	+	-
32A	-		+	
38A	+	+	+	-
39A	-		-	
40A	+	+		+
41A	+	-	-	+
46A	+	+		-
47A	+	+	+	-

Table 6.4 Assays on isolates of *B. thuringiensis* derived from heat shock selection to identify the presence of the *cry*IB(a) gene; B. *cereus*-type enterotoxin and β-exotoxin.

Isolate number	cryIB(a) gene	enterotoxin ELISA	enterotoxin RPLA	β-exotoxin
A	-			
B	-		+	
C	-		+	
F	-			-
G	-		+	-
H	-		+	
I	-			-
J	-			+
K	-		+	-
L	-		+	+
M	-		+	+
N	-		+	
O	-		+	+
P	-		+	
Q	-			-
R	-		+	-
S	-		+	
T	-		-	-
U	-		+	-
V	-			-
260	-		+	-
W	-		+	
261	-		+	
262	-		+	
263	+	+		
264	-		+	+
265	-		+	+
266	-			-
267	-		+	-
268	-		+	+
269	-		+	+
270	-		+	-
271	-		+	
169	+	+		
183	-		+	+
185	-		-	
201	-			-
O	-		-	
200	-		-	+
T	-			

Table 6.4(cont.) Assays on isolates of *B. thuringiensis* derived from heat shock selection to identify the presence of the *cry*IB(a) gene; B. *cereus*-type enterotoxin and β-exotoxin.

Isolate number	cryIB(a) gene	enterotoxin ELISA	enterotoxin RPLA	β-exotoxin
J	+	+	+	+
K	-		-	-
123	+	+		
27	+	+		+
28	-		-	
178	+	+		+
122	-		+	-
128	+	+		+

Table 6.5 Summary of frequencies of toxin occurrence (*cry*IB(a) presence; enterotoxin absence; exotoxin absence) in novel isolates of *B. thuringiensis* obtained from penicillin selection and from heat shock only.

Toxin occurrence	Isolates from penicillin selection	Isolates from heat shock only
*cry*IB(a)	35/ 48 = 73%	7/ 42 = 17%
entero$^-$	8/ 48 = 17%	5/ 33 = 15%
exo$^-$	16/ 38 = 42%	17/ 29 = 59%
*cry*IB(a) and *entero*$^-$	2/ 35 = 6%	0/ 7 = 0%
*cry*IB(a) and *exo*$^-$	14/ 35% = 40%	0/ 4 = 0%
entero$^-$ and *exo*$^-$	3/ 7 tested = 43%	2/ 3 tested = 67%
*cry*IB(a) + *entero*$^-$ + *exo*$^-$	zero	zero

From Table 6.5 it is possible to derive an estimate of the frequency with which a strain might be isolated with the desired genotype (*cry*IB(a)+, *entero*$^-$, *exo*$^-$):

<u>For isolates derived by the penicillin method</u>:

100 x 0.475 (positives) x 0.73 (*cry*IB(a)+) x 0. 7 (*entero*$^-$) x 0.42 (*exo*$^-$) = 0.25%

In other words 1 strain out of 40 colonies with the appearance of *B. thuringiensis* should produce the fly-toxic δ-endotoxin but not the enterotoxin nor the exotoxin.

<u>For isolates derived by heat shock only method:</u>

100 x 0.21 (positives) x 0.17 (*cry*IB(a)+) x 0.15 (*entero*$^-$) x 0.59 (*exo*$^-$ = 0.3%

In other words 1 strain out of 323 colonies with the appearance of *B. thuringiensis* should produce the fly-toxic δ-endotoxin but not the enterotoxin nor the exotoxin.

These calculations depend on there being no linkage between the genes encoding the δ-endotoxin and either of the other two. Since the crystal and exotoxin genes (at least) are plasmid-encoded the question of genetic linkage is somewhat different from that in higher organisms which also undergo chromosomal recombination during reproduction. Chi-Square analysis of the penicillin isolates shows that there is no dependence between the frequency of the appearance of the δ-endotoxin gene and either of the other two ($p < 0.05$). In the case of the enterotoxin gene the probability that there is no linkage with the *cry*IB(a) gene is, however, only just outside the 5% limit.

The frequency with which the ability to produce enterotoxin occurred (in the isolates tested and with the kits used) was 80%. This is in close agreement with the comparable frequency in *B.cereus* of 75% (Prof. Granum, pers. comm.). This emphasizes the similarity between the two 'species' and the caution required in assessing the safety of *B. thuringiensis* as an insecticide applied to crops close to consumption (see Chapter 3).

As the tables, above, show, we did not find a single isolate with the desired genotype (*cry*IB(a)+, *entero*$^-$, *exo*$^-$). Our results indicate, nevertheless, that, theoretically, this is a goal which is be very realistic to achieve.

6.3 Discussion

The availability of the techniques developed and applied in this project greatly facilitated the screening of environmental samples for isolates of *B. thuringiensis* with several, specific characteristics. The initial screening programme (Chaper 2) was a very hit and miss process. By investigating aspects of the fundamental science of a project, which also had very applied aspects, we were able, very rapidly, to repeat the process in spite of having very specific requirements in terms of the molecular characteristics of the strain(s) sought.

The techniques required to isolate an acceptable, insecticidal strain have been demonstrated in this chapter.

Chapter Seven

Formulation of *Bacillus thuringiensis* pesticide and field trial

Strains of *Bacillus thuringiensis* had initially been isolated and shown in the laboratory to produce ICPs which killed the larvae of a variety of species of blowfly. At this stage the goal of the project was to be able to treat fish with the biological insecticide, prior to drying in the sun, thereby protecting them from attack by insects. The next objective had, therefore, been to develop a formulation in which to apply the pesticide to cleaned and split fish. The product from this work was then to be tested, on post-harvest fish in Africa, to gain knowledge of its efficacy and applicability under field conditions. Numerous difficulties were encountered in trying to arrange field trials in Ghana and Malawi. South Africa was also considered as a potential site. In this respect the Food-Tek department of the CSIR in South Africa were helpful and were also identified as potential pilot-scale producers of the biopesticide using their 150 litre fermenter capacity.

Due to the concerns over the safety of food treated with *B. thuringiensis* which this project had raised (Chapter 3) the final objective was changed, as previously mentioned, towards the end of its life-span, to the use of our pesticide in a bait. The formulation developed for use on pre-dried fish became obsolete. A summary of this portion of the project is, nevertheless, included below for completeness.

