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THE DEVELOPMENT AND EVALUATION OF BACULOVIRUS FORMULATIONS FOR THE BIOLOGICAL CONTROL OF THE AFRICAN COTTON BOLLWORM

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A dissertation submitted to the Faculty of Science, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science.

Johannesburg, 2008

DECLARATION

I declare that this dissertation is my own unaided work. It is being submitted for the Degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university.

_____

Michelle Lilly Grant

_____ day of _____ 2009

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ABSTRACT

Wettable powder and suspension formulations of a *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus (HearSNPV) biopesticide were developed for the control of the African cotton bollworm, *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae).

Greenhouse trials of an unformulated suspension of HearSNPV were conducted on tomato plants artificially infested with *H. armigera* larvae to determine the efficacy of the virus without formulation additives. Two greenhouse trials were carried out. An application rate of 1.00 x 10^{13} occlusion bodies per hectare (OBs/ha) significantly reduced the number of larvae per plant compared to the control group in greenhouse trial 1, and application rates ranging between 4.80 x 10^{11} to 4.80 x 10^{12} OBs/ha significantly reduced the number of larvae compared to the control group in greenhouse trial 2. In terms of pest reduction, the HearSNPV treatments in greenhouse trial 2 were comparable to a commercially available biopesticide (Dipel, containing *Bacillus thuringiensis*).

The effect of the purity, in terms of bacterial contamination, of the inoculum used to infect *H. armigera* insects was compared at three different storage temperatures (4, 25 and 37 °C) for three different storage periods (7, 30 and 90 days). No significant difference was found between the bacterial counts of the homogenates prior to storage. However, the total bacterial aerobic counts increased on storage and were highest for homogenates prepared from the crude inoculum which averaged 5.16 log cfu/mg, compared to 3.92 log cfu/mg and 2.90 log cfu/mg for the purified and control (sterile distilled water) inoculums respectively. The contaminating bacteria were identified using 16S rDNA sequence analysis, and found to be a *Bacillus* and *Enterococcus* species. This suggests that bacterial contamination should be minimized from the start of baculovirus production, since the microbial load can increase on storage.

Additives, namely Instant Starch (IS) and Xanthan Gum (XG) were used to prepare HearSNPV suspensions and these were evaluated under accelerated storage conditions, corresponding to storage at room temperature for two years. The IS suspension completely lost its insecticidal activity after storage, while the XG and Unformulated (UF) suspensions were 4.8 and 3 times respectively, less insecticidal after storage. The microbial load decreased from an initial total aerobic count of 8.0 log cfu/ml each, to 3.24 log cfu/ml, 6.86 log cfu/ml and 4.26 log cfu/ml for the IS, UF and XG suspensions respectively. Two bacterial genera were isolated from these samples, namely *Bacillus* and *Paenibacillus*. The pH of the suspensions remained near neutral, with the exception of the IS suspension, which had a pH of approximately 3.5 after storage. The XG suspension displayed the best stability on storage, followed by the UF suspension.

A spray-dryer was used to develop a wettable baculovirus powder and Response Surface methodology was used to optimize the process. Two carriers, namely Polysaccharide-MS and Polysaccharide-WM were used and inlet temperature, air speed and feed flow rate were used as the model factors. The response factors monitored were powder yield (mg/ml), active ingredient yield of the occlusion bodies (OBs/mg), and moisture content (%). The effect of spray-drying on the microbial load of the samples was also examined. The optimal conditions for Polysaccharide-WM were determined to be an inlet temperature of 130 °C, air speed dial setting of 45 and feed flow rate of 9 ml/minute. According to the predictive models obtained, this would give a powder yield of 337.5 mg, active ingredient yield of 8.0×10^5 OBs/mg and moisture content of 4.8 %. The optimal conditions for Polysaccharide-MS were determined to be an inlet temperature of 130 °C, air speed dial setting of 45 and feed flow rate of 9 ml/minute. According to the predictive models obtained, this would give a powder yield of 110.9 mg/ml, active ingredient yield of 1.0×10^6 OBs/mg and moisture content of 4 %. Spray-drying reduced the microbial load of the sample four-fold.

Baculovirus biopesticides in the form of suspensions and wettable powders were developed and evaluated in this project and will form the foundation for the

development of commercial biopesticides for the control of the African cotton bollworm.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
GV	Granulovirus
HearSNPV	*Helicovpera armigera* single nucleocapsid nucleopolyhedrovirus
LD	Lethal dose
NPV	Nucleopolyhedrovirus
OB	Occlusion body
UV	Ultra-violet

CHAPTER 1

General introduction

1.1 Introduction

The African cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is a highly polyphagous and widespread insect pest, which causes enormous economic losses to many crops (Fitt, 1989). It has been ranked as the most important insect pest in South Africa (Moran, 1983), primarily causing damage to cotton, citrus, sorghum and tomato crops (Hofs et al., 2006; Moore et al., 2004; Sivasupramaniam et al., 2007). Moore et al. (2004) reported damage by *H. armigera* to citrus fruit in South Africa to be between 26 and 36 % of the crop.

Chemical insecticides such as pyrethroids, quinolphos, monocrotophos and endosulfan have been used to control *H. armigera,* but their use is becoming less effective because of the resistance that *H. armigera* have developed to them (Tatchell, 1997). Chemical insecticide use is also becoming less appropriate because of a concern for consumers' food safety and for the environment. This makes the use of biological pesticides an attractive alternative for insect pest control (Tatchell, 1997). Currently, the only registered biopesticide available for the control of *H. armigera* is *Bacillus thuringiensis* var. *kurstaki* (Moore et al., 2004).

Baculoviruses (family Baculoviridae) are generally highly selective pathogens of insects belonging to the orders Lepidoptera and Hymenoptera (Fuxa, 2004). Two genera in the family are the *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV). The virions are occluded in a crystalline protein matrix composed of polyhedrin in NPVs and granulin in GVs (Funk et al., 1997), which enables the virions to remain viable in the environment for many years. Larvae of *H. armigera* become infected with the virus after ingesting occlusion bodies contaminating their food source and this results in disease of the larvae and, depending on the dose, their death (Federici, 1997).

Members of the baculovirus family make attractive agents for the biological control of *H. armigera* because of their high pathogenicity, narrow host range, persistence in the environment and safety to vertebrates, plants and the environment. They can also be processed, formulated and applied to crops using conventional equipment (Cory and Hails, 1997; Khetan, 2001; Miller et al., 1983). *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus (HearSNPV) was tested on citrus in South Africa and offered protection to the fruit by reducing damage between 75 and 84 % (Moore et al., 2004).

Several environmental factors have been found to inactivate baculoviruses namely, temperature, leaf surface exudates, sun, wind and rain (Chang et al., 1998; Jones et al., 1997; Jones et al., 1993). However, additives can been used to protect the baculovirus from some of these environmental factors, thereby improving their persistence and helping to maintain their insecticidal activity (Jones et al., 1997; Tamez-Guerra et al., 2000b).

Jones and Burges (1998) identified the basic purposes of formulations: formulations should stabilize the microorganism during production, distribution and storage; assist in the handling and application of the microorganism; protect the microorganism from environmental factors that may inactivate it; and enhance the activity of the microorganism at the target site. The challenge is to address all four of the above points to produce a product that is practical as well as economical (Behle et al., 2003; Jones and Burges, 1998).

Baculoviruses can be formulated as wettable powders, granules, or as liquid suspensions. Additives may need to be incorporated to reduce the growth of unwanted microbial populations, to prevent separation during storage and to enhance application. These additives can be added to formulated products during processing, or they can be added to spray tank mixes prior to application (Behle et al., 2003; Jones et al., 1997; Lasa et al., 2008; Tamez-Guerra et al., 2000b).

Additives such as lignin and pregelatinized corn flour have been used in spray-dried formulations of baculovirus occlusion bodies to stabilize them during storage and to protect them from UV once applied to a crop (Tamez-Guerra et al., 2000b; Tamez-Guerra et al., 2002).

Many formulation ingredients, such as sugar, provide more than one benefit to biological pesticides. Sugar has been found to have phagostimulatory effects on the insect, and it is thought to play a role in protecting the occlusion body when it dries on the crop. The challenge is to combine the advantageous effects of formulation ingredients without compromising on cost, efficacy and protection from adverse environmental conditions (Behle et al., 2003).

1.2 The African cotton bollworm

The African cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is an insect pest of many crops including tomato, sunflower, corn, pulse, maize, tobacco, pigeonpea, chickpea and cotton. It is the most widespread agricultural pest and occurs in New Zealand, the Middle East, Europe, Africa, India, Asia and many Pacific Islands (Bués et al., 2005; Fitt, 1989; Talekar et al., 2006; Zhou et al., 2000).

The adults lay their eggs on plant foliage, and since the larvae preferably feed on plant structures that are high in nitrogen such as the cotton bolls, corn ears, tobacco buds and sorghum heads, they penetrate the fruit and feed inside it, which impacts crop yield directly.

The successfulness of *H. armigera* as a population, and therefore as a pest to crops can be attributed to many characteristics, including polyphagy, high mobility, high fecundity and a facultative diapause (Fitt, 1989). The larvae of *H. armigera* have well developed detoxification systems, which enables them to feed on a wide range of plants. They are also able to migrate when local conditions for reproduction become poor (Fitt, 1989). *H. armigera* has become a very important

pest over a wide geographical range due to its polyphagous nature (Zhou et al., 2000).

Populations of *H. armigera* were previously controlled by chemical insecticides, but the efficacy of many chemical insecticides has been reduced, since *H. armigera* developed resistance to them (Srinivas et al., 2004). *Helicoverpa armigera* populations in the cotton and pulse growing regions of Central and Southern India have been shown to have developed resistance to the insecticides pyrethroids, quinolphos, monocrotophos and endosulfan. This resistance was found to be due to a combination of the over expressing P-glycoprotein, decreased sensitivity to acetylcholinesterases and higher levels of esterases and phosphatases (Srinivas et al., 2004).

Bumble bees are used as pollinators in some crops such as long-season tomato and sweet pepper, and chemical insecticides are incompatible with these pollinators, making biopesticides essential in these crop systems (Tatchell, 1997).

Populations of *H. armigera* larvae in Pakistan were shown to have a level of tolerance to new chemical insecticides developed in the late 1990's. This resistance was thought to be due to cross-resistance from mechanisms developed to older chemical insecticides (Ahmad et al., 2003).

The development of resistance to these chemical insecticides resulted in more frequent application and the use of harder chemicals to control *H. armigera* populations (Fitt, 1989). This became a huge concern for consumers from a food safety aspect, making the use of biopesticides more attractive (Tatchell, 1997).

1.3 Baculoviruses

It has been proposed that the Baculoviridae family be divided into four new genera, namely the alphabaculoviruses, betabaculoviruses, gammabaculoviruses and deltabaculoviruses in order to separate the hymenopteran and dipteran baculoviruses from the lepidopteran baculoviruses (Theilman and Blissard, 2008). However, currently the Baculoviridae family is divided into two genera, namely

the *Nucleopolyhedrovirus* (NPV) and the *Granulovirus* (GV). NPVs are large polyhedron-shaped structures ranging from 0.15 to 15 μm in size that contain many virions, whereas the GVs have smaller occlusion bodies ranging from 0.3 to 0.5 μm, containing a single virion. The nucleocapsids contained within the virions are rod-shaped and contain double-stranded DNA of between 80-180 kbp in size (Funk et al., 1997; van Regenmortel et al., 2000).

Baculoviruses are generally highly selective pathogens of insects belonging to the orders Lepidoptera and Hymenoptera (Fuxa, 2004). The virions are occluded in a crystalline protein matrix composed of polyhedrin in NPVs and granulin in GVs (Funk et al., 1997), which enables the virions to remain viable in the environment for many years. Soil samples have shown that approximately 1 % of NPV occlusion bodies of the Douglas-fir tussock moth (*Orgyia pseudotsugata*) were still active after 41 years in the soil, enough to infect tussock moth larvae and start an epizootic (Thompson et al., 1981).

The narrow host range of baculoviruses can be explained by several factors. The protein matrix that encloses the virions is only dissolved in high alkaline environments (pH 8-11), such as the midgut of lepidopteran hosts. Baculoviruses exhibit tissue specificity and this tropism varies according to the host species. Some baculoviruses are able to infect and replicate in many insect hosts, whereas others are limited to replication in specific insect species (Miller and Lu, 1997).

1.3.1 Mode of infection

Larvae of the insect host become infected with NPV after ingesting occlusion bodies contaminating their food source, and once the occlusion bodies have dissolved in the insect's midgut due to its alkalinity, the virions are released. The virions pass through the peritrophic membrane and the virion envelope fuses with receptors in the midgut vesicles (van Regenmortel et al., 2000). The virion is able to enter host cells by direct membrane fusion. Specific virus attachment sites on the host cells are indicated by two major factors namely (a) saturable attachment of the virus to host cells and (b) virus competition for limited receptor sites. The

receptor in lepidopteran larval midguts is protein in nature and the alkalinity provides optimum conditions for fusion of the virion to the midgut receptors (Horton and Burand, 1993).

The nucleocapsids enter the microvilli and travel to the nucleus where replication occurs. A non-occluded phenotype of the baculovirus, known as the budded virus, disseminates viral infection throughout the tissues of the host to produce a systemic infection and once most of the cells are infected, the basal lamina, which may be weakened by virus enzymes, loses its integrity and ruptures, resulting in death of the larvae. The rupturing of the insect host's cuticle releases the occlusion bodies into the environment (Federici, 1997; Fuxa, 2004).

1.4 Biopesticides

1.4.1 Biopesticide formulation

It has been shown that baculoviruses have the ability to provide long term control of lepidopteran pests. This is due to their high pathogenicity, narrow host range, persistence in the environment, safety to vertebrates and plants and because they can be formulated and applied to crops with conventional equipment (Cory and Hails, 1997; Khetan, 2001; Miller et al., 1983). Thompson et al. (1981) reported that in 1938, the Douglas-fir tussock moth population was suppressed by NPV within one year.

Baculoviruses only replicate in living cells, therefore have to be produced either in cell culture (*in vitro*) or in live insect hosts (*in vivo*) (Khetan, 2001). Burges et al. (1998a) notes that *in vitro* production is approximately 10 times more expensive than *in vivo* production and that there are also difficulties with the scaling-up of *in vitro* production. The *in vivo* production of baculoviruses is more practical for large-scale production, but can result in microbial contamination from microorganisms associated with the insects (Grzywacz et al., 1997; Podgwaite et al., 1983).

Formulation additives can be used to inhibit microbial contamination as well as protect baculoviruses from adverse environmental factors, enhance storage stability and maximize application efficiency (Jones et al., 1997; Lasa et al., 2008; Tamez-Guerra et al., 2002). The formulations used with pathogens, that infect the insect perorally, need to be palatable to the insect and be applied evenly to the insect pest's food source. It also needs to be persistent and remain viable for a time period sufficient for the insect to consume a lethal dose (Burges and Jones, 1998a).

The aim of formulation is to combine the advantageous effects of formulation ingredients without compromising on cost, efficacy and protection from adverse environmental factors (Behle et al., 2003).

Biopesticides should aim to have a two year shelf-life, as is required by commercial chemical pesticides. The shelf-life of a microbial agent can be improved by adding ingredients which increase the stability and shelf-life of the product (Jones and Burges, 1997).

Wettable powder or granules can be prepared by freeze-drying, air-drying or spray-drying. Freeze-drying is carried out by freezing the product and evaporating the water under vacuum to produce powder or granules. A spray-dried product is prepared by spraying a suspension of the microbial agent into a hot air stream in an expansion chamber. The temperature of the air stream is approximately 120 °C, but the microbial agent is only exposed to the inlet temperature for a short time period, and is kept cool by the evaporation of the water, thus non-delicate micro-organisms remain viable in this process. This process has the advantage that it may kill contaminating microorganisms in the product (Jones and Burges, 1997). A suspension that is air-dried has a great chance of becoming contaminated, therefore freeze-drying or spray-drying are the preferred methods (Jones and Burges, 1997).

The insecticidal activity of NPV wettable powders has been shown to be retained at a moisture content of less than 10 % (Tamez-Guerra et al., 2002), however Burges and Jones (1998a) suggest that the moisture content of wettable powders or granules should be 5 % or less to ensure good storage of the product (Burges and Jones, 1998a). Airtight containers should be used to keep the moisture content below 5 %, because free water in the product allows the growth of contaminants, whose metabolites may have adverse effects on the microbial agent (Jones and Burges, 1997).

Dry formulations are easier to handle and store than liquid formulations due to their reduced weight and package size, however they may be dusty, posing an inhalation risk, and may require agents to keep them dry during storage (Seaman, 1990). Baculovirus occlusion bodies have been microencapsulated in cornstarch or lignin by spray-drying to provide improved field performance and storage stability (Tamez-Guerra et al., 2002).

In respect of liquid suspensions, water or oil have mainly been used as carriers of microbial agents, although the growth of contaminating microorganisms in water based concentrates has made water an inferior carrier (Jones and Burges, 1997). The growth of contaminating microorganisms can be suppressed by maintaining the pH of the suspension between 4 and 6. Additives can be used to inhibit contaminants, however the suppression of contaminants by medically significant antibiotics must be avoided to prevent the development of resistance by human and other vertebrate pathogens (Jones and Burges, 1997).

Microbial agents can be kept suspended in the liquid suspensions with the use of thickeners, such as gums and thixotropic agents, however surfactants will need to be included to aid flowability for application (Jones and Burges, 1997). It is important that wettable powders and suspensions mix evenly with any diluents prior to application. Dispersants and surfactants can be added to aid mixing, break the surface tension at the liquid/solid interface and to prevent settling of the microorganisms (Jones et al., 1997).

Anti-evaporants or humectants can be added to suspensions which use water as a carrier to prevent evaporation of the droplet once it has been sprayed onto the target site (Jones et al., 1997). Some sprayer types require the spray liquid to have a certain viscosity and not contain large particles such as insect debris which could block nozzles. Additives to improve the efficacy and persistence of microbial agents can be added into formulated products or be added to spray tank mixes prior to application (Jones et al., 1997).

1.4.2 Efficacy of formulated biological pesticides

A commercial formulation of codling moth, *Cydia pomonella L.*, granulovirus was proven to be a valuable alternative for codling moth control in organic apple orchards. The virus remained highly effective for the first 24 hours resulting in an insect mortality of 94 %, and moderately effective after 72 hours with an insect mortality of 71 %, under dry, sunny conditions and the virus remained active for 14 days in protected areas such as the calyx of the fruit (Arthurs and Lacey, 2004).

It has been found that spray-dried formulations with potassium lignate and pregelatinized corn flour, and lignin in combination with pregelatinized corn flour provided protection from UV to *Anagrapha falcifera* multiple nucleocapsid nucleopolyhedrovirus (*Af*MNPV) after simulated rainfall and UV exposure on greenhouse-grown cotton plants (Tamez-Guerra et al., 2000b). The ratio of dry solid formulation ingredients to virus is an important factor in producing a biopesticide, as it has been shown that adding too much lignin can reduce the storage stability of the virus (Behle et al., 2003). Some formulation ingredients, such as sugar, can decrease the concentration required to obtain 50 % mortality of the insect pest because it acts as a phagostimulant and may also protect virus activity when it dries on the crop (Behle et al., 2003). Tamez-Guerra et al. (2002) also found sugar improved the storage stability of a baculovirus.

1.5 Objectives

Developing a HearSNPV biopesticide that has good storage stability may improve its potential as a commercial biopesticide for the control of the African cotton bollworm, *Helicoverpa armigera*.

The main objective of this study was to develop and evaluate baculovirus formulations for the biological control of the African cotton bollworm.

The specific aims of this study were:

1. To determine the efficacy of unformulated HearSNPV against the African cotton bollworm on tomato plants in greenhouse trials.
2. To determine the effect of inoculum purity, in respect of bacterial contamination, on the microbial load of insect homogenates.
3. To develop and evaluate novel HearSNPV suspension formulations for control of the African cotton bollworm.
4. To develop a HearSNPV wettable powder using a multi-factorial optimization strategy.

CHAPTER 2

The efficacy of unformulated *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus (HearSNPV) against African cotton bollworm in greenhouse trials

2.1 ABSTRACT

Greenhouse trials of *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus (HearSNPV) were conducted on tomato plants artificially infested with African cotton bollworm larvae to determine the efficacy of the virus without formulation additives. Two greenhouse trials were carried out, the first in December 2005 and the second in March 2007. An application rate of 1.00 x 10^{13} OBs/ha significantly reduced the number of larvae per plant compared to the control group in greenhouse trial 1, and application rates ranging between 4.80 x 10^{11} to 4.80 x 10^{12} OBs/ha significantly reduced the number of larvae compared to the control group in greenhouse trial 2. HearSNPV treatments in greenhouse trial 2 did not differ significantly to a treatment with a commercially available biopesticide (Dipel: active ingredient *Bacillus thuringiensis*) which reduced the larval infestation by 95 % (1.08/1.14). The efficacy of the unformulated suspension of HearSNPV justifies future work with formulation additives and provides baseline data for future greenhouse trials.

2.2 INTRODUCTION

The African cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is a widespread insect pest of many crops including tomato, sunflower, corn, pulses and cotton (Bués et al., 2005; Fitt, 1989; Zhou et al., 2000). It has been named the most important pest of the lepidopteran order in South Africa (Bell and McGeogh, 1996).

The larvae's preference of feeding on plant structures that are high in nitrogen, such as the cotton bolls, corn ears and sorghum heads, results in them impacting crop yields directly causing enormous economic losses (Fitt, 1989). Commercial losses are incurred in tomatoes for example, because early instar larvae, which are tiny, bore into tomatoes and go unnoticed until the fruits are peeled for canning (García, 2006).

The use of natural enemies as biopesticides has been encouraged by international concerns for a reduction in pesticides in the environment, as well as by the development of resistance to chemical insecticides by target pests (Ahmad et al., 2003; Martin et al., 2005; van Lenteren, 2000). Baculoviruses are known to be ubiquitous in the environment and naturally control insect populations, therefore they have been utilized as an alternative to chemical insecticides (Behle et al., 2003).

An advantage of using biological control agents, such as baculoviruses, is that fewer treatments may be necessary than with chemical insecticides to keep the insect pest below the damage threshold because the virus can replicate and therefore persist in the environment (Fuxa, 1991; Moscardi, 1999).

HearSNPV has been effective in laboratory assays against third instar *H. armigera* with a LD_{50} of 157 OBs/larva (data not shown). Studies have shown that there are differences between the efficacy of NPVs in the laboratory and in the field and these differences are ascribed to the virus being consumed in a much shorter time

period in the laboratory as well as other factors such as temperature differences in the field (Daoust and Roome, 1974; Sun et al., 2002).

Some of these differences in the field can be overcome by additives that add several benefits to improve the efficacy of the virus such as facilitating leaf coverage, improving palatability and protection from UV (Burges and Jones, 1998a; Cisneros et al., 2002).

Several greenhouse and field trials have been conducted using baculoviruses against insect pests on many different crops, and proved to be successful. For example, efficacy trials for the registration of Spod-X, a formulation of *Spodoptera exigua* NPV, was successful in reducing the beet armyworm on garden pea, Chinese kale, shallot and table grape in Thailand (Kolodny-Hirsch et al., 1997).

A reduction in African cotton bollworm populations on tomato and citrus plants was achieved with HearNPV (Moore et al., 2004). The *Heliothis zea* baculovirus effectively controlled *H. virescens* populations on cotton, corn and grain sorghum (Ignoffo et al., 1965) and *Heliothis armigera* larvae numbers on sorghum were reduced by a NPV isolated from *Heliothis* larvae (Daoust and Roome, 1974). A HearNPV isolated from field-collected *H. armigera* larvae in southern India was more effective in controlling the chick-pea pod-borer (*Helicoverpa armigera*) on chickpea than the chemical insecticide endosulfan which is commonly used for this pest (Cherry et al., 2000).

Studies on the efficacy of baculoviruses enhanced with formulation additives have been promising such as flour-based additives to aid phagostimulation (Williams et al., 2004) and spray-dried baculoviruses with additives such as lignin and pregelatinized corn flour (Behle et al., 2003; Tamez-Guerra et al., 2000b).

Formulations of HearSNPV using thickening and gelling agents, are being developed as biopesticides for the control of the African cotton bollworm in our

laboratory. Although thickening and gelling agents are commonly used in the food industry, they have not been used in agriculture, before developing formulated HearSNPV biopesticides, it is important to assess the insecticidal activity of the biopesticide in its unformulated form.

Although formulation ingredients can improve the efficacy of a biopesticide, an unformulated product showing poor activity is not likely to become effective on formulation and hence it was decided to determine the efficacy of the biopesticide in its unformulated form before investing resources in developing formulated versions. Tomato plants, which are targeted by the African cotton bollworm, and are one of the crops that this biopesticide is being developed for were used to test the efficacy of the unformulated HearSNPV.

2.3 MATERIALS AND METHODS

2.3.1 *Helicoverpa armigera* larvae

Helicoverpa armigera eggs were initially obtained from the Agricultural Research Council, Plant Protection Research Institute (ARC-PPRI) in Pretoria, South Africa and were reared in the laboratory at 28 °C at a relative humidity of 70 % and a 12 hour day night cycle on an artificial wheatgerm diet (Bot, 1966).

2.3.2 HearSNPV preparation

Helicoverpa armigera single nucleocapsid nucleopolyhedrovirus (HearSNPV) isolate used in the greenhouse trials was initially obtained from Dr. Gustav Bouwer. Third instar larvae (5 day-old) were inoculated with a lethal dose of 2 x 10^5 OBs/larva by allowing larvae to feed on diet plugs of the artificial wheatgerm diet that had been surface-contaminated with the virus (Evans and Shapiro, 1997). Insects that did not consume their contaminated diet were excluded from the study. Cadavers of diseased insects were harvested and stored at -20 °C.

A crude extract of HearSNPV was prepared by homogenizing cadavers in sterile distilled water using a tissue homogenizer for 6 minutes. The homogenate was filtered through one layer of miracloth with a pore size of 22 – 25 μm

(Calbiochem, catalogue number 475855) to remove large insect debris. The concentration of OBs in the crude extract was quantified using a haemocytometer. The crude extract was diluted with sterile distilled water to the required concentration.

2.3.3 Tomato plants

Tomato plants grown in pots in a greenhouse were used for the trials. The plants were spaced so that they didn't touch each other in order to prevent insects migrating between the plants.

2.3.4 Determination of oviposition preference by the moths

Cages made of insect gauze covering a metal frame were placed over five of the tomato plants that contained flowers. A breeding pair of *H. armigera* moths was placed into each cage and the position of eggs on the plant was monitored to ascertain the oviposition preferences of the moths. This would assist us to more accurately inoculate the plants with larvae.

2.3.5 Greenhouse trial 1

Twenty five neonates were artificially inoculated onto each plant one day prior to spraying to allow them to establish themselves. They were inoculated onto the top half of the tomato plant on new shoots containing leaves, near flowers and on the calyx of fruit. Only a single application of the virus was made to the plants. Four replicates of eight plants were treated per treatment over two days, spraying two replicates per day, using the following treatments:

1) Control (sterile distilled water)
2) No-spray control
3) HearSNPV at a rate of 10^{10} OBs/ha
4) HearSNPV at a rate of 10^{11} OBs/ha
5) HearSNPV at a rate of 10^{12} OBs/ha
6) HearSNPV at a rate of 10^{13} OBs/ha

The plants were sprayed using a Mist Blower (Solo Port 423). Plants were treated outside the greenhouse to avoid contaminating other plants inside and were sprayed at a rate of 500 l/ha. Water sensitive paper was stapled to 1 plant per replicate to ensure that the droplets covered the plants evenly for all the replicates. The number of surviving larvae on each plant was scored seven days post treatment by destructive sampling to ensure that no surviving larvae that had bored into the flowers or fruit were overlooked. The temperature and humidity of the greenhouse during the trial were recorded.

2.3.6 Greenhouse trial 2

This trial was set-up slightly differently to greenhouse trial 1. *Helicoverpa armigera* neonate larvae were reared on an artificial wheatgerm diet overnight and then inoculated onto tomato plants. Twenty larvae were put onto each plant in groups of three to four on the top half of the tomato plants on branches coming off the main stem which carried leaves, tomatoes or flowers or a combination of these. Larvae were allowed to adapt to their new environments for 2 days and plants were sprayed on the 2nd day in the afternoon.

Five treatments were used to spray four replicates of nine plants per treatment. The plants were sprayed outside the greenhouse to avoid contaminating other plants inside. The treatments used were:

1) Control 1 (no-spray)
2) HearSNPV 4.8 x 10^{12} OBs/ha
3) HearSNPV 9.6 x 10^{11} OBs/ha
4) HearSNPV 4.8 x 10^{11} OBs/ha
5) Control 2 (Commercially available product Dipel (active ingredient: *Bacillus thuringiensis*) at a rate of 500 g/ha as per manufacturer's recommendations).