A field trial was planned and carried out in Sri Lanka to test the performance of our pesticide as a bait. Dr Keith Jones of NRI but currently seconded to CARE International in Sri Lanka was instrumental in facilitating this.

Since this work frequently involved simultaneous testing, development and data-gathering the outputs are presented together in a 'Results' section: there is no 'Methods and Materials' section.

7.1 Results

7.1.1 Formulation of *Bacillus thuringiensis* for application to post-harvest fish

The majority of this work was completed by Dr Andrew Rosenthal of Oxford Brookes Universty. The section below is based, largely, upon his report of 20th February 1995.

The intention was to develop a pesticide formulation of *B. thuringiensis* which might be manufactured and utilised in developing countries on a self-sustaining basis. Requirements to fulfil this included the availability of all the raw materials and technology either in the country of use or producible therein with indigenous materials.

The use of a spray was initially considered but was rejected because it required the use of specialist equipment and it was thought that dipping would give better penetration to all of the surfaces of the fish.

Experience of dipping fish, in emulsion-based pesticides, results in a rapid contamination of the dipping solution, which can lead to wastage if the batch is discarded in favour of a fresh one. It was, therefore, felt desirable that the formulation should possess a reasonable viscosity such that the mix adheres to the surface of the fish. Thus the formulation can be reduced (because more mixture adsorbs to the surface) resulting in less wastage if the batch becomes soiled and is discarded. It was thought that desirable properties of the formulation were that it should:

be a food grade dip;
2. be easily and cheaply produced with locally available materials;
3. be able to suspend the particulate *B. thuringiensis* crystal and spore suspension;
4. provide adequate viscosity to coat the surface of the fish;
5. be stable when packaged and stored in a tropical climate;
6. reconstitute with a minimum of equipment;
7. be simple to apply;
8. not hinder the drying process;
9. not alter the organoleptic properties of the food (in its final state)
10. provide some protection from ultra violet radiation.

The intention was to use a pre-gelatinised starch, available in the country of manufacture. At a suitable concentration, such a material would provide a suitable viscosity to suspend the spores and ICPs of *B. thuringiensis* and coat the surface of the fish. A pre-gelatinised starch (believed to be cassava) was obtained from National Starch (product B38). Attempts to disperse this starch with water proved difficult due to the development of clumps when water was added. To overcome this clumping the dry starch powder was dispersed with varying amounts of dry salt. Equal parts of salt and starch was found to achieve a dispersible concentration.

Relative viscosity of the mixture was determined using a 'U' tube viscometer. It was found that the starch viscosity seemed to increase with time. It was decided to mill the starch and thereby reduce the particle size enabling more rapid rehydration. The sample was milled and the viscosity of a mix was found to remain more constant. On further enquiries to National Starch it was found that the starch was in fact a pregelatinised corn starch and that a similar product with a smaller particle size (product B37) was available.

Starch B37 mixed with equal quantities of salt were found to disperse well in water at a concentration of starch of 3% and produced a solution with a relative viscosity about double that of water. Bearing in mind that during the dipping procedure a fair degree of agitation is achieved, such a solution should keep the insecticidal particles (being about 1μm) in suspension.

Some attention was paid to the particle size of the salt being used for dispersing the starch. It was thought that a fine powder would act to aid dispersal more effectively. This, however, proved not to be the case and the use of milled salt actually lead to clumping of the mixture during reconstitution.

It had been intended to pack the starch/ salt/ insecticide mix in flexible plastic vacuum packs. Ideally a foil base plastic laminate would prevent the uptake of moisture and would prevent damage from UV. Unfortunately such materials may require importing an expensive laminate into the country of manufacture.

Instructions for use of B. thuringiensis mix:

1. Fill a clean bucket with 5 litres of clean, potable water.
2. Using a whisk or a narrow spoon, stir the water while gradually adding the dry powder. Try to break up any clumps that may form. Use mix immediately.
3. Wash and drain the fish prior to dipping (especially if salted overnight).
4. Immerse fish in the mix. Lift out and drain excess *B. thuringiensis* mix back into the bucket.
5. Place fish on racks to dry .

(a) Dipping and drying experiments

Several trials were carried out to determine the amount of dip solution which was taken up during dipping. Bearing in mind that the product was to be used on fish which had been split and opened out to reveal a larger surface area, white fish fillets were chosen. In the first trial approximate surface areas were determined by simple geometry to gain an idea of surface coverage. Subsequent trails looked at uptake of the mix on a weight basis and then followed the drying process using a laboratory tray dryer.

1. Four fillets with an approximate surface area of 473 cm^2 were weighed (±1 g), dipped, drained and reweighed. During dipping the weight increased from 342 g to 360 g. The 18 g weight gain was assumed to be due to an even deposition over the entire surface area, suggesting an uptake of 0.038 g cm^{-2}. Knowing from bioassay data that a final concentration of 0.1 mg cm^{-2} of *B. thuringiensis* spore suspension was required it was calculateded that a concentration of 0.26% dried *B. thuringiensis* fermentation media would be needed in pesticide mixture when reconstituted.

2. Two drying trials were done in which the fish fillets were designated control or test. The control were weighed, dipped in water, reweighed and placed on the trays of the laboratory tray dryer. The test treatments were weighed, dipped in a 3% starch and 3% salt mix (without spores and crystals of *B. thuringiensis*), reweighed and placed on the trays so as to gain a balanced exposure to different positions within the dryer.

Results (not shown) showed that the extent and time-course of drying were effectively identical between test and control fillets. This showed that in applying the dip no

retardation of the drying process would be incurred. This would otherwise delay the process and negate the purpose of the dip to a greater or lesser extent.

Concurrent experiments of our own showed that the use of a 1% solution of gum arabic fulfilled the same performance criteria as the starch. It was thought, furthermore, that it was a more readily available ingredient, and therefore cheaper, than pre-gelatinised starch would be in Africa. Use of native starch was impossible because it was very difficult to dissolve without it forming insoluble clumps.

7.1.2 Field trial in Sri Lanka

7.1.2.1 Experimental conditions

The experiments were carried out at the University of Ruhuna which is in the south of the island, about $^1/_2$ km from the Indian Ocean. My counter-part in the Department of Zoology was Dr Hemanthe Erdinsinghe who kindly assisted me and allowed me to enlist technical help.

Before my arrival the area in which I worked was baited with fish scraps which were left out to attract flies. The species present included those from the Family Calliphoridae, subfamily Calliphorinae, for example *Chrysomya* spp.; Family Calliphoridae, subfamily Sarcophaginae and Family Muscidae.