The plants were treated using a hollow cone nozzle at a pressure of 2 bar and a rate of 400 l/ha. Plants were sprayed on two opposite sides of the plant and water sensitive paper was randomly put onto some of the plants in each replicate to

ensure the spray coverage was similar for each treatment. The surviving larvae on each plant were scored 7 days post spraying and destructive sampling was carried out as per greenhouse trial 1. The temperature and humidity of the greenhouse during the trial were recorded.

2.3.7 Statistical analysis

Analysis of variance (ANOVA) was used to determine whether there was a significant difference between the treatments. The Bonferonni post hoc test was used to compare the means at the 95 % significance level. SAS Enterprise Guide 3.0.1.396 was used to perform all analyses.

2.4 RESULTS

The *H. armigera* moths preferentially oviposited eggs on the underside of leaves and some on top of the leaves on the top half of the tomato plant near flowers and fruit.

Application of HearSNPV to tomato plants in greenhouse trial 1 resulted in a decrease in the number of surviving larvae per tomato plant at concentrations of 1.0×10^{11} to 1.0×10^{13} OBs/ha (Figure 2.1). Although a trend was observed where an increase in nucleopolyhedrovirus (NPV) concentration resulted in a decrease in the mean number of surviving larvae ($F_{2, 94} = 3.77$), a significant difference ($P < 0.05$) was only found for the 1.00×10^{13} OBs/ha treatment which reduced the number of larvae per plant by 63 % (0.66/1.04) compared to the untreated control group. No statistical difference ($P < 0.05$) was observed between the no-spray control group and the control group sprayed with sterile distilled water. On average, the temperature ranged between 15 and 28 °C, and the relative humidity ranged between 50 and 83 % daily.

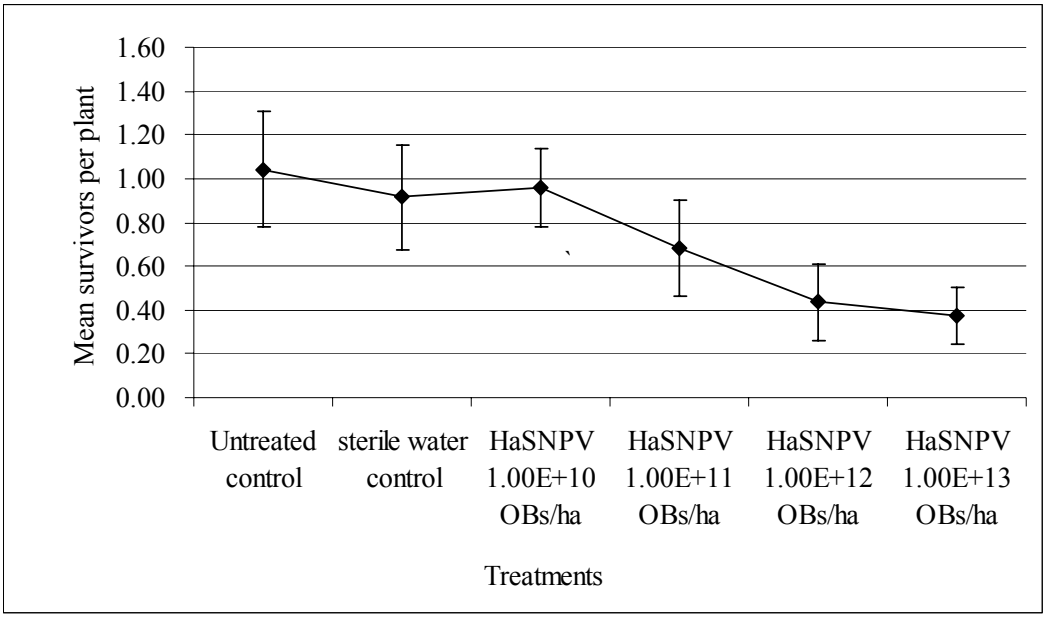


Figure 2.1 Mean number of surviving larvae per plant for greenhouse trial 1. A statistical difference ($P < 0.05$) was found between the application rate of 1.00 x 10^{13} OBs/ha and the control treatment. The control treatment represents the sterile distilled water treatment. Error bars represent standard errors.

Since there was no difference between the no-spray control and the control sprayed with sterile distilled water in greenhouse trial 1, the no-spray controls were used in the next trial. In greenhouse trial 2, there was a significant decrease ($F_{4, 179} = 12.24$) in the mean surviving larvae per plant on the treated plants (Figure 2.2). The 4.80 x 10^{11}, 9.60 x 10^{11} and 4.80 x 10^{12} OBs/ha treatments reduced the number of larvae per plant by 75 % (0.86/1.14), 85 % (0.97/1.14) and 97 % (1.11/1.14) respectively compared to the control group (1.14). No significant difference was observed between the different HearSNPV application rates. The number of surviving larvae in the second control group (treated with Dipel), did not differ significantly from any of the virus applications. On average, the temperature ranged between 17 and 41 °C, and the relative humidity ranged between 29 and 88 % daily.

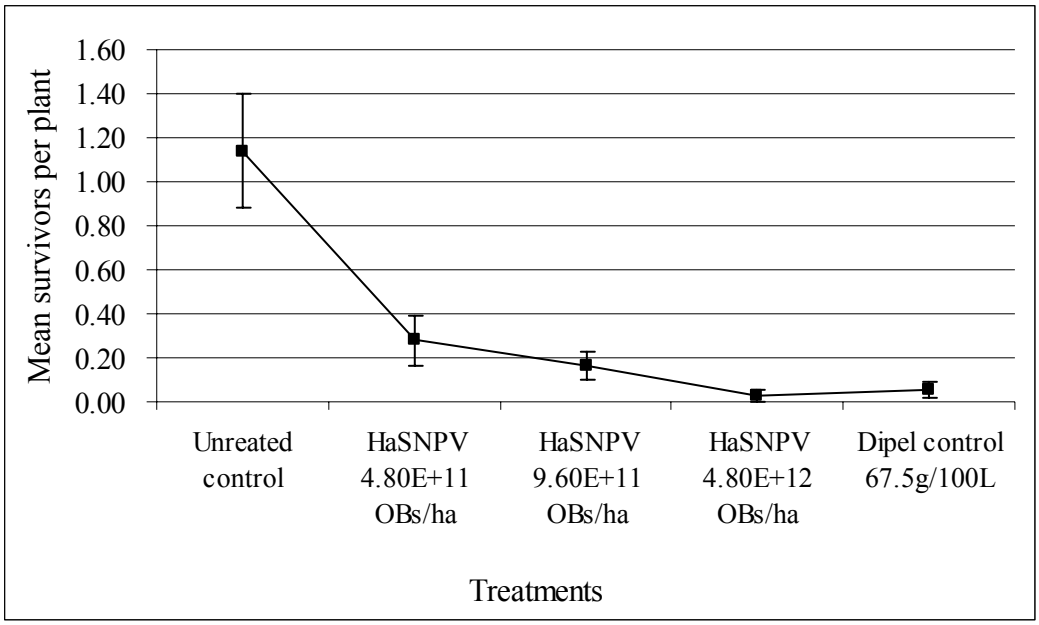


Figure 2.2 Mean number of surviving larvae per plant for greenhouse trial 2. All treatments were statistically different ($P < 0.05$) from the control. Error bars represent standard errors.

2.5 DISCUSSION

These greenhouse trials show a clear trend where an increase in HearSNPV application rate resulted in a reduction of *H. armigera* larvae per tomato plant seven days post treatment. A significant reduction in *H. armigera* infestation per plant was observed at the highest dose in greenhouse trial 1 and for all the HearSNPV doses used in greenhouse trial 2. This is comparable to a study by Moore et al. (2004), who found a South African isolate of HearSNPV to be effective in reducing larvae of *H. armigera* on tomato plants in a greenhouse. The South African isolate of HearSNPV used in our greenhouse trial was an uncharacterised South African isolate which proved to be highly virulent compared to other South African isolates (G. Bouwer, unpublished data). No significant difference was found between the doses used by Moore et al. (2004) either, however they did find that mortality of the larvae occurred faster at the higher doses.

The results of these two greenhouse trials are similar to those of an experiment in which a nucleopolyhedrovirus (NPV) isolated from *Heliothis zea* was effective in reducing *H. zea* populations at a concentration of 9.96 x 10^{11} OBs/acre (2.46 x 10^{12} OBs/ha) on sorghum plants and 6 x 10^{11} OBs/acre (1.48 x 10^{12} OBs/ha) on corn and cotton (Ignoffo et al., 1965). *Heliothis virescens* numbers were also effectively reduced using the same NPV at a rate of 6 x 10^{11} OBs/acre (1.48 x 10^{12} OBs/ha) on cotton plants (Ignoffo et al., 1965).

The results of field trials of beet armyworm infestations on garden pea, Chinese kale, shallot and table grape in Thailand are also comparable to the greenhouse trials carried out in our study. The beet armyworm infestations were reduced by between 75 and 100 % by a *Spodoptera exigua* NPV formulation (tradename Spod-X) applied at concentrations ranging between 3.1 to 12.5 x 10^{11} OBs/ha (Kolodny-Hirsch et al., 1997).

Many of the insects that were artificially inoculated onto the tomato plants were seen to be predated on by natural enemies in the greenhouse such as ants and spiders. The impact of natural enemies on *H. armigera* populations in a field experiment in Kenya showed that ant densities of 25 per plant caused a reduction in the number of 2nd and 3rd instar larvae by three to five times (Denberg et al., 1997). Moore et al. (2004) also found a high reduction in *H. armigera* larvae on control plants and attributed it to natural mortality and larvae boring into plant parts, thus not being detected. The presence of predatory insects in our study suggests that this virus is not overtly detrimental to non-target insects such as ants and spiders.

Baculovirus applications should be timed so that they target early instars of the larvae to minimize damage to crops because later instar larvae are less susceptible to the virus (Bianchi et al., 2002; Moscardi, 1999). Application timing is also crucial when targeting a pest that bores into fruit or plant parts, thereby escaping infection (Moscardi, 1999). The NPV biopesticide used in this study was used

against early instars of *H. armigera* and higher doses would need to be used to control later instar larvae which are less susceptible to the virus.

The highest HearSNPV dose used in greenhouse trial 2 showed very similar control to the commercial biopesticide (Dipel), indicating that the HearSNPV was as effective as Dipel, which is encouraging for the commercial potential of this virus.

The data presented here indicate that the unformulated HearSNPV biopesticide used in this study is effective against larvae of *H. armigera* on tomato plants and provides baseline data for future greenhouse trials. It also justifies further work using novel formulations of this virus, which could enhance the storage stability and field persistence of the biopesticide.

CHAPTER 3

The effect of *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus (HearSNPV) inoculum purity on the microbial load of insects used for *in vivo* production

3.1 ABSTRACT

The effect of HearSNPV inoculum purity used for the *in vivo* production in *Helicoverpa armigera* larvae was evaluated. Homogenates prepared from insect cadavers infected with three inoculums (sterile distilled water, purified and crude HearSNPV) were compared at three different storage temperatures (4, 25 and 37 °C) for three different storage periods (7, 30 and 90 days). Standard culture-based methods were used to detect and enumerate the bacterial populations in the homogenates and representative isolates were identified using 16S rDNA. The bacterial contaminants isolated, were *Bacillus* and *Enterococcus*. No significant difference was found between the microbial loads of the insect homogenates prior to storage. The inoculum purity significantly affected the total aerobic counts on storage, which averaged 5.16 log cfu/mg for the crude inoculum, compared to 3.92 log cfu/mg and 2.90 log cfu/mg for the purified and sterile water inoculums respectively. The *Bacillus* counts were significantly higher when the homogenates were stored at 37 °C (3.63 log cfu/ml) compared to storage at 4 °C (1.11 log cfu/ml) and the *Enterococcus* counts were significantly higher when the homogenates were stored at 4 °C (3.73 log cfu/ml) compared to storage at 37 °C (0.78 log cfu/ml). Storage time (90 days) significantly decreased the anaerobic counts of the sample from 3.93 log cfu/mg to 2.25 log cfu/mg. This study confirms that care should be taken to minimize the bacterial contamination from the start of baculovirus production, since the microbial load can increase during storage.

3.2 INTRODUCTION

Baculoviruses are well known to cause epizootics in lepidopterans and have been used as biocontrol agents as an alternative to chemical insecticides (Hails, 1997; Szewczyk et al., 2005). One of the genera of the *Baculoviridae* family is *Nucleopolyhedrovirus* (NPV), most of which produce large occlusion bodies (OBs) ranging 0.15 - 15µm in size. These OBs enclose the virions in a protein matrix of polyhedrin, which allows them to survive outside their host, in the soil and on plants for years (Funk et al., 1997; Miller, 1997; van Regenmortel et al., 2000).

The OBs initiate infection when the insect ingests them, where the alkaline conditions of the midgut dissolves the protein matrix, releasing the virions. Baculoviruses have been found to have a high pathogenicity toward insect pests, a narrow host range, and are persistent in the environment (Fuxa, 1991; Miller et al., 1983; Szewczyk et al., 2005). They have only been found to cause disease in arthropods, so are safe to vertebrates and plants in the environment (Miller, 1997). These factors make baculoviruses attractive agents for the biological control of lepidopteran insects (Fuxa, 2004; Miller et al., 1983).

In vivo propagation is generally used when producing a baculovirus biopesticide because this is the most economical and effective way to propagate the virus. However, the use of live insects presents the potential of bacterial contamination due to the high numbers of normal microbial flora associated with their guts. (Bignell, 1984; Broderick et al., 2004; Charpentier et al., 1978; Grzywacz et al., 1997; Podgwaite et al., 1983; Xiang et al., 2006).

Bacterial contamination may reduce the shelf-life of the biopesticide as a result of bacterial metabolic by-products which could change environmental conditions such as pH (Burges and Jones, 1998a) as well as pose a potential threat to other organisms in the environment. The microorganisms associated with NPV production have been examined previously and species of *Bacillus*, *Staphylococcus*, *Streptococcus*, *Enterococcus*, Enterobacteriaceae, *Aspergillus*

and yeasts were found to be the most frequent species in samples (Grzywacz et al., 1997; Lasa et al., 2008; Podgwaite et al., 1983).

Microorganisms that could pose a potential health risk to humans include *Bacillus cereus*, for example, which causes food poisoning when foods contain a concentration of more than 10^6 bacteria per gram (Goepfert, 1976; U.S. Food and Drug Administration, 2001). Another source of food poisoning is *Staphylococcus aureus* which produces enterotoxins if ingested (Baer et al., 1976). Enterobacteriaceae are commonly used as indicator species of faecal contamination (Fishbein et al., 1976; Prescott et al., 1990).

It has been suggested that when propagating virus *in vivo*, insects should be harvested before death or should be stored at a low temperature post-harvest to minimize contaminating bacteria, however early harvesting could result in partly formed occlusion bodies which may result in reduced environmental persistence (Burges and Jones, 1998b).

A purified inoculum of the virus can be prepared using rate zonal centrifugation to minimize contaminating microorganisms, however, this is a time-consuming process and as much as 30 % of virus has been found to be lost by using this procedure (Kelly and Entwistle, 1988). Therefore, purifying NPV on a mass scale using rate zonal centrifugation which is labour intensive and results in the loss of occlusion bodies could be cost prohibitive (Kelly and Entwistle, 1988). A crude extract of the virus can be used as an inoculum, but it may contain a higher concentration of contaminating microorganisms, which could adversely affect the biopesticide.

It is extremely important that the biopesticide remains viable during storage. A minimum shelf-life of 2 years is required for commercial chemical pesticides, and biopesticides should aim at a similar shelf-life. Metabolic waste products of contaminating microorganisms could change the pH of the product, and possibly

inactivate the baculovirus since the polyhedrin matrix of the OBs dissolves in alkaline conditions (Burges and Jones, 1998a; Burges and Jones, 1998b).

Helicoverpa armigera single nucleocapsid nucleopoyhedrovirus (HearSNPV) (family Baculoviridae) is a pathogen of the African cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) which is a polyphagous insect pest that causes enormous economic losses to crops such as cotton, soybeans, tobacco and the pulses (Fitt, 1989). *Helicoverpa armigera* has developed resistance to many chemical insecticides such as pyrethroids, quinolphos, monocrotophos and endosulfan (Tatchell, 1997). There is thus a demand for alternative control measures. We wish to develop a HearSNPV biopesticide that can be used to control *H. armigera* and want to know what effect inoculum purity would have on the microbial load of infected insects when using *in vivo* propagation.

In this study, the aim was to determine whether inoculum purity has a large effect on the microbial load of insects, and whether this microbial load is exacerbated by storage time and storage temperature.

3.3 MATERIALS AND METHODS

3.3.1 *Helicoverpa armigera* culture

Eggs were initially obtained from the Agricultural Research Council, Plant Protection Research Institute (ARC-PPRI) in Pretoria, South Africa. The insects were maintained at 28 °C at a relative humidity of 70 % and a 12 hour photoperiod. Eggs were sterilized in 0.2 % (v/v) sodium hypochlorite and once the neonates hatched (after 2 days) they were transferred onto an artificial wheatgerm diet (Bot, 1966) and reared individually in vials.

3.3.2 HearSNPV propagation

Third instar larvae (5 day-old) were inoculated with a dose of 2 x 10^5 OBs/larva by allowing larvae to feed on diet plugs of the artificial wheatgerm diet that had been surface-contaminated with the virus (Evans and Shapiro, 1997). Insects that

had not consumed their diet plug were excluded from the study. Cadavers of diseased insects were harvested upon death, weighed and stored at -20 °C.

3.3.3 Inoculum preparation

Two levels of inoculum purity were used to infect the larvae, namely a purified inoculum of HearSNPV and a crude inoculum of HearSNPV.

Purified HearSNPV inoculum preparation

The purified HearSNPV inoculum was prepared using rate zonal centrifugation. The thawed cadavers were homogenized using a mortar and pestle in sterile distilled water and filtered through three layers of muslin cloth. Debris was removed by three low-speed centrifugation runs of the filtrate. The supernatant was subjected to centrifugation at 19 000 g for 30 minutes to sediment the viral OBs, which were then washed twice in sterile distilled water and subjected to centrifugation at 19 000 g for 30 minutes to sediment them. Aliquots of this sediment were resuspended in sterile distilled water and were subjected to rate zonal centrifugation through a 40 to 65 % (w/w) discontinuous sucrose gradient and centrifuged at 49 000 g for 1 hour and 30 minutes. A thick, cloudy band which formed at the 60 % interface was removed and washed twice in sterile distilled water by differential centrifugation at 21 000 g for 30 minutes. The resultant OB sediment was run through a second sucrose gradient and washed twice as above and the purified OBs were resuspended in sterile distilled water and stored at 4 °C. All centrifugation steps were carried out at 4 °C.

Crude HearSNPV inoculum preparation

A crude inoculum of HearSNPV was prepared by inoculating third instar larvae with purified HearSNPV using the same method as for propagation of the virus. Cadavers of diseased larvae were harvested and stored at -20 °C until use. Cadavers were thawed and homogenized in sterile distilled water using a tissue homogenizer for 6 minutes. The homogenate was filtered through one layer of miracloth with a pore size of 22 – 25 μm (Calbiochem, catalogue number 475855)

to remove large insect debris. The crude extract was stored at 4 °C for a maximum of seven days until use.

3.3.4 Preparation of insect homogenates

Three groups of insect homogenates were prepared for the experiment by infecting third instar larvae using the diet surface contamination method (Evans and Shapiro, 1997), namely:

1. Homogenate of cadavers infected with a purified inoculum of HearSNPV. This group of insects was infected with a lethal dose of 2 x 10^5 OBs/larva of the purified inoculum of HearSNPV.

2. Homogenate of cadavers infected with a crude inoculum of HearSNPV. This group of insects was infected with a lethal dose of 2 x 10^5 OBs/larva of crude inoculum of HearSNPV.

3. Homogenate of uninfected cadavers. This group of insects was mock-infected with sterile distilled water (and used as a control group).

Three replicates of insects were infected on different days. Once these larvae had consumed their diet plugs, they were transferred back onto their artificial wheatgerm diet and upon death, 18 - 20 larvae were randomly harvested from each group, weighed and stored at -20 °C. Insects that did not consume the contaminated diet were excluded from the study.

These cadavers were defrosted and homogenized in 150 ml of sterile distilled water using a mechanical homogenizer for 6 minutes and aliquoted into sterile tubes. One set of tubes was analysed for the initial (time 0) microbial load and the rest were stored in the dark at three different temperatures namely, 4 , 25 and 37 °C and their microbial load analysed at 7, 30 and 90 days of storage at these temperatures.

3.3.5 pH of homogenates

The pH of an aliquot of each homogenate was taken using a Metrohm 744 pH meter.

3.3.6 Microbial load determination

Total aerobic counts of the homogenates were done using Plate Count Agar (Merck). Anaerobic counts were determined by plating the samples onto Plate Count Agar (Merck) and incubating them in anaerobic jars with anaerobic sachets (Oxoid, AnaeroGen^{TM}). In addition, microbes associated with the homogenates were screened for and enumerated using the following selective media: MacConkey Agar and Salmonella Shigella Agar (Merck) as well as Eosin Methylene Blue Agar and Brilliant Green Agar (Oxoid) were used for coliforms, *Salmonella* and *Shigella* species; Mannitol Salt Agar and Baird Parker Agar (Merck) for *Staphylococcus aureus*; PEMBA Agar (Oxoid) was used for *Bacillus* species and Columbia Blood Agar for *Streptococcus* species (Bridson, 1990). Spore counts were performed by heating an aliquot of the sample at 80 °C for 30 minutes with agitation every 10 minutes to kill the vegetative cells and the spores were quantified using plate count agar. The presence of yeasts and moulds were detected using Dichloran Rose Bengal Chloramphenicol agar (DRBC agar). The microbial load counts were calculated in colony forming units per milligram of larva in the homogenate for each count type.

Enrichment procedures were followed for the detection of *Salmonella*, *Shigella* and *Staphylococcus* species. For *Salmonella* and *Shigella,* primary enrichment was carried out by incubating samples in Lactose Broth at 37 °C for 24 hours, followed by secondary enrichment in Tetrathionate Broth at 37 °C for 24 hours and then plated onto MacConkey agar and Salmonella Shigella agar (Merck) and Eosin Methylene Blue agar and Brilliant Green agar (Oxoid). For *Staphylococcus* detection, primary enrichment was carried out in Brain Heart Infusion broth at 37 °C for 2 hours, followed by secondary incubation in Brain Heart Infusion broth containing 20 % NaCl at 37 °C for 24 hours, and then plated onto Mannitol salt agar and Baird Parker agar.

The presence of yeast and moulds were determined with Dichloran Rose Bengal Chloramphenicol (DRBC) Agar. Since phosphate buffered saline was used as a diluent for the other media, it was used as a diluent for the DRBC media as well rather than peptone water as recommended by the US FDA (2001). Trials were conducted to confirm that the change of diluent did not make a difference in the number of organisms that grew on the media. All plates were incubated at 37 °C for 24 hours except for the DRBC agar which was incubated at 25 °C for five days.

For each type of medium used in the microbial load determination, duplicate plate counts were performed.

3.3.7 Identification of isolates

Isolates were sub-cultured and cryopreserved at -80 °C. The isolates were grown overnight in Nutrient Broth at 37 °C for analysis. Gram stains were carried out with representative colonies and their morphology observed under a light microscope (Olympus BX41). DNA was extracted from representative colonies using a RTP® Spin Bacteria DNA Mini Kit from Invitek as per the manufacturer's instructions.

PCR was performed with one of the representative colonies to amplify the 16S rDNA region for sequencing. Approximately 1300 bp of the 16S rDNA region was amplified using the universal primers 63f and 1387r (Marchesi et al., 1998) with the Expand Long Template PCR enzyme with proof-reading activity (Roche). The PCR mixtures contained 200 µm dNTPs, 1.75 mM $MgCl_2$, 3.75 U enzyme, and 0.300 µM of each primer in a final reaction volume of 50 µl. The reactions were amplified in an Eppendorf Mastercycler® ep thermocycler using the following cycles: 95 °C for 5 min; 30 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 min; and a final cycle of 72 °C for 5 min. Samples were sent to the Specialist Sequencing Service, Department of Molecular and Cell Biology, University of Cape Town, for sequencing. The following sequencing primers were used:

R3 (5' CACGAGCTGACGACAICCATGC 3')

F3 (5’ GCCAGCAGCCGCGGTAATAC 3’)
R5 (5’ GCATGGITGTCGTCAGCTCGTG 3’)
F38 (5’ CTGGCTCAGGA(CT)GAAGCGTG 3’).

The sequences obtained were submitted to BLAST on the National Centre for Biotechnology Information (NCBI) database to screen for similar sequences. Type strains of sequences with high sequence similarities to the unknown bacterial isolate were downloaded for the phylogenetic analyses. Multiple sequence alignments of these type strains and were performed with ClustalX2.0.3 as implemented in MEGA version 2.1 (Kumar et al., 2001).

The same procedure was followed for the *Enterococcus* isolate alignment as done for the *Bacillus* isolate. A double stranded sequence was obtained for each isolate and a single sample of each isolate was used for sequencing.

A phylogenetic tree was constructed for each genus with the aligned sequences using MEGA version 2.1 (Kumar et al., 2001) using the neighbor-joining method of Saitou and Nei (1987). In the neighbor-joining analyses, the Jukes' and Cantor's one-parameter model was used. The statistical reliability of the tree was determined by using bootstrapping analysis (1000 replications).

3.3.8 Statistical analysis

Analysis of variance (ANOVA) was used to determine whether inoculum purity, storage temperature or storage time significantly ($P < 0.05$) affected the mean counts ($\log_{10}$ cfu/mg of larva). The means were compared using the Bonferonni post hoc test at the 95 % confidence level. SAS Enterprise Guide 3.0.1.396 (SAS Institute Inc., Cary, NC, USA) was used to perform the statistical analysis.

3.4 RESULTS

3.4.1 Microbial load of inoculums

The initial inoculums used to infect *H. armigera*, namely the purified HearSNPV inoculum and the crude HearSNPV inoculum contained total aerobic counts of 2.43 x 10^3 cfu/ml and 2.24 x 10^7 cfu/ml respectively. This gives a bacterium to OB ratio of 4.85 x 10^{-6}:1 for the purified HearSNPV and 0.05:1 for the crude HearSNPV.

3.4.2 Microbial load of the insect homogenates prior to storage

Prior to storage, the total aerobic counts were higher for the homogenate of insects infected with the crude inoculum, compared to the homogenates of insects infected either with the pure inoculum or sterile distilled water, however they did not differ statistically (Table 3.1).

Table 3.1 The effect of inoculum purity on the microbial load of insect homogenates prior to storage.

Count type	Counts (log cfu/mg)			F value
	Sterile distilled water	Purified HearSNPV	Crude HearSNPV	
Aerobic	2.22 ± 1.99	2.45 ± 1.24	4.17 ± 1.82	$F_{2, 8} = 0.85$; P = 0.4715
Anaerobic	2.25 ± 1.17	2.21 ± 1.28	4.15 ± 1.12	$F_{2, 8} = 0.87$; P = 0.4667
Bacillus	0.45 ± 0.45	0.90 ± 1.47	2.05 ± 1.49	$F_{2, 8} = 0.63$; P = 0.5645
Spores	0.73 ± 0.73	Not detected	0.98 ± 1.11	$F_{2, 8} = 0.55$; P = 0.6024
Enterococcus	2.19 ± 1.14	0.12 ± 0.90	2.24 ± 1.74	$F_{2, 8} = 0.07$; P = 0.9320

Means are followed by standard errors. No significant difference *(P < 0.05)* was found for a particular count type between the different homogenates prior to storage.

3.4.3 Stored insect homogenates

The effect of the three main factors (inoculum type, storage temperature and storage time) on the microbial load of the samples was investigated independently, and then the effect of the interaction between these factors was

considered. In cases where the effect of the interaction between the main factors was not significant, data were pooled to elucidate the overall effects of the main factors.

A general trend was observed, with the inoculum type significantly affecting the microbial counts of the homogenates (Table 3.2). The homogenate prepared from cadavers of insects infected with crude HearSNPV inoculum contained significantly higher total aerobic, anaerobic, *Bacillus* and spore counts than the homogenates prepared from cadavers of insects infected with sterile distilled water. However, these counts did not differ significantly between the crude inoculum and the purified inoculum, or between the purified inoculum and the sterile distilled water control. Inoculum types did not statistically affect the *Enterococcus* counts.

Table 3.2 The effect of inoculum purity on the microbial load of insect homogenates on storage.