The trial area used was a concreted square of about 3 metres square which was open on one side and covered over-head. Wire fencing was put up to keep out lizards and cats etc. An adjoining laboratory with running water and electronic balance was provided.

The day time temperature was regularly about 30°C and the humidity high but not measured. The experimental area was covered so the occasional rain showers had no effect. Since baits are the designated means of using the insecticide it would be necessary to design pots with lids to prevent them from being filled with rainwater. Obviously the lid would have to be raised above the lip of the pot to allow access to adult flies. Scattering the bait on the ground would not be so effective. Germination of the spores takes place and would have a detrimental effect if the bait were not contained, see section (c)(ii), below.

7.1.2.2 Experimental design

Various strains and formulations of *B. thuringiensis* were tested both as baits and as coatings of whole fish. The latter experiments were carried out in case this might ever become viewed as a potential route for application again. Six replicates of each dosage for the baits were prepared and distributed randomly in the test area. Experiments were repeated at least twice. Baits were prepared to a volume of 25ml and presented in 50ml plastic pots. Lids were either punctured to provide aeration, if the larvae were to be forced to remain in the pot, or a hole (~ 2cm square) was cut in the lid to allow

adults access to the bait. Neonate larvae were either counted (20/ replicate) and placed in position or else adults were allowed to lay eggs at will, as stated below.

The insecticide used was grown in a fermenter using BGM as the medium. The cultures were centrifuged for 40 min at 8,000 r.p.m.(Sorval G.S.A. rotor). The particulate matter was washed twice in distilled water and freeze-dried. It was stored in sealed plastic bags themselves sealed inside bags containing silica gel as a desiccant. The strains used were 13B (exotoxin-producer); 25A (non-exotoxin-producer), obtained from the last screening programme (Chapter 6) and *B. thuringiensis* subsp. *tenebrionis* (negative control producing neither exotoxin nor a δ-endotoxin which kills any fly larvae).

7.1.2.3 Results

(a) dipping whole, gutted, split ' sardine-sized' fish with a suspension of spores and crystals in gum arabic solution (1.5%) is not effective. This is even at an incorporation rate of (40% w/v) freeze-dried ICPs and spores.Adults were allowed to lay eggs at will and the preferred sites were seen to be in the head or, to a much lesser extent, in crevices in the flesh. Essentially the sites which were dark and slower to dry out were favoured. In these sites larvae were able to avoid the insecticide sufficiently to mature to such a size where, from laboratory work, we know that they are effectively resistant to the insecticide.

This finding is of interest bearing in mind that it was the initial objective of the project. Effectively the problem is that experienced in agricultural applications such as against the European corn borer: the larva has to ingest a lethal dose of toxin before it burrows under the surface, where the insecticide is confined, and hence escapes exposure.

(b) the formulation that was found to be most effective as a bait was homogenised fish (50% w/v) incorporated with insecticidal powder at the desired rate and made into a thick paste with a hydrated, complex carbohydrate. Gum arabic was not good at producing a paste of high enough viscosity and the amounts needed would be prohibitively expensive. For this reason an un-purified, cheaply available ingredient was sought. Semolina was selected since, when boiled (50g in 200ml water for 5min) a very thick paste was produced. This both bound the ingredients together and stopped the paste from drying out quickly. By homogenising the fish the larvae were exposed to the insecticide at all times: the problem with dipping fish was that the behaviour of the larvae lead them to avoid the insecticide until they had grown too big to be affected by it.

Oviposition occurred on the bait in the presence of gutted fish. It seemed to be at least as attractive a site as the fish. This is, of course, crucial if the bait is to have any use whatsoever. The consistency of the bait had to be semi-solid: if it were at all liquid the adults would still feed on it but would not lay eggs on it. This is important with respect to point (iv), below.

(c) very high incorporation rates were necessary to have any effect on even the first instars of the indigenous species of blowfly. Incorporation of insecticide up to 1.5% (w/v) had an insignificant effect compared to the control. Since there was a limited amount of freeze-dried insecticide available the dose of strain 25A was increased to 10% (w/v). (Freeze-dried powder from more than 70l of culture media were used in the course of this trial).This produced a 95% kill rate compared to the control (10% w/v spores and crystals of *B. thuringiensis* subsp. *tenebrionis*). The mortality in the controls was always less than 10%.

Due to the high content of insecticide in the baits there was insufficient remaining to set up replicated assays using lower doses and thereby construct a dose-response curve. Observations from a very limited number of intermediate doses indicated that the necessary incorporation rate seems to be little short of 10%.

(d) a major problem was encountered at the high inclusion rates. The bait quickly liquified due to protease production from germinating spores: the conditions of high temperature, moisture and protein availability are ideal conditions to trigger outgrowth from spores. The 'soup-like' consistency of the bait that resulted was attractive to adults for feeding but not for oviposition. Refinement of the bait was therefore necessary. It was found that putting a layer of coconut fibre (1-2cm) in the bottom of the pots before adding the bait partially soaked up the liquid as it formed. Air spaces were also formed and the semi-solid nature of the bait retained. It also acted as a wick to stop the top of the bait from drying out. In this way the bait was maintained in an attractive state for oviposition. In different parts of the world other locally available and cheap alternatives to the coconut fibre should be available.

Inhibition of germination of the spores could be effected by increasing the acidity of the bait to pH 4. This was not tested but would almost certainly make the bait less attractive to flies. Alternatively a specific inhibitor of germination could be added such as 4-hydroxymethylparabenzoic acid. This would increase the cost of the insecticide and would decrease its efficacy: the germination of the spores and ensuing septicaemia enhances the toxic effect of the insecticidal crystal proteins and, for some species is obligatory for lethality to occur. Laboratory experiments have shown (Fig. 2.2) that vegetative cells have a comparable potentiating effect on the lethality to maggots of the crystals as do spores. Presumably the near neutral pH value of the gut allows them to survive until they can initiate infection when the epithelial cells of the lumen of the gut have been ruptured by the δ-endotoxin (Knowles and Dow, 1993).

(e) the exotoxin-positive strain, 13B, has a higher specific potency than strain 25A. This is somewhat irrelevant for two reasons:

(i) the ability to produce β-exotoxin is unacceptable in any strain of *B. thuringiensis* other than for research purposes;

(ii) the exotoxin producer gave rise to feeding inhibition as evidenced by larvae migrating to the lid of closed containers. In open containers (as baits must be) larvae would be free to leave the bait by crawling away. This would be completely counter-productive to the purpose of the bait. Feeding inhibition or avoidance was not noticeable with the non-exotoxin producing strain.