Count type	Counts (log cfu/mg)			F value
	Sterile distilled water	Purified HearSNPV	Crude HearSNPV	
Aerobic	2.90 ± 0.43^{a}	$3.92 \pm 0.46^{a,b}$	5.16 ± 0.18^{b}	$F_{8, 80} = 7.52$; P = 0.0013
Anaerobic	2.11 ± 0.43^{c}	$3.22 \pm 0.43^{c,d}$	3.62 ± 0.38^{d}	$F_{8, 80} = 4.24$; P = 0.0195
Bacillus	1.43 ± 0.44^{e}	$2.52 \pm 0.54^{e,f}$	2.51 ± 0.47^{f}	$F_{8, 80} = 6.96$; P = 0.0012
Spores	1.22 ± 0.42^{g}	$2.52 \pm 0.53^{h,i}$	3.67 ± 0.49^{i}	$F_{8, 80} = 6.87$; P = 0.0022
Enterococcus	1.89 ± 0.42^{j}	1.95 ± 0.44^{j}	3.95 ± 0.42^{j}	$F_{8, 80} = 0.87$; P = 0.4229

Means are followed by standard errors. Means in the same row that are followed by the same letter are not significantly different ($P < 0.05$).

The mean aerobic and anaerobic counts decreased on storage time, but increased in respect of the *Bacillus* and spore counts (Table 3.3). However, the effect of storage time was only significant for the anaerobic counts, which decreased significantly after 90 days of storage.

Table 3.3 The effect of storage time on the microbial load of insect homogenates.

Count type	Counts (log cfu/mg)			F value
	7 days	30 days	90 days	
Aerobic	4.41 ± 0.34^{a}	3.92 ± 0.41^{a}	3.65 ± 0.48^{a}	$F_{8, 80} = 0.86$; P = 0.4295
Anaerobic	3.93 ± 0.36^{b}	2.77 ± 0.39^{b}	2.25 ± 0.47^{c}	$F_{8, 80} = 5.13$; P = 0.0091
Bacillus	2.21 ± 0.53^{d}	2.56 ± 0.50^{d}	3.14 ± 0.49^{d}	$F_{8, 80} = 0.97$; P = 0.3843
Spores	2.14 ± 0.49^{e}	2.45 ± 0.53^{e}	2.86 ± 0.53^{e}	$F_{8, 80} = 0.66$; P = 0.5235

Means are followed by standard errors. Means in the same row that are followed by the same letter are not significantly different ($P < 0.05$).

Storage temperature did not significantly affect the total aerobic counts of the homogenates, but it did affect the other counts (Table 3.4). The anaerobic counts were significantly lower at 37 °C storage compared to 4 °C, whereas the *Bacillus* and spore counts were significantly higher, compared to 4 °C for both 25 and 37 °C (Table 3.4).

Table 3.4 The effect of storage temperature on the microbial load of insect homogenates.

Count type	Counts (log cfu/mg)			F value
	4 °C	25 °C	37 °C	
Aerobic	3.68 ± 0.43^{a}	4.54 ± 0.35^{a}	3.75 ± 0.44^{a}	$F_{8, 80} = 1.33$; P = 0.2721
Anaerobic	3.56 ± 0.42^{d}	$3.37 \pm 0.43^{d,e}$	2.03 ± 0.38^{e}	$F_{8, 80} = 4.81$; P = 0.0120
Bacillus	1.11 ± 0.35^{f}	3.43 ± 0.51^{g}	3.36 ± 0.52^{g}	$F_{8, 80} = 7.61$; P = 0.0012
Spores	0.64 ± 0.22^{h}	3.21 ± 0.53^{i}	3.56 ± 0.54^{i}	$F_{8, 80} = 11.56$; P = <.0001

Means are followed by standard errors. Means in the same row that are followed by the same letter are not significantly different ($P < 0.05$).

The *Enterococcus* counts were affected by an interaction between storage time and storage temperature ($F_{8, 80} = 2.81$; $P = 0.0342$) (Table 3.5). The counts remained constant when stored at 4 °C, but decreased significantly over time when stored at 25 °C. The *Enterococcus* counts were present in significantly

lower numbers at 37 °C compared to 4 °C, and their numbers did not change significantly on storage.

Table 3.5 The effect of the interaction of storage time and storage temperature on the *Enterococcus* counts.

Storage time	Counts (log cfu/mg)		
	4 °C	25 °C	37 °C
7 days	3.77 ± 0.78^{a}	3.83 ± 0.74^{a}	$1.03 \pm 0.42^{a,b}$
30 days	3.83 ± 0.74^{a}	$1.67 \pm 0.60^{a,b}$	0.50 ± 0.44^{b}
90 days	3.59 ± 0.70^{a}	0.00^{b}	$0.82 \pm 0.57^{a,b}$

Means are followed by standard errors. Means that are followed by the same letter are not significantly different ($P < 0.05$).

Moulds were detected in the samples, however the counts did not differ significantly ($F_{2, 8} = 1.32$; $P = 0.1955$) in any of the samples and, when present, were in low concentrations (less than 1.00 cfu/mg of homogenate).

3.3.4 pH of the homogenates

The same model was used to analyze the pH as the microbial load, where the effect of the three main factors on the pH of the homogenates (inoculum type, storage temperature and storage time) were investigated independently, as well as the effect of the interaction between these factors. In cases where the effect of the interaction between the main factors was not significant, data was pooled to elucidate the overall effects of the main factors.

The pH of the homogenates prepared from insects infected with the sterile distilled water, purified HearSNPV and crude HearSNPV inoculums were 6.04, 5.75 and 5.41 respectively, prior to storage. On storage, only the interaction of two of the main factors namely inoculum type and storage temperature significantly affected the pH of these homogenates ($F_{2, 8} = 2.26$; $P < 0.1$), thus the data for time was pooled (Table 3.6).

The interaction between inoculum type and storage temperature significantly increased the pH for the homogenates prepared using the pure and crude inoculum's when stored at 25 or 37 °C. No difference was observed between the homogenates for any inoculum type when stored at 4 °C.

Table 3.6 The effect of storage temperature and inoculum type on the pH of insect homogenates.

	pH		
Inoculum type	4 °C	25 °C	37 °C
Sterile distilled water	4.92 ± 0.25[a]	4.75 ± 0.35[a]	5.10 ± 0.40[a]
Purified HearSNPV	4.80 ± 0.17[a]	6.23 ± 0.51[b]	6.41 ± 0.53[b]
Crude HearSNPV	4.66 ± 0.13[a]	6.56 ± 0.44[b]	6.78 ± 0.42[b]

Means are followed by standard errors. Means that are followed by the same letter are not significantly different ($P < 0.1$).

3.3.5 Identification of isolates

The phylogenetic tree constructed using the 16S rDNA sequences in Figure 3.1, shows that the *Bacillus* isolate does not cluster closely with any of the type strains it was compared to, and stands alone in the tree. The highest sequence similarity of 97.4 % was found between the *Bacillus* isolate and the reference strain *Bacillus velezensis* CECT 5686^T.

The phylogenetic tree constructed with the 16S rDNA sequences for the *Enterococcus* isolate in Figure 3.2 shows that it does not cluster closely with any of the *Enterococcus* type strains it was compared to, however, the sequence identity matrix (Table 3.8) shows a 99.3 % sequence similarity with *Enterococcus moraviensis* LMG 19486^T.

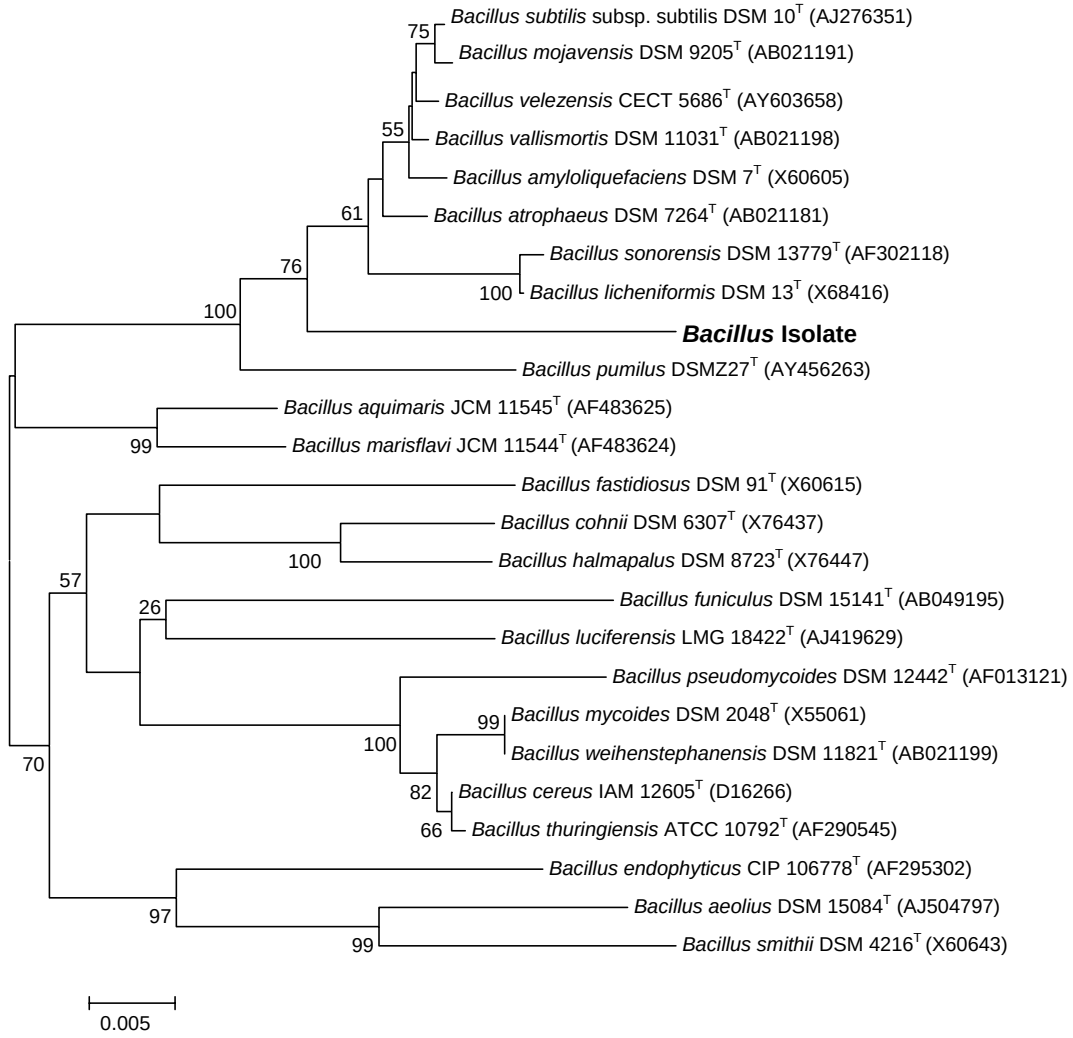


Figure 3.1 Unrooted neighbour-joining tree showing the phylogenetic position of the *Bacillus* isolate to other *Bacillus* type strains based on 16S rDNA gene sequence comparisons (Ruiz-García et al., 2005). Bootstrap values greater than 50 % are shown at the nodes (1000 replications). Scale bar indicates substitutions per nucleotide position.

Table 3.7 Calculated sequence similarity (%) of the 16S rDNA gene sequence of the *Bacillus* isolate and type strains of *Bacillus*.

	***Bacillus* Isolate**	*B. mojavensis* DSM 9205^T	*B. vallismortis* DSM 11031^T	*B. subtilis* subsp. *subtilis* DSM 10^T	*B. velezensis* CECT 5686^T	*B. amyloliquefaciens* DSM 7^T	*B. atrophaeus* DSM 7264^T	*B. pumilus* DSMZ27^T	*B. licheniformis* DSM 13^T	*B. sonorensis* DSM 13779^T	*B. aquimaris* JCM 11545^T	*B. fastidiosus* DSM 91^T	*B. marisflavi* JCM 11544^T	*B. cohnii* DSM 6307^T	*B. halmapalus* DSM 8723^T	*B. luciferensis* LMG 18422^T	*B. funiculus* DSM 15141^T	*B. pseudomycoides* DSM 12442^T	*B. mycoides DSM 2048T*	*B. weihenstephanensis* DSM 11821^T	*B. cereus* IAM 12605^T	*B. thuringiensis* ATCC 10792^T	*B. endophyticus* CIP 106778^T	*B. aeolius* DSM 15084^T	*B. smithii* DSM 4216^T
***Bacillus* Isolate**																									
B. mojavensis DSM 9205^T	97.1																								
B. vallismortis DSM 11031^T	97.2	99.7																							
B. subtilis subsp. *subtilis* DSM 10^T	97.2	99.8	99.7																						
B. velezensis CECT 5686^T	97.4	99.6	99.8	99.8																					
B. amyloliquefaciens DSM 7^T	97.2	99.5	99.7	99.5	99.6																				
B. atrophaeus DSM 7264^T	96.9	99.4	99.5	99.4	99.4	99.4																			
B. pumilus DSMZ27^T	95.6	97.1	97.3	97.2	97.2	97.2	97.5																		
B. licheniformis DSM 13^T	96.2	98.8	98.6	98.8	98.5	98.6	98.8	96.8																	
B. sonorensis DSM 13779^T	96.0	98.6	98.5	98.6	98.4	98.5	98.8	96.8	99.8																
B. aquimaris JCM 11545^T	94.6	96.0	95.8	96.1	95.8	95.8	96.2	95.2	96.6	96.4															
B. fastidiosus DSM 91^T	93.8	94.5	94.5	94.6	94.5	94.6	94.5	94.5	94.4	94.5	95.0														
B. marisflavi JCM 11544^T	94.5	95.6	95.8	95.7	95.6	95.6	96.0	95.6	96.2	96.1	98.5	95.1													
B. cohnii DSM 6307^T	93.2	94.3	94.4	94.4	94.4	94.4	94.5	94.9	94.5	94.7	95.1	96.0	95.3												
B. halmapalus DSM 8723^T	93.7	94.4	94.5	94.5	94.5	94.5	94.5	94.7	94.6	94.6	95.5	96.0	95.6	98.2											
B. luciferensis LMG 18422^T	93.5	94.4	94.4	94.5	94.5	94.6	94.7	94.5	95.0	94.8	95.1	95.1	95.4	96.0	96.2										
B. funiculus DSM 15141^T	92.6	93.5	93.4	93.5	93.4	93.5	93.8	95.0	93.9	93.9	94.9	94.9	94.8	95.4	95.1	95.5									
B. pseudomycoides DSM 12442^T	93.2	93.8	93.8	93.9	93.8	93.8	94.4	93.7	94.1	94.1	94.7	93.6	95.4	94.5	94.5	95.0	94.8								
B. mycoides DSM 2048^T	93.7	94.5	94.5	94.5	94.5	94.5	94.8	94.5	94.9	94.7	95.2	94.2	95.7	95.0	95.1	95.6	95.3	98.1							
B. weihenstephanensis DSM 11821^T	93.7	94.5	94.5	94.5	94.5	94.5	94.8	94.5	94.9	94.7	95.2	94.2	95.7	95.0	95.1	95.6	95.3	98.1	100.0						
B. cereus IAM 12605^T	93.9	94.9	94.9	95.0	94.9	94.8	95.2	94.8	95.1	95.0	95.5	94.6	96.2	95.1	95.6	95.9	95.6	98.6	99.4	99.4					
B. thuringiensis ATCC 10792^T	93.8	94.8	94.8	94.9	94.8	94.7	95.1	94.7	95.1	94.9	95.4	94.5	96.1	95.0	95.5	95.8	95.5	98.5	99.5	99.5	99.9				
B. endophyticus CIP 106778^T	93.8	94.4	94.2	94.3	94.2	94.4	94.0	93.3	94.6	94.6	95.6	94.5	95.2	95.1	94.6	94.7	93.9	93.3	93.8	93.8	93.9	93.8			
B. aeolius DSM 15084^T	92.6	93.7	93.5	93.8	93.5	93.7	93.9	93.7	94.3	94.3	95.4	94.0	95.1	94.1	93.2	93.8	93.9	93.3	93.8	93.8	93.9	93.8	95.1		
B. smithii DSM 4216^T	92.4	93.3	93.2	93.4	93.2	93.4	93.3	93.2	93.8	93.9	94.7	94.0	94.8	94.2	93.2	93.5	93.8	93.0	93.7	93.7	93.6	93.5	95.1	96.8	

* B = *Bacillus;* See figure 3.1 for the NCBI Accession numbers for the reference strains.