7.2 Discussion

The effectiveness of the biopesticide has been proved under field conditions. Estimates at present on the cost of the bait (about £0.30/ 100ml for just producing the dried product in U.K. alone) appear to be prohibitive. As discussed in Chapter Eight there is promise in producing the insecticide at a local level with a completely different economic analysis potentially attendant upon this.

It had been thought, prior to the field trial, that the bait could be scattered on the ground. In view of the liquification that takes place, section 7.1.2.3(d), this would now seem not to be recommended. The leaching away of insecticide and the hastening of drying out would probably have a very detrimental effect on the efficacy of the bait. The use of containers for the bait, ideally of specialist design, have been shown to be necesssary. The number of pots of bait that would have to be available in order to produce a noticeable effect in decreasing losses at a fish processing site requires further investigation. This would require further investigation in the field since it would seem to be impossible to predict from laboratory studies.

The use of a bait removes the need to consider UV protectants in the formulation. Since the insecticide is mixed in with the other components of the bait it is protected from sunlight. In practice it was found that the insecticide used to dip fish also did not need UV protectants. The behaviour of the adults dictated that eggs were layed in locations hidden from light and the larvae were also strongly negatively phototactic. Thus, wherever the insecticide was required to work it was protected from UV degradation. Dipping was a good method of applying the formulation to these regions.

Chapter Eight

Conclusions

This project necessitated the acquisition of a large amount of novel information in a previously unexplored area of biological control. We have managed to isolate and characterise strains of *B. thuringiensis* with a previously unreported larvicidal activity *viz.* against tropical blowflies.

The second group of target insects in the initial objectives of this project were the carnivorous beetles typified by *Dermestes maculatus*. These remained unaffected by every previously reported strain of *B. thuringiensis* active against any species of coleopteran and representatives from nearly all of the other sub-families of δ-endotoxin classified to date. From this extensive survey we were able to recommend that this was not a goal of the project which had any realistic likelihood of success. No report to date gives any evidence to the contrary.

As a result of our rigorous concerns for the safety of the intended beneficiaries of this project its goals became altered. The particular obstacle to the implementation of the initial goal was the production of *B. cereus*-type enterotoxin by all of our bacterial isolates. Further investigations have widened the scope of this enquiry and have raised possible ramifications for the use of all commercial preparations of *B. thuringiensis* on crops intended for imminent human consumption.

During the course of this project we gained or applied techniques for rapid and efficient isolation and screening of environmental samples for strains of *B. thuringiensis* with particular molecular characteristics. This has allowed the informed prediction to be made that a safe strain with the desired insecticidal activity could now, quite readily, be isolated from Nature.

The production of mutants of various isolates of *B. thuringiensis* in order to remove the ability to synthesize β-exotoxin was ultimately unsuccessful. Fermentation studies with these mutants nevertheless produced information of potential value. They seemed, in some media, to have gained the ability to produce more insoluble protein than their parents. It was not verified whether an increase in ICP production was being witnessed. Were this found to be the case it would offer a very promising avenue of further investigation which could result in decreasing the production cost of the insecticide. In addition the choice of fermentation medium was also shown to have a profound effect on the yield of insoluble protein. Taken together one might expect a five-fold increase in specific production of insecticide (mg ICP/ litre of fermentation medium) based upon a judicious choice of production organism and fermentation medium. In view of the high estimate for the cost of currently producing an effective bait such an investigation might make the difference between viability and abandonment of this approach.

The field trial proved the potential effectiveness of a bait in killing larvae of the blowfly species endemic to Sri Lanka. It is not intended that the use of any insecticide

applied in such a way should be a 'cure-all' for the reduction of losses which initiated this project. Other improvements in good manufacturing practice such as removing wastes which could act as a potential breeding sites for blowflies and beetles could have a profound effect. Even details such as the design of the container in which any bait might be held are important: final instar fly larvae like to leave the food source in which they have grown and, ideally, burrow into the ground just before pupation. Any larvae which have survived to that stage would, possibly, have some resistance to the insecticide. In preventing them from exiting the pot the propagation of their resistance could be prevented by the routine destruction of the spent bait. A design such as a ruff of bristles at the top of the container, facing inwards, perpendicular to the sides, would allow the adults in but probably prevent (large) larvae from leaving.

The investigation of the production of *B. thuringiensis* by simple technology was rudimentary and brief. Published reports leave no doubt this this is feasible, cost-effective and socially acceptable in many countries. Probably one of the greatest assets of biological control in less developed countries is that it can be placed in the hands of the users or, if not, their near neighbours. It is exactly in this direction that the project has become forced to look. With hind-sight this is, perhaps, fortuitous: it is unlikely that the cost, logistics and infrastructure required to produce and transport a high quality biotechnological product could have been supported by its intended beneficiaries.

As a regional industry the use of this insecticide does have the potential for sustained success. The specific activity of the insecticide produced will be much lower than that from a sophisticated CSTR. Adjustment to the composition of the formulation could accommodate this. Manipulation of the components of the fermentation, as discussed above, could also mitigate this decrease in specific activity. Were the ability to produce *B. thuringiensis* initiated in appropriate regions the industry could be made more cost effective by alternating production of insecticide for use on post harvest fish to that for agricultural purposes. Once the ability to produce one strain of the organism is in place little modification is required to produce a strain with a different spectrum of toxicity e.g. against lepidopteran larvae. Increasing the scale of operation and optimising the usage of time and equipment in this way would would decrease unit costs throughout.

At its inception this project was considered adaptive in nature. It had been believed that an insecticide with a world-wide and long-established usage in agriculture could be transferred, more or less directly, to protect post-harvest fish. Largely from a concern for the well-being of the beneficiaries this was shown to be ill-advised. Knowledge resulting from fundamental research into the biochemistry and genetics of this organism, initiated at the beginning of the project, has enabled a safe alternative means for its application to be suggested. Studies have shown that the new approach could be effective under field conditions.

Further work is needed to integrate the use of the bioinsecticide baits developed and demonstrated here into a reviewed regime for the processing and sundrying of fish in the tropics if losses due to insects are to be decreased. Studies on the most cost-effective mean of producing the pesticide using low-level technology is also required

It has been suggested that small businesses producing bio-pesticides active against a variety of pests could be the most promising means of sustaining the introduction of this biopesticide. Knock-on effects of this policy might be that:

i) wealth would be created and health would improve through decreasing exposure to harmful chemicals and increased food security;

ii) the use of environmentally-damaging chemical pesticides in agriculture might also diminish with the introduction of the ability to produce appropriate strains of *B. thuringiensis* cheaply and locally and

iii) areas where the population suffers from insect-borne diseases such as malaria and river-blindness would be able to produce strains of *B. thuringiensis* active against the water-inhabiting larvae of the vectors propagating these diseases (Youdeowei and Service, 1983)

References

Adams, L.F., Visick, J.E. and Whiteley, H.R. (1989) A 20 kilodalton protein is required for efficient production of the *Bacillus thuringiensis* subsp. *israelensis* 27 kilodalton crystal protein in *Escherichia coli*. J.Bact. 171: 521-530.

Ambridge, E.M. & Haines, I.H. (1987) "Some aspects of pesticide use and human safety in Southeast Asia." In: Proceedings, 11th International Congress on Plant Protection, vol II, pp219-227, Manilla, Philippines, October 5th-9th, 1987.

Amos, T.G. (1968) Some laboratory observations on the rates of development, mortality and oviposition of *Dermestes frischii*. J. Stored. Prods. Res. 4: 108-17.

Becker, N. (1990) Microbial control of mosquitoes and black flies. In: Proceedings of the 5th International Colloquium on invertebrate pathology and microbial control (Ed. D. Pinnock) pp.298-302, Adelaide, Australia, 20th-24th August, 1990, Society of Invertebrate Pathology.

Bond, R.P.M., Boyce, C.B.C., Rogoff, M.H. & Sheih, T.R. (1971) The thermostable exotoxin of *Bacillus thuringiensis*. In: Microbial Control of Insects and Mites, (Eds. H.D. Burgess & N.W. Hussey) pp.275-303, Academic Press, New York.

Bone, E.J., and Ellar, D.J. (1989) Transformation of *Bacillus thuringiensis* by electroporation. FEMS Microbiol. Letts. 58:171-178.

Borgstrom, G. (1962) Fish in world nutrition. In: Fish as Food (Ed.Borgstrom, G.) pp. 267-360. New York: Academic Press.

Brousseau, R., Saint-Onge, A., Prefontaine G., Masson, L. and Cabana, J. (1993) Arbitrary primer polymerase chain reaction, a powerful method to identify *Bacillus thuringiensis* serovars and strains. Appl. Env. Microbiol. 59: 114-119.

Brizzard and Whiteley (1988) Nucleotide sequence of an additional crystal protein gene cloned from *Bacillus thuringiensis* subsp. *thuringiensis*. Nucleic Acid Res.16: 4168-4169.

Buchanan, R.L. and Schultz, F.J. (1994) Comparison of the Tecra VIA kit, Oxoid BCET-RPLA kit and CHO cell culture for the detection of *Bacillus cereus* diarhoeal enterotoxin. Letts. in Appl. Microbiol. 12: 353-356.

Bulla, L.A., Bechtel, D.B., Kramer, K.J. and Shethna, Y.I. (1980) Ultrastructure, physiology and biochemistry of *Bacillus thuringiensis*. CRC Crit. Rev. Microbiol. 8: 147-205.

Burges, H.D. (1981) Safety, safety testing and quality control of microbial pesticides; In: Microbial Control of Pests and Plant Disease (1970-1980) (Ed. Burges, H.D.) pp 737-767. London and New York: Academic Press.

Carlton, B.C. and Gonzalez, J.M. (1985) The Genetics and Molecular Biology of *Bacillus thuringiensis*; In: Molecular Biology of the Bacilli, Vol. 2 (Ed. Dubnau, D.,), pp. 211-249. New York: Academic press.

Carozzi, N.B., Kramer, V. C., Warren, G. W. Evola, S. and Koziel, M. G. (1991) Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. Appl. Environ. Microbiol. 57: 3057-3062.

Cerón, J., Ortíz, A., Quintero, R., Güereca, L. and Bravo, A. (1995) Specific PCR primers directed to identify *cryI* and *cryIII* genes within a *Bacillus thuringiensis* strain collection. Appl. Environ. Micro. 61: 3826-3831.

Day, T.L., Tatani, S.R., Notermans, S. and Bennett, R.W. (1994) A comparison of ELISA and RPLA for the detection of *Bacillus cereus* diarrhoeal enterotoxin. J. Appl Bacteriol. 11: 9-13.

Dent, D.R. (1993) The use of *Bacillus thuringiensis* as an insecticide. In: Exploitation of Microorganisms (Ed. Jones, D.G.), pp.19-44, Chapman and Hall, London.

Diouf, N. (1980) Trial on protection of dried fish by pyrethrum and solar energy. paper presented at FAO Expert Consultation on Fish Technology in Africa, Dar es Salaam, Tanzania, 11th-15th Feb. 1980.

Donovan, W.P., Gonzalez, J.M., Gilbert, M.P and Dankocsik, C. (1988) Isolation and characterisation of EG2158, a new strain of *Bacillus thuringiensis* toxic to coleopteran larvae, and nucleotide sequence of the toxin gene. Mol. Gen. Genet. 214: 365-372.

Drobniewski, F.A (1994) The safety of *Bacillus* species as insect vector control agents; J. Appl. Bacteriology 76: 101-109.

Dulmage, H.T. (1971) Production of δ-endotoxin by eighteen isolates of *Bacillus thuringiensis* serotype 3 in fermentation media. J. Inv. Pathol. 18: 353-358.

Duncan, C.L. and Strong, D. H. (1969) Ileal loop fluid accumulation and production of diarrhea in rabbits by cell-free products of *Clostridium perfringens*. J. Bacteriol. 100: 86-94.

Hofte, H. and Whiteley, H.R. (1989) Insecticidal proteins of *Bacillus thuringiensis* Microbiol. Rev. 53: 242-255.

Esser, J.R. (1991) Biology of Chrysomya megacephala (Diptera: Caliphoridae) and reduction of losses that it causes to salted-dried fish in south east Asia. Bullet.Entomol. Res.81:33-47.

Esser, J.R. (1992) Reduction of insect infestation and losses of traditionally processed salted-dried fish in Thailand.Investigations into treatment of salted-dried fish with insecticides.Unpublished overseas assignment report 2.Overseas Development Administration.London.

Esser, J.R. (1994) Reduction of insect infestation and losses of traditionally processed salted- dried fish in Thailand. Investigations into treatment of salted-dried fish with insecticides. Unpublished overseas assignment report 3. Overseas Development Administration. London.