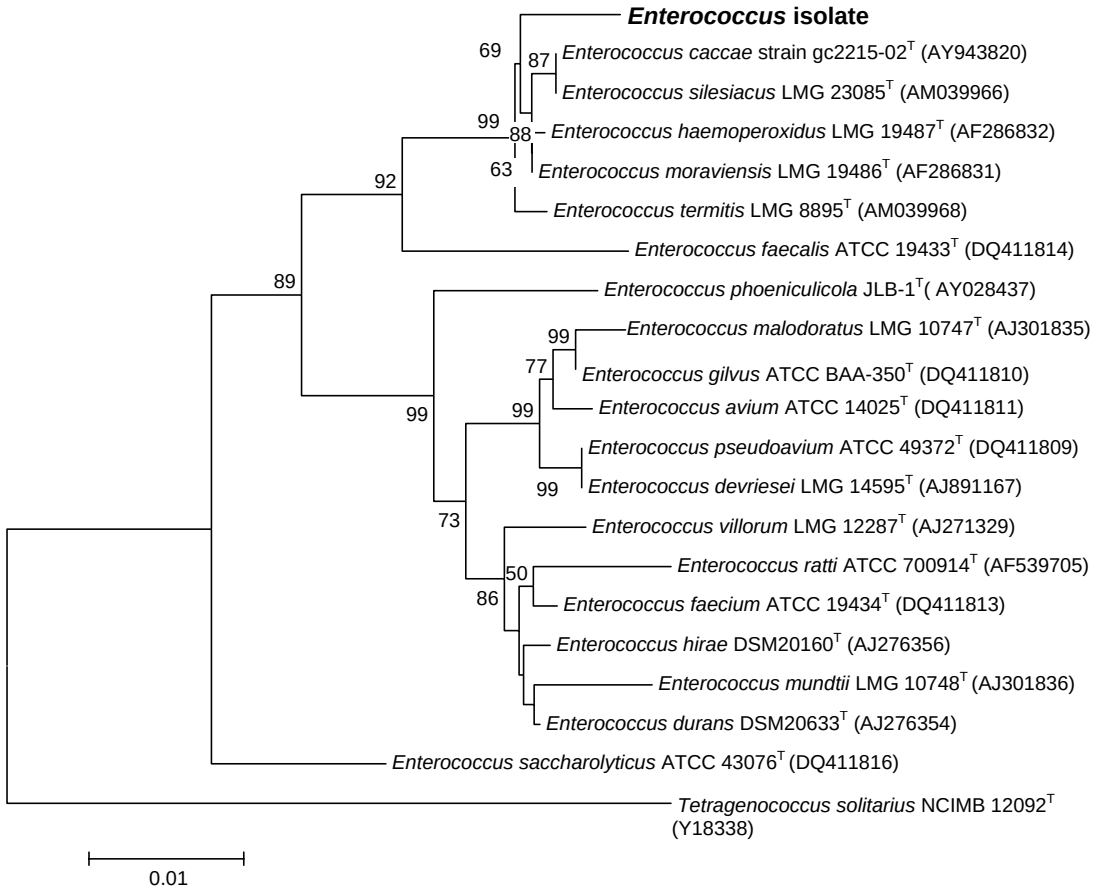


Figure 3.2 Neighbour-joining tree showing the phylogenetic position of the *Enterococcus* isolate to other *Enterococcus* type strains based on 16S rDNA gene sequence comparisons. The tree was rooted using *Tetragenococcus solitarius* NCIMB 12092^T as an outgroup (Sukontasing et al., 2007). Bootstrap values greater than 50 % are shown at the nodes (1000 replications). Scale bar indicates substitutions per nucleotide position.

Table 3.8 Calculated sequence similarity (%) of the 16S rDNA gene sequence of the *Enterococcus* isolate and type strains of *Enterococcus*.

	***Enterococcus* isolate**	*E. caccae* strain gc2215-02^T	*E. durans* DSM20633^T	*E. faecalis* ATCC 19433^T	*E. faecium* ATCC 19434^T	*E. haemoperoxidus* LMG 19487^T	*E. hirae* DSM20160^T	*E. moraviensis* LMG 19486^T	*E. mundtii* LMG 10748^T	*E. phoeniculicola* JLB-1^T	*E. ratti* ATCC 700914^T	*E. silesiacus* LMG 23085^T	*E. termitis* LMG 8895^T	*E. villorum* LMG 12287^T	*E. devriesei* LMG 14595^T	*E. pseudoavium* ATCC 49372^T	*E. gilvus* ATCC BAA-350^T	*E. malodoratus* LMG 10747^T	*E. avium* ATCC 14025^T	*E. saccharolyticus* ATCC 43076^T	*Tetragenococcus solitarius* NCIMB 12092^T
***Enterococcus* isolate**																					
E. caccae strain gc2215-02^T	99.1																				
E. durans DSM20633^T	96.6	97.0																			
E. faecalis ATCC 19433^T	97.3	97.6	96.3																		
E. faecium ATCC 19434^T	96.4	96.8	99.7	96.5																	
E. haemoperoxidus LMG 19487^T	99.2	99.8	97.1	97.7	96.9																
E. hirae DSM20160^T	96.6	97.0	99.8	96.3	99.6	97.1															
E. moraviensis LMG 19486^T	99.3	99.8	97.2	97.7	97.0	99.9	97.2														
E. mundtii LMG 10748^T	95.9	96.3	99.2	95.8	98.9	96.4	99.0	96.5													
E. phoeniculicola JLB-1^T	96.4	96.8	98.3	96.0	98.0	96.9	98.1	97.0	97.5												
E. ratti ATCC 700914^T	95.8	96.4	98.9	95.7	99.0	96.5	98.8	96.6	98.1	97.7											
E. silesiacus LMG 23085^T	99.1	100.0	97.0	97.6	96.8	99.8	97.0	99.8	96.3	96.8	96.4										
E. termitis LMG 8895^T	99.1	99.5	97.1	97.5	96.9	99.6	97.1	99.7	96.7	96.9	96.3	99.5									
E. villorum LMG 12287^T	96.2	96.6	99.1	95.9	99.1	96.7	99.2	96.7	98.6	97.9	98.4	96.6	96.7								
E. devriesei LMG 14595^T	95.7	96.2	98.8	95.7	98.5	96.2	98.6	96.3	98.1	98.2	97.9	96.2	96.2	98.6							
E. pseudoavium ATCC 49372^T	95.7	96.2	98.8	95.7	98.5	96.2	98.6	96.3	98.1	98.2	97.9	96.2	96.2	98.6	100.0						
E. gilvus ATCC BAA-350^T	95.9	96.3	98.8	95.7	98.6	96.4	98.8	96.5	98.2	98.1	97.9	96.3	96.4	98.6	99.5	99.5					
E. malodoratus LMG 10747^T	95.6	96.0	98.5	95.4	98.3	96.1	98.5	96.2	97.9	97.7	97.6	96.0	96.1	98.2	99.2	99.2	99.7				
E. avium ATCC 14025^T	95.8	96.2	98.7	95.7	98.6	96.3	98.7	96.4	98.2	97.8	97.8	96.2	96.3	98.3	99.4	99.4	99.6	99.3			
E. saccharolyticus ATCC 43076^T	95.9	96.1	96.4	96.2	96.4	96.2	96.4	96.2	95.9	96.2	95.6	96.1	96.2	96.7	96.7	96.7	96.7	96.4	96.7		
Tetragenococcus solitarius NCIMB 12092^T	91.5	92.1	91.8	92.4	92.0	92.0	91.9	92.1	91.1	91.7	92.3	92.1	91.8	91.8	91.9	91.9	92.0	91.7	92.1	93.3	

* E = *Enterococcus;* See figure 3.2 for the NCBI Accession numbers for the reference strains.

3.5 DISCUSSION

Contaminants have been noted to possibly be one of the most important problems of baculovirus production (Lasa et al., 2008), because contaminating microorganisms can be carried through to the final product. The inoculum's used in this study had a bacteria to OB ratio of 2.43 x 10^{-6}:1 for the purified inoculum compared to 0.05:1 for the crude inoculum. This bacteria:OB ratio is much lower than the bacteria:virus ratio of 47:1 obtained in a similar study where *Spodoptera littoralis* NPV was produced in Egypt, however the authors noted that they had power cut problems and the freezers in which their samples were stored were often subjected to repeated defrosting (Grzywacz et al., 1997).

In this study, the inoculum purity did not initially have a significant effect on the microbial load of the homogenates, but as expected, storage introduced a significantly higher microbial load to the homogenate prepared from cadavers of insects infected with crude inoculum, compared to the homogenate of insects mock-infected with sterile distilled water. Interestingly, the microbial load of the homogenates prepared from insects infected with the purified inoculum did not differ statistically to the homogenates prepared using the crude inoculum or the control homogenates on storage.

The bacterial species isolated from the homogenates were identified as an *Enterococcus* species and a *Bacillus* species. Storage time and storage temperature had the effect of reducing the anaerobic and *Enterococcus* counts, but increasing the *Bacillus* and spore counts. Although the ratio of the bacterial composition changed, the overall aerobic counts remained relatively consistent. Lasa et al. (2008) also determined that the microbial populations changed on storage, with a decrease in *Enterococcus* counts when stored at 25 or -20 °C, and an increase in their Enterobacteriaceae counts. However, their *Bacillus* counts remained constant at all storage temperatures.

The findings of other studies on the microbial populations associated with lepidopterans isolated more species of bacteria than in this study. The microbial

contamination associated with NPV produced in Egypt isolated species of *Bacillus*, *Enterococcus*, *Acinetobacter*, *Klebsiella*, *Staphylococcus* and *Actinomycetes* (Grzywacz et al., 1997). The authors of this study suggested that the contamination arose from personnel working with the insects as well as the microorganisms associated with the insects. Another study on the microorganisms in production lots of NPV isolated several *Bacillus* species, of which *Bacillus cereus* was the most dominant, as well as species of *Enterobacter*, *Micrococcaceae*, *Streptococcaceae*, *Pseudomonadaceae*, *Actinomycetaceae*, *Neisseriaceae*, *Cryptococcaceae* and *Moniliaceae* (Podgwaite et al., 1983).

Broderick et. al., (2004) isolated species of *Pseudomonas*, *Enterobacter*, *Pantoea*, *Serratia*, *Bacillus*, *Staphylococcus*, *Paenibacillus*, *Enterococcus*, *Rhodococcus* and *Microbacterium*. Xiang et. al., (2006) detected species of *Enterococcus*, *Leuconostoc*, *Lactococcus*, and *Acinetobacter.* However, they noted that the *Enterococcus* and *Lactococcus* species were the common species of the gut microbial flora of the *H. armigera* used in their study.

The differences in the number of species detected in these studies could be attributed to the different lepidopteran species used as well as whether the insects used were field-collected or laboratory-reared cultures. For example, Xiang et al. (2006) noted that the small number of the gut bacteria detected in their *H. armigera* larvae was as a result of the narrow range of food their insects were raised on. Broderick et al. (2004) demonstrated that the microbial diversity of Gypsy moth midguts was influenced by their diet. This shows that insects raised on a standardized laboratory diet could have a very different microflora compared to insects feeding on plant leaves containing different nutrients and bacteria associated with them. This effect of diet on microorganisms was also proposed by a study on the microbial communities of grasshopper guts, which suggested that diet may be a major factor contributing to differences in microorganisms associated with the insects (Mead et al., 1988). Laboratory techniques used in rearing the insects and the techniques used in the *in vivo* propagation of the virus

could also affect the microbial community associated with the insects, such as the introduction of human pathogens by laboratory personnel (Grzywacz et al., 1997).

Phylogenetic analysis based on 16S rDNA was done to confirm the identification of the colonies that grew on the media, and determine which type strains they cluster with. Based on this analysis, both the *Bacillus* and *Enterococcus* isolates were confirmed at the genus level, but they did not cluster closely with any of the type strains they were compared to and stand alone in the phylogenetic trees. Further characterization and analysis using the full 16S rDNA sequence could be carried out to identify the isolates to species level.

Bacillus, a gram positive and spore-forming bacterium, is ubiquitous and found in a large variety of foods (Goepfert, 1976). Further investigation determined that the *Bacillus* species isolated was introduced through the wheatgerm which is one of the ingredients used to prepare the insect diet. This indicates that care must be taken to minimize contaminating microorganisms in the insect diet when propagating NPV *in vivo*, as they can be carried through to the final product. This is consistent with a study on the larval Gypsy moth midgut which found that insect diet significantly affected the microbial diversity of the midgut (Broderick et al., 2004). Contaminating microorganisms could possibly be prevented from being carried through to the final product by using antimicrobials in the insect diet (Frederick and Caesar, 1999).