Esser, J.R., Hanson, S.W. & Taylor, K.D.A., Cox, J.R., Evans, N.J. & Walker, D.J. (1986) Evaluation of insecticides to protect salted-dried marine catfish from insect infestation during processing and storage.Investigation of losses associated with the production and storage of cured fish and squid in Indonesia.Unpublished joint report Humberside College of Higher Education, Grimsby and Tropical Development Research Institute, London, UK.

Esser, J.R. (1994) Reduction of insect infestation and losses of traditionally processed salted-dried fish in Thailand. Investigations into treatment of salted-dried fish with exotoxin. Unpublished overseas assignment report. Overseas Development Administration. London.

Fietelson, J.L., Payne, J. and Kim, L. (1992) *Bacillus thuringiensis*: Insects and beyond. Bio/ Technology. 10: 271-275.

Endo, Y & Nishiitsutsuji-Uwo, J. (1980) Mode of action of *Bacillus thuringiennsis* δ-endotoxin: histopathological changes in the silkworm midgut. J. Invert. Pathol. 36: 90-103.

Esser, J.R., Hanson, S.W. & Taylor, K.D.A., Cox, J.R., Evans, N.J. & Walker, D.J. (1986) Evaluation of insecticides to protect salted-dried marine catfish from insect infestation during processing and storage.Investigation of losses associated with the production and storage of cured fish and squid in Indonesia.Unpublished joint report Humberside College of Higher Education, Grimsby and Tropical Development Research Institute, London, UK.

FAO. (1970) Smoke curing of fish. FAO Fisheries Report, No.88. Food and Agriculture Organization of the United Nations, Rome.

FAO. (1971) Equipment and methods for improved smoke-drying of fish in the Tropics. FAO Fisheries Report, No.88. Food and Agriculture Organization of the United Nations, Rome.

FAO. (1981) The prevention of losses in cured fish. Fisheries Technical Paper Number FIIU/T219. Food and Agriculture Organization of the United Nations, Rome.

FAO. (1989) A field guide to the types of insects and mites infesting cured fish. Fisheries Technical Paper No. 303. Food and Agriculture Organization of the United Nations, Rome.

FAO/WHO (1970) Evaluations of some pesticide residues in food. The Monograph. Report of Joint Meeting of FAO Working Party of Experts and WHO Expert Group on Pesticide Residues. FAO/PL: 1969/M/17/1. WHO/FOOD ADD./70.38.

Faust, R. M. (1975) Evidence for plasmid-associated crystal toxin production in *Bacillus thuringiensis* subsp. *israelensis*. Plasmid 9: 98-103.

Fisher, R. and Rosner, L. (1959) Toxicology of the microbial insecticide, Thuricide. J Agric. Food Chem. 7: 686-688.

Golob, P., Cox, J.R., and Kilminster, K. (1987) Evaluation of insecticide dips as protectants of stored dried fish from dermestid beetle infestation. J. Stored. Prods. Res. 23: 47-56.

Gonzalez, J.M., Dulmage, H.T. and Carlton, B.C (1981) Correlation between specific plasmids and δ endotoxin production in *Bacillus thuringiensis*. Plasmid 5: 351-365

Granum, P.E. (1994) *Bacillus cereus* and its toxins. J. Appl. Bacteriol. Symp. Suppl. 76: 61S- 66S.

Granum, P.E., Brynestad, S. and Kramer, J. M., (1993) Analysis of enterotoxin production by *Bacillus cereus* from dairy products, food poisoning incidents and non-gastrointestinal infections. Int. J. Food Microbiol. 17: 269 - 279

Hadley, W.M., Burchiel, S.W., McDowell, T.D., Thilsted, J.P., Hibbs, C.M., Horton, J.A., Day, P.W., Friedman, M.B. and Stoll, R.E. (1987) Five month oral (diet) toxicity/ infectivity study of *Bacillus thuringiensis* insecticides in sheep. Fund. Appl.Toxicol. 8: 236-242.

Hameed, A.A and Landen, R. (1994) Studies on *B. thuringiensis* strains isolated from Swedish soils: insect toxicity and production of *B. cereus* diarrhoeal-type enterotoxin; World J. of Microbiol. Biotechnol. 10: 406-409.

Herbert, D., Phipps, P.J. and Strange, R.E., (1971) Chemical analysis of microbial cells. Methods in Microbiol. 5B: 209-344.

Hodgman, T.C., Ziniu, Y., Ming, S., Sawyer, T., Nicholl, C.M. and Ellar, D.J. (1993) Characterization of a *Bacillus thuringiensis* strain which is toxic to the housefly *Musca domestica*. FEMS Microbiol. Letts 114: 17-22.

Höfte, H., and Whiteley, H. R. (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. 53: 243-255.

Hofte, H., van Rie, J., Jansens, S., van Houtven, A., Vanderbruggen, H. and Vaeck, M.(1988) Monoclonal antibody analysis and insecticidal spectrum of three types of lepidopteran-specific insecticidal crystal proteins of *Bacillus thuringiensis*. Appl. Env. Microbiol. 54: 2010-2017.

Howe, R.W. (1965) A summary of estimates of optimal and minimal condition for population increase of some stored products insects. J. Stored. Prods. Res. 1: 177-84

Indrasith, L. S., Suzuki, N., Ogiwara, K., Asano, S. and Hori, I. (1992) Activated insecticidal crystal proteins from *Bacillus thuringiensis* serovars killed adult house flies. Letts. Appl. Microbiol.14:174-177.

Jackson, S.G., Goodbrand, R.B., Ahmed, R. amd Kasatiya, S. (1995) *Bacillus cereus* and *Bacillus thuringiensis* isolated in a gastroenteritis outbreak investigation. Letts. Appl. Microbiol. 21: 103-105.

Jarrett, P and Stephenson, M. (1990) Plasmid transfer between strains of *B. thuringiensis* infecting *Galleria mellonella* and *Spodoptera littoralis*. Appl. and Env. Microbiol. 56:1608-1614.

Johnson, C. and Bishop, A.H. (1996) A technique for the effective enrichment and isolation of *Bacillus thuringiensis*. FEMS Microbiol. Letts. 142: 173-177.

Johnson, C. & Esser, J.R. (1996) Investigation into the efficacy of salting as a means of controlling blowfly infestation in traditionally processed fish in the tropics. Report for the Overseas Development Administration, May 1996, Project Number R5795.

Jones, K.A., Westby, P.J.A. & Jeger, M.J. (1993) The exploitation of microorganisms in the developing countries of the tropics. In: Exploitation of Microorganisms (Ed. D.G. Jones) pp.343-370. London: Chapman and Hall.

Karel, M. (1973) Recent research and development in the field of low and intermediate moisture foods. Crit. Rev. Food Technol. 3: 329-358.

Karel, M. (1974) Fundamentals of dehydration processes. In: Advances in Preconcentration and Dehydration of Foods(Ed. Spicer A.), pp. 49-60. London: Applied Science Publishers.

Karel, M. (1975) Dehydration of foods. In: O.R. Fenema (Ed) Principles of Food Science, Part 2, Physical Principles of Food Preservation pp.328-331. New York: Marcel Dekker.

Knowles B.H. and Dow, J.A.T. (1993). The crystal δ-endotoxins of *Bacillus thuringiensis*: models for their mechanism of action on the insect gut. Bioessays. 15: 469-475.

Knowles, B.H. and Ellar, D.J. (1986); Characterisation and partial purification of a plasma membrane receptor for *Bacillus thuringiensis* var. *kurstaki* lepidopteran specific δ endotoxin; J. Cell. Sci. 83: 89-101.

Knowles, B.H. and Ellar, D.J. (1987) Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis* δ endotoxins with different specificity; Biochim. Biophys. Acta 924: 509-518.

Kordyl, E. (1976) Some protective measures against insect infestation of dried fish in Africa.In: Procedings of the conference on handling and marketing of tropical fish, London, 5th-9th July 1976. Tropical Products Institute.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Lamanna, C. and Jones, L. (1963) Lethality for mice of vegetative and spore forms of *Bacillus cereus* and *Bacillus cereus*-like insect pathogens injected intraperitoneally and subcuteaneously. J. Bacteriol. 85: 532- 535.

Lawson, J.R. & Gemmell, M.A. (1985) The potential role of blowflies in the transmission of taeniid tapeworm eggs. Parasitology 91:129-143.

Lerici, C.R., Pinnavaia, G., Dalla Rosa, M. & Bartolucci, L. (1985) Osmotic dehydration of fruit: Influence of osmotic agents on drying behaviour and product quality. J. Food. Sci. 50: 1217-1219.

Levinson, B. L., Kasyan, K. J., Chiu, S. S., Currier, T. C. and Gonzalez, J.M. (1990) Identification of β-exotoxin production, plasmids encoding β-exotoxin, and a new exotoxin in *Bacillus thuringiensis* by using high-performance liquid chromatography. J.Bacteriol. 172: 3172-3179

Leung, H.K. (1987) Influence of water activity on chemical reactivity. In: Water Activity: Theory and Applications to Food. (Ed. Currier, T.C.) pp27-50. New York: Marcel Dekker.

Li, J., Carroll, J. and Ellar, D.J. (1991) Crystal structure of insecticidal δ endotoxin from *Bacillus thuringiensis* at 2.5A resolution. Nature 353: 815-821.

Li, R. S., Jarrett, P. and Burges, H. D. (1987) Importance of spores, crystals, and δ-endotoxins in the pathogenicity of different varieties of *Bacillus thuringiensis* in *Galleria mellonella* and *Pieris brassicae*. J. Invert. Pathol. 50: 277-284.

Lisanski, S.G., Quinlan, R. and Tassoni, G. (1993) *Bacillus thuringiensis* production handbook. CPL Press.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Lund, T. and Granum, P.E. (1996) Characterisation of a non-haemolytic enterotoxin complex from *Bacillus cereus* isolated after a foodborne outbreak. FEMS Microbiol. Letts. 141:151-156.

Madden, J.L., Anggawati, A.M. & Indriati, N. (1995) Impact of insects on the quality and quantity of fish and fish products in Indonesia. In: Fish Drying in Indonesia Proceedings (Champ, B.R. Ed.) No 59, pp. 97-106. Canberra: Australian Centre for Agricultural Research.

Mandel, M. and Higa, A. (1970) Calcium-dependent bacteriophage DNA infection. J. Molec. Biol. 53: 159-162.

Maniatis, T, (1989) In 'Molecular cloning: a laboratory manual' (Eds. Sambrook, J., Fritsch, E.F. and Maniatis, T., 2nd edition) New York: Cold Spring Harbour Laboratory Press.

Martin, P.A. and Travers, R.S. (1989) Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. Appl. Environ. Microbiol. 55: 2437-2442.

Mathen, C., Unnikrishnan Nair, T.S. & Ravindranathan Nair, P. (1992) Effect of vegetable oils on insect infestation during storage of dry cured fish. Fish. Technol. 29: 48-52.

McGaughey (1985) Insect resistance to the biological insecticide *Bacillus thuringiensis*. Science 229:195-195.

McGaughey,W.H. and Beeman, R.W. (1988) Resistance of *Bacillus thuringiensis* in colonies of the Indian meal moth and almond moth (Lep.: Pyralidae). J. Econ. Entomol. 80: 1122-1126..

McLellan, R.H. (1963) The use of a pyrethrum dip as protection for drying fish in Uganda. Pyrethrum Post 7: 8-10.

Meadows, M. P., Ellis, D. J., Butt, J., Jarrett, P. and Burges, H. D. (1992) Distribution, frequency and diversity of *Bacillus thuringiensis* in an animal feed mill Appl. Environ. Microbiol. 55: 2437-2442.

Melnick, J.L. & Penner, L.R. (1947) Experimental infection of flies with human poliomyelitis virus. Proc. Sco. exp. Biol. 65: 342.

Meynell, P.J. (1978) Fish processing and preservation in Malawi. Tropical Products Institute unpublished report. London: TPI.

Miyasono, M., Inagaki, S., Yamamoto, M., Ohba, K., Ishiguro, T., Takeda, R. and Hayashi, Y. (1994) Enhancement of δ-endotoxin activity by toxin-free spore of *Bacillus thuringiensis* against the diamondback moth, *Plutella xylostella*. J. Invert. Pathol..63:111-112.

Morris, O.N., Converse, V., Kanagaratnam, P. and Davies, J.S. (1996) Effect of cultural conditions on spore-crystal yield and toxicity of *Bacillus thuringiensis* subsp. *aizawai* (HD 133). J. Inv. Pathol. 67: 129-136.

Morris, R.F. & Andrews, D. (1968) Investigations into the use of pyrethrum and other insecticides for the control of the blowfly *Calliphora terraenovae* (Macq.), infesting light-salted cod in Newfoundland. Pyrethrum Post 9: 9-12.