Enterococcus species are gram positive facultative anaerobes and are common inhabitants of insect guts (Carra et al., 2002; Cox and Gilmore, 2007; Grzywacz et al., 1997; Tholen et al., 1997; Walsh and Webster, 2003). Broderick et al. (2004) found *Enterococcus faecalis* present in all of their Gypsy moth larval midguts. *Enterococcus* species can grow at a high pH of 8-10, making the alkaline midgut of the insect a suitable environment, which it acidifies with its metabolism (Broderick et al., 2004; Manero and Blanch, 1999). It is thought that by decreasing the pH of the midgut, the *Enterococcus* species may be providing protection to the insect host since pathogens such as NPV and *Bacillus*

thuringiensis require an alkaline environment to be activated (Broderick et al., 2004; Wilson and Benoit, 1993).

The pH of the homogenates could be affected by the metabolic by-products of the contaminating microorganisms and if conditions became alkaline, could dissolve the occlusion body of the virus. The mean pH of homogenates prepared using cadavers of insects infected with crude HearSNPV had a significantly higher pH than the homogenates prepared with cadavers of insects infected with sterile distilled water. Higher pH values were also seen at storage at 37 °C, compared to storage at 25 °C and 4 °C. The homogenates containing these high pH values correspond to samples containing high *Bacillus* counts. This indicates that the metabolic by-products of the *Bacillus* species in the samples may be causing this increase in pH.

No vertebrate pathogens such as *Shigella*, *Salmonella* or *Staphylococcus* were detected in the homogenates or in the enrichment experiments of the homogenates, nor were coliforms detected. This is consistent with other studies, where with the exception of the occasional *Staphylococcus* being detected, human pathogens have not been found (Grzywacz et al., 1997; Podgwaite et al., 1983; Shapiro, 1982; Xiang et al., 2006). The *Staphylococcus* was thought to be introduced into the products by personnel working with the *in vivo* propagation of the baculovirus (Grzywacz et al., 1997).

The fungi detected in some of the homogenates was present in low concentrations and was not consistently detected in the samples. It is possible that spores from the air contaminated some of the samples. Their mean counts did not increase during storage, which may indicate that the homogenates did not provide a suitable environment or nutrient source for growth of the fungus. Yeast was detected in the study by Grzywacz et al. (1997), as well as by Lasa et al. (2008), but Grzywacs et al. (1997) state that very few yeasts have been isolated from insects.

Bacterial contamination should be minimized from the start of the baculovirus production process by maintaining strict hygiene procedures and harvesting diseased insect cadavers before their microbial load becomes too great (Grzywacz et al., 1997). The data presented in this study indicate that the purity of a NPV inoculum used for the *in vivo* propagation of a baculovirus does not statistically affect the microbial load of the product at production, but can increase the microbial load when stored at 25 or 37 °C.

CHAPTER 4

The development and evaluation of novel *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus (HearSNPV) suspension formulations for control of the African cotton bollworm

4.1 ABSTRACT

Three suspension formulations of *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus (HearSNPV, Family Baculoviridae) were developed and evaluated, namely Instant Starch (IS), Xanthan Gum (XG) and Unformulated (UF). Their shelf-life stability was evaluated under accelerated storage conditions correlating to two years storage at room temperature on the basis of their microbial load, insecticidal activity and pH. A separate low temperature storage stability test was also carried out. The IS and XG suspensions remained suspended at 4 °C after seven days. The IS suspension completely lost its insecticidal activity after storage at the accelerated storage conditions, while the XG and UF suspensions were 4.8 and three times respectively, less effective after 12 weeks of accelerated storage. The suspensions initially contained total aerobic plate counts of approximately 8.0 log cfu/ml each, and this decreased to 3.24 log cfu/ml for the IS suspension, 6.86 log cfu/ml for the UF suspension and 4.26 log cfu/ml for the XG suspension. Two species of bacteria were isolated from the samples and were analysed using 16S rDNA analysis to be a *Bacillus* isolate, which had a 100 % sequence similarity to *Bacillus thuringiensis* and *Bacillus cereus*, and a *Paenibacillus* isolate, which had a 99.5 % sequence similarity to *Paenibacillus motobuensis*. The pH of the suspensions remained near neutral, with the exception of the IS suspension, where the pH remained low, starting at 3.77 and dropping to 3.11 over the 12 week period. The XG suspension displayed the best stability on storage, followed by the UF suspension.

4.2 INTRODUCTION

When using a pathogen as a biopesticide, several factors need to be kept in mind to provide a successful product for storage and use in the field. These factors include producing consistent results; maintaining stability during storage and after application to crops; correct and convenient delivery of the virus to the crop so that the insect ingests a lethal dose; an aesthetically acceptable product with no clumping or flocculation; and competitive production costs (Fuxa, 1991; Jones and Burges, 1997; Lisansky, 1997; Miller et al., 1983). Farmers require an efficient product that is easily dispersed in a tank, preferably with few tank mixes (Seaman, 1990).

Deterioration of biopesticide pathogens occur mainly as a result of high temperatures, adverse pH, enzymes, surfactants and moisture content of the product (Burges and Jones, 1998a). A shelf-life of two years is currently required for commercial chemical pesticides (Jones and Burges, 1997). In order to compete with this market, biopesticides should aim at a similar shelf-life.

Contaminating bacteria arising from the microbes associated with the insects during *in vivo* propagation of the virus may be detrimental to the quality of the product as well as other organisms in the environment in which it will be dispersed (Podgwaite et al., 1983). The registration requirement for biopesticides in some countries allow a limit of 10^7 colony-forming units (c.f.u) per gram of biopesticide, and the Environmental Protection Agency in the USA allows 2×10^8 c.f.u per gram of biopesticide (Burges and Jones, 1998a).

Formulation additives and processing conditions may counteract some of the causes of deterioration of the active ingredient. Additives can provide a range of advantages to the biopesticide including phagostimulation, facilitation of leaf coverage, improved adhesion to plants, UV protection and shelf-life stability. However, some additives such as surfactants, which may be beneficial for the application of the product, may have adverse effects on the active ingredient. A compromise often needs to be reached between the positive and negative effects

that additives may have on the active ingredient. Additives necessary for field application that may be detrimental to the active ingredient should rather be included as tank mixes to minimize any damage (Burges and Jones, 1998a; Jones and Burges, 1997).

According to Jones and Burges (1997), the pH of the product should be maintained between 4 and 6 to prevent the growth of most contaminating organisms while not being detrimental to the active ingredient. Additives such as sugar, sorbic acid, sodium benzoate and antibiotics can also be used to inhibit contaminating microorganisms. The use of medically important antibiotics should be avoided however, since they could promote the development of resistance by pathogenic microorganisms when sprayed into the environment (Jones and Burges, 1997).

Baculovirus biopesticides have been prepared with several additives to act as feeding stimulants and provide UV protection such as sugar, pregelatinized flour, starch, ground maize cob, maize oil, boric acid and optical brighteners (Castillejos et al., 2002; Cisneros et al., 2002; Dougherty et al., 1996; Méndez et al., 2002). Gums, algins and oils have been used to keep active ingredients in suspension during storage as well as to aid resuspension prior to use (Jones and Burges, 1997). The viscosity of the suspension should be guided by the settling rate of the active ingredient particles, but should not be too viscous to use on application (Jones and Burges, 1998).

The shelf-life of a product can be evaluated by accelerated storage studies in which the product is stored at an elevated temperature to predict the stability of the product over time (Corradini and Peleg, 2007; Mizrahi, 2004). This method is commonly used in the food industry because they often need to determine the shelf-life of a product in a short time (Mizrahi, 2004). One mechanism of evaluating the shelf-life of a product is to subject it to the worst-case conditions that it could encounter. Although this would underestimate the shelf-life of the product, it would provide a conservative safety margin (Man, 2004).

Two promising polysaccharides were selected out of a range of additives used in preliminary studies to develop a baculovirus biopesticide with an acceptable shelf-life. The two polysaccharides used were an Instant Starch and a Xanthan Gum. Both polysaccharides act as thickeners and were found to keep the baculovirus suspended over time and did not encourage visible growth of bacterial contaminants.

These additives were used in suspension formulations with *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus (HearSNPV, Family Baculoviridae) and evaluated on the basis of microbial load, insecticidal activity and pH, which were monitored at accelerated storage conditions corresponding to two years of storage at room temperature.

4.3 MATERIALS AND METHODS

4.3.1 *Helicoverpa armigera* culture

Eggs were initially obtained from the Agricultural Research Council, Plant Protection Research Institute (ARC-PPRI) in Pretoria, South Africa. The insects were maintained at 28 °C at a relative humidity of 70 % and a 12 hour photoperiod. Eggs were sterilized in 0.2 % (w/v) sodium hypochlorite and once the neonates hatched they were transferred onto an artificial wheatgerm diet (Bot, 1966) in individual vials because larvae are cannibalistic under crowded conditions.

4.3.2 HearSNPV propagation

Third instar larvae (5 day-old) were inoculated with a dose of 2 x 10^5 OBs/larva by allowing larvae to feed on diet plugs of the artificial wheatgerm diet that had been surface-contaminated with the virus (Evans and Shapiro, 1997). Cadavers of diseased insects were harvested and stored at -20 °C.

4.3.3 Suspension formulation preparation

The suspensions were prepared using a crude extract of HearSNPV. The crude extract was prepared by thawing diseased cadavers and homogenizing them in

sterile distilled water using a tissue homogenizer for 6 minutes. The homogenate was filtered through one layer of miracloth with a pore size of 22 - 25 μm (Calbiochem, catalogue number 475855) to remove large insect debris. The crude extract was stored at 4 °C for a maximum of seven days until use.

The suspension formulations were prepared in a mechanical mixer by adding the dry ingredient to the crude HearSNPV extract while agitating at a rotational speed of 7 500 rpm and mixing for six minutes. The suspension contained a final concentration of 5.0 x 10^8 OBs/ml.

Three suspensions were prepared:

1. Unformulated (UF): 5.0 x 10^8 OBs/ml HearSNPV suspended in sterile distilled water.
2. Instant Starch (IS): 5.0 x 10^8 OBs/ml HearSNPV suspended in 3 % (w/v) Instant Starch.
3. Xanthan Gum (XG): 5.0 x 10^8 OBs/ml HearSNPV suspended in 0.2 % (w/v) Xanthan Gum.

4.3.4 Low temperature storage stability

The low temperature storage stability of each suspension formulation was determined by CIPAC method 1999 MT39.3. Basically, each suspension formulation was stored in a graduated cone shaped glass centrifuge tube at 4 °C for seven days and the volume of any separated matter recorded. Due to a shortage of sample, 90 ml samples were used instead of the 100 ml specified by CIPAC method 1999 MT39.3.

4.3.5 Accelerated storage conditions

Aliquots of the suspension formulations were stored at 35 ± 2 °C for 12 weeks in 100 ml glass bottles as per CIPAC method 1999 MT 46.3 accelerated storage procedure to give two-year storage data. This storage temperature was selected from the range given because it was closest to the optimum temperature for most

microorganisms expected to be associated with the product. The bottles were sealed with plastic screw-caps and stored in the dark.

4.3.6 Evaluation of suspensions at accelerated storage conditions

Each suspension formulation was evaluated before storage (time 0), followed by evaluation of the stored (35 °C) samples at four week intervals to monitor any changes in them upon storage. One sample was removed from the accelerated storage conditions every four weeks and allowed to reach room temperature before being analysed.

Lethal dose assays

Ranging bioassays were performed for the UF suspension prior to the storage stability evaluation in order to determine baseline lethal doses (LD_{30} and LD_{70}). This was done by allowing second instar larvae (3 day-old) to feed for 24 hours on diet plugs of artificial wheatgerm diet that had been surface-contaminated with the virus (Evans and Shapiro, 1997). Larvae that consumed the entire diet plug were returned to their vial containing wheatgerm diet. Mortality was scored seven days post infection and the LD_{30} and LD_{70} doses were calculated for the UF suspension using Probit analysis.

Each suspension was used to infect three replicates of 24 larvae with the LD_{30} and LD_{70} doses estimated from the UF suspension using the surface-contamination method as described above. Mortality was scored seven days post infection and the median lethal dose (LD_{50}) was estimated for each suspension to compare their insecticidal activity.

Microbial load of stored suspensions

Serial dilutions of each suspension formulation in phosphate buffered saline were plated onto Plate Count Agar (Merck) and differential media. The Plate Count Agar was used to determine total aerobic counts; chromogenic UTI agar (Oxoid) was used to detect *Bacillus* species, *Enterococcus* species, *E. coli*, coliforms and *Proteus* species. The presence of yeasts and moulds were detected using

Dichloran Rose Bengal Chloramphenicol agar (DRBC agar). Spore counts were determined by heating a 500 µL aliquot of the suspension formulation at 80 °C for 30 minutes with agitation at 10 minute intervals and serial dilutions of the sample were plated onto plate count agar. All inoculated media were incubated at 37 °C for 24 hours, except for the DRBC agar which was incubated at 25 °C for five days before being evaluated.

Bacterial isolates were sent to Inqaba Biotechnical Industries (Pty) Ltd, Hatfield, South Africa for 16S rDNA sequencing to identify the species. The sequences obtained were submitted to BLAST on the National Centre for Biotechnology Information (NCBI) database to screen for similar sequences. Type strains of these sequences were downloaded for the phylogenetic analyses. Multiple sequence alignments of these type strains were performed with ClustalX2.0.3 as implemented in MEGA version 2.1 (Kumar et al., 2001). The *Bacillus* isolate was aligned using 1177 bases. The same procedure was followed for the *Paenibacillus* isolate, except that 1100 bases were used for the alignment.

A phylogenetic tree was constructed for each genus with the aligned sequences using MEGA version 2.1 (Kumar et al., 2001) using the neighbor-joining method of Saitou and Nei (1987). In the neighbor-joining analyses, the Jukes' and Cantor's one-parameter model was used. The statistical reliability of the tree was determined by using bootstrapping analysis (1000 replications).

pH of suspension formulations

The pH was determined for each suspension formulation using a Metrohm 744 pH meter.