Olley, J.(1961). Pyrethrum in the fish meal industry. Pyrethrum Post. 6: 9-10.

Osuji, F.N.C. (1975) Recent studies on the infestation of dried fish in Nigeria by *Dermestes maculatus* and *Necrobia rufipes* with special reference to the Lake Chad district. Trop. Stored Prod. Inf. 29:21-32.

Osuji, F.N.C. (1975) Some aspects of the biology of *Dermestes maculatus* Degeer in dried fish. J. Stored. Prods. Res. 24: 167-79.

Peferoen, M. (1991) *Bacillus thuringiensis* in crop protection. Agro-Industry Hi-Tech 6: 5-9.

Passmore, R. & Eastwood, M.A. (1986) Protein energy malnutrition. In Davidson and Passmore Human Nutrition and Dietetics (Eds. Passmore, R.and Eastwood, M.A.) 8th edition, chapter 29, pp 279-291.

Powell, K.A. (1993) The commercial exploitation of microorganisms in agriculture In Exploitation of Microorganisms (Ed. Jones, D.G.) Chapter 14, pp.343-370. London: Chapman and Hall.

Ray, D.E. (1991) Pesticides derived from plants and other organisms. In: Handbook of pesticide toxicology (Eds. Hayes W.J. and Laws E.R. Jr.) pp.585-636. San Diego: Academic Press.

Rusul, G. & Yaacob, N.H. (1995) Prevalence of *Bacillus thuringiensis* in selected foods and detection of enterotoxin in selected foods using TECRA-VIA and BCET-RPLA. Int. J. Food Microbiol. 25: 131-139.

Salama H.S. and Morris, O.N. (1993) The use of *Bacillus thuringiensis* in developing countries. 237-249. In *Bacillus thuringiensis*, an environmental biopesticide: theory and practice. (Eds. Entwhistle, P.F., Cory, J.S., Bailey, M.J. and Higgs, S.). Chichester: John Wiley.

Salama, H.S., Foda, M.S., Dulmage, H.T. and El-Sharaby, A. (1983) Novel fermentation media for production of δ-endotoxin for *Bacillus thuringiensis.* J. Inv. Pathol. 41: 8-19.

Scoggins, J.K. and Tauber, O.E. (1951) The bionomics of *Dermestes maculatus.* Larval and pupal development at different moisture levels and on various media. Ann. Entomol. Soc. Am. 44: 544-50.

Sebasta, K., Farkas, J. and Horska, K. (1981) Thuringiensin the β-exotoxin of *Bacillus thuringiensis.* In: Microbial control of pests and plant diseases 1970-1980 (Ed. Burges, H.D) pp. 249-281. New York: Academic Press.

Siegel, J.P. and Shadduck, J. A. (1990) Clearance of *Bacillus sphaericus* and *Bacillus thuringiensis* ssp. *israelensis* from mammals. J. Econ. Entomol. 83: 347-355.

Siegel, J.P., Shadduck, J.A. and Szabo, J. (1987) Safety of the entomopathogen *Bacillus thuringiensis* var. *israelensis.* J. Econ. Entomol. 80: 717-723.

Somme, L. and Gjessing, E.T. (1963) Insecticides for protection against blowflies in the stockfish industry. Pyrethrum Post. 7: 3-7.

Stewart, G. S. A. B., Johnstone, K., Hagelberg, E. and Ellar, D.J. (1981) Commitment of bacterial spores to germinate. A measurement of the trigger reaction. Biochem. J. 198: 101-106.

Stone, T.B., Sims, S.R.and Marrone, P.G. (1989) Selection of resistance of tobacco budworm to a genetically engineered *Pseudomonas fluorescens* containing the delta-endotoxin of *Bacillus thuringiensis* subsp. *kurstaki* J. Invert. Pathol. 53: 228-234.

Thomas, W.E. and Ellar, D.J. (1983) *Bacillus thuringiensis* var. *israelensis* crystal δ-endotoxin: effects on insect and mammalian cells *in vitro* and *in vivo*. J. Cell Sci. 60:181-197.

van Nguyen, A. (1995). Ph.D. thesis. University of Cambridge.

Vogel (1961) A text-book of quantitative inorganic analysis, 3rd Edition. Nitrogen: pp. 256-257; Phosphate: p.810. London: Lowe and Brydone.

Walker, D.J. (1986) Report on a visit to Indonesia to present recommendations of the joint Humberside College/TDRI project to reduce post-harvest losses in cured marine catfish. Report R1311(S) Tropical Development and Research Institute, London, UK.

Walker, D.J. (1987) A review of the use of contact insecticides to control post-harvest insect infestation of fish and fish products. FAO Fish. Circ.804: 19.

Walker, D.J. (1988) Control of insect infestation in fishery products in less developed countries. In: Morrissey, M.T. (Ed.) Postharvest Fishery Losses. pp.163-180. Proceedings of an International Workshop held April 12th-16th 1987 at the University of Rhode Island, Kingston, RI. ICMRD.

Walker, D.J. & Donegan, L. (1984) Spray application of insecticides to protect sundrying fish from blowfly infestation during the wet season in Malawi. Internat. Pest Control 26: 132-137.

Walker, D.J. & Evans, N.J. (1984) Report on two visits to the Gambia to evaluate the use of insecticides in reducing losses in traditionally cured fish. Report R1245(S) Tropical Development and Research Institute, London, UK.

Vaeck, M., Raynaerts, A., Höfte, H., Jansens, S., de Beuckleer, M., Dean, C., Zabeau, M., Van Montagu, M. & Leemans, J. (1987) Transgenic plants protected from insect attack. Nature 328: 33-37.

Walsh, J. (1986) River blindness, a gamble pays off. Science 232: 922-5

WHO (1973) Evaluations of some pesticide residues in food: The Monographs. WHO Pesticide Residue series, No 2, WHO, Geneva.

Young, J. & Esser, J.R. (1992) Marketing sun dried fish in Indonesia. The case for adding insecticides to add value. In: Proceedings of the 4th Asean Food Conference, Jakarta, February 17-21, 1992.

Youdeowei, A. and Service, M.W.(1983) Pest and vector management in the tropics. London: Longman.

Yu, S.Y. (1995) Salted dried fish in Southeast Asia. In: Fish Drying in Indonesia Proceedings (Ed. Champ, B.R.) No 59, pp. 97-106. Australian Centre for Agricultural Research, Canberra.