4.3.7 Statistical analysis

Probit regression, using the method of Williams (1986), was used to calculate the lethal doses for each of the suspension formulations at the different time intervals in order to compare their insecticidal activity. Analysis of variance (ANOVA) was used to determine whether the microbial load in the suspension formulations

significantly ($P < 0.05$) differed between the suspensions over time. The Bonferonni post hoc test was used to compare the means at the 95 % significance level. SAS Enterprise Guide version 3.0.1.396 (SAS Institute Inc., Cary, NC, USA) was used to perform all analyses.

4.4 RESULTS

4.4.1 Low temperature stability of liquid formulations

After seven days at 4 °C, the IS suspension remained fully suspended, except for a 1 % clear layer on top of the suspension. Unlike the XG suspension, which remained fully suspended, the Unformulated (UF) suspension settled out, with the top 88 % consisting of a clear liquid, followed by a 2 % layer of fine debris and the remaining 10 % settled out as coarse debris in the bottom of the glass tube. The suspensions were examined again after 14 days, and the XG suspension was still in suspension, while the IS suspension had settled out in the bottom 28 % of the glass tube. The UF suspension remained unchanged from the seven day evaluation.

4.4.2 Suspension evaluation at accelerated storage conditions

Lethal dose assays

On the basis of the median lethal doses estimated from the LD_{30} and LD_{70} bioassays, the insecticidal activity of the formulations decreased on storage (Table 4.1). The IS formulation completely lost its insecticidal activity after four weeks of accelerated storage, whereas the UF suspension's insecticidal activity decreased two-fold and the XG suspension decreased 2.4 times compared to their initial insecticidal activity. This decrease continued over the 12 week period, with the UF sample's insecticidal activity three times less and the XG sample's 4.8 times less than their respective initial insecticidal activity.

Table 4.1 Median lethal doses calculated for each suspension formulation stored under accelerated storage conditions.

Sample	Time (weeks)	LD_{50} (OBs/larva) (95 % CL)
IS	0	205 (108 - 286)
IS	4	0
IS	8	0
IS	12	0
UF	0	229 (74 – 364)
UF	4	467 (372 – 665)
UF	8	373 (265 – 567)
UF	12	726 (548 – 1435)
XG	0	158 (95 - 215)
XG	4	383 (303 – 500)
XG	8	350 (269 – 460)
XG	12	759 (483 – 19 911)*

IS, Instant Starch; UF, Unformulated; XG, Xanthan Gum. LD_{50} = median lethal dose. 95 % CL = 95 % confidence limit.

*In the case where the LD_{30} and LD_{70} bioassays gave results below 50 % mortality, the median lethal dose was extrapolated, which is indicated by larger confidence levels for sample XG at 12 weeks of storage.

Microbial Load

No statistical difference ($F_{11, 35} = 16.46$, $P < 0.05$) was found at time 0 between the mean total aerobic counts of the three suspension formulations, however the microbial load differed for the formulated suspensions compared to the unformulated suspension on accelerated storage (Table 4.2). The microbial load of the three suspensions initially (time 0) contained a mean total aerobic count of approximately 8.0 log cfu/ml, which generally decreased for all three suspensions on storage, however, the UF suspension's counts were significantly higher than that of the XG and IS counts (Table 4.2).

Table 4.2 Total aerobic plate counts (log cfu/ml) of suspensions stored at 35 °C for 12 weeks.

Sample	Storage time (weeks)			
	0	**4**	**8**	**12**
IS	8.25 ± 0.02^{a}	4.44 ± 0.10^{b}	4.71 ± 0.25^{b}	3.24 ± 0.15^{b}
UF	8.36 ± 0.02^{a}	6.63 ± 0.04^{c}	7.72 ± 0.11^{c}	6.86 ± 0.14^{c}
XG	8.30 ± 0.03^{a}	5.21 ± 0.65^{b}	4.13 ± 0.22^{b}	4.26 ± 0.19^{b}

IS, Instant Starch; UF, Unformulated; XG, Xanthan Gum. $F_{11, 35} = 16.46$, ($P < 0.05$). Means are followed by standard errors. Means followed by the same letter are not significantly different.

Two species were isolated from the suspension formulations, namely a *Bacillus* species and a *Paenibacillus* species. The mean *Bacillus* counts ($F_{11, 35} = 256.77$) decreased on storage for all suspensions but the counts were not significantly different prior to storage (Table 4.3). The UF suspension counts were significantly higher than the IS and XG suspension counts for each storage time interval. No *Bacillus* was detected in the XG suspension after 12 weeks of storage.

Table 4.3 *Bacillus* counts of suspensions stored at 35 °C for 12 weeks.

Sample	Accelerated storage time (weeks)			
	0	**4**	**8**	**12**
IS	5.26 ± 0.09^{a}	3.81 ± 0.19^{b}	3.11 ± 0.08^{c}	2.88 ± 0.39^{c}
UF	5.35 ± 0.03^{a}	6.44 ± 0.08^{d}	7.50 ± 0.09^{e}	6.84 ± 0.15^{d}
XG	5.31 ± 0.02^{a}	4.27 ± 0.05^{b}	4.04 ± 0.31^{b}	0.00

IS, Instant Starch; UF, Unformulated; XG, Xanthan Gum. Means are followed by standard errors. Means followed by the same letter are not significantly different, $F_{11, 35} = 256.77$, ($P < 0.05$).

The *Paenibacillus* counts ($F_{11, 35} = 5.73$) decreased over time for all three suspension formulations, and was not detected after 12 weeks of storage in the UF sample, but was present in low concentrations (± 3 log cfu/ml) in the IS and XG samples (Table 4.4).

Table 4.4 *Paenibacillus* counts of suspensions stored at 35 °C for 12 weeks.

Sample	Accelerated storage time (weeks)			
	0	**4**	**8**	**12**
IS	8.11 ± 0.03^{a}	3.53 ± 0.41^{b}	2.66 ± 0.51^{b}	2.52 ± 0.00^{b}
UF	8.17 ± 0.03^{a}	0.00^{c}	0.00^{c}	0.00^{c}
XG	8.17 ± 0.09^{a}	2.94 ± 0.10^{b}	2.86 ± 0.58^{b}	2.26 ± 1.96^{b}

IS, Instant Starch; UF, Unformulated; XG, Xanthan Gum. Means are followed by standard errors. Means followed by the same letter are not significantly different, $F_{11, 35} = 5.73$, ($P < 0.05$).

The spore counts decreased on storage for the IS and XG suspensions, but increased in respect of the UF suspension (Table 4.5). These counts do not correlate directly with the *Bacillus* and *Paenibacillus* counts, but this discrepancy may be because the *Bacillus* and *Paenibacillus* counts would include vegetative cells as well as spores.

Table 4.5 Spore counts of suspensions stored at 35 °C for 12 weeks.

Sample	Accelerated storage time (weeks)			
	0	**4**	**8**	**12**
IS	4.32 ± 0.08^{a}	$2.72 \pm 0.17^{a, b}$	$1.86 \pm 1.64^{b, c}$	$2.90 \pm 0.34^{a, c}$
F	4.29 ± 0.26^{a}	$6.32 \pm 0.08^{a, d}$	7.66 ± 0.10^{d}	6.42 ± 0.36^{d}
XG	4.24 ± 0.12^{a}	$3.85 \pm 0.43^{a, c}$	$3.71 \pm 0.20^{a, c}$	$1.48 \pm 1.28^{b, c}$

IS, Instant Starch; UF, Unformulated; XG, Xanthan Gum. Means are followed by standard errors. Means followed by the same letter are not significantly different, $F_{11, 35} = 15.04$, ($P < 0.05$).

Moulds were detected in low concentrations of between zero and 1.8 log cfu/ml in each of the suspensions. No statistical difference ($F_{11, 35} = 5.60$, $P < 0.05$) was found for the fungi counts in any of the suspensions and they were not detected after 12 weeks of storage.

The phylogenetic tree constructed using the 16S rDNA sequences in Figure 4.1 shows that the *Bacillus* isolate clusters closely with *Bacillus thuringiensis* and *Bacillus cereus*. Based on the 16S rDNA analysis, the *Bacillus* isolate had the

highest sequence similarity of 100 % to both *Bacillus thuringiensis* and *Bacillus cereus*.

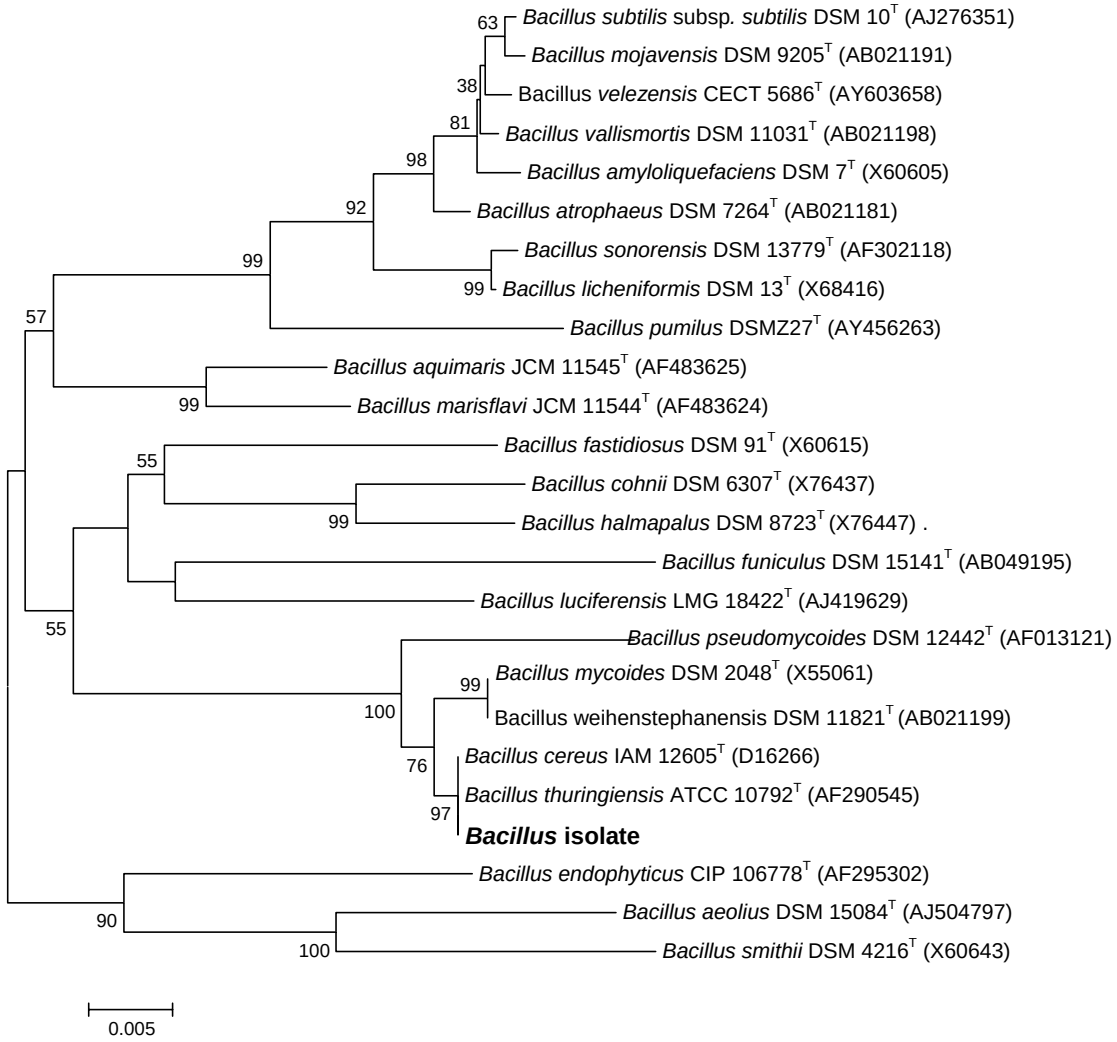


Figure 4.1 Unrooted neighbour-joining tree showing the phylogenetic position of the *Bacillus* isolate compared to other *Bacillus* type strains based on 16S rDNA gene sequence comparisons (Ruiz-García et al., 2005). Bootstrap values greater than 50 % are shown at the nodes (1000 replications). Scale bar indicates substitutions per nucleotide position.

Table 4.6 Calculated sequence similarity (%) of the 16S rDNA gene sequence of the *Bacillus* isolate and type strains of *Bacillus*.

	***Bacillus* isolate**	*B. vallismortis* DSM 11031^T	*B. mojavensis* DSM 9205^T	*B. subtilis subsp. subtilis* DSM 10^T	*B. velezensis* CECT 5686^T	*B. amyloliquefaciens* DSM 7^T	*B. atrophaeus* DSM 7264^T	*B. pumilus* DSMZ27^T	*B. licheniformis* DSM 13^T	*B. sonorensis* DSM 13779^T	*B. aquimaris* JCM 11545^T	*B. fastidiosus* DSM 91^T	*B. marisflavi* JCM 11544^T	*B. cohnii* DSM 6307^T	*B. pseudomycoides* DSM 12442^T	*B. halmapalus* DSM 8723^T	*B. luciferensis* LMG 18422^T	*B. funiculus* DSM 15141^T	*B. mycoides* DSM 2048^T	*B. weihenstephanensis* DSM 11821^T	*B. cereus* IAM 12605^T (D16266)	*B. thuringiensis* ATCC 10792^T	*B. endophyticus* CIP 106778^T	*B. aeolius* DSM 15084^T	*B. smithii* DSM 4216^T
***Bacillus* isolate**																									
B. vallismortis DSM 11031^T	94.6																								
B. mojavensis DSM 9205^T	94.6	99.6																							
B. subtilis subsp. subtilis DSM 10^T	94.7	99.6	99.8																						
B. velezensis CECT 5686^T	94.6	99.7	99.5	99.7																					
B. amyloliquefaciens DSM 7^T	94.5	99.6	99.4	99.4	99.5																				
B. atrophaeus DSM 7264^T	95.0	99.4	99.3	99.3	99.4	99.3																			
B. pumilus DSMZ27^T	94.6	97.0	96.7	96.9	96.9	96.8	97.2																		
B. licheniformis DSM 13^T	94.9	98.4	98.6	98.6	98.3	98.4	98.6	96.4																	
B. sonorensis DSM 13779^T	94.7	98.2	98.4	98.4	98.1	98.2	98.6	96.4	99.8																
B. aquimaris JCM 11545^T	95.1	95.6	95.8	95.9	95.6	95.6	96.0	95.0	96.4	96.2															
B. fastidiosus DSM 91^T	94.5	94.4	94.5	94.6	94.5	94.6	94.4	94.6	94.3	94.5	94.6														
B. marisflavi JCM 11544^T	95.8	95.5	95.3	95.4	95.3	95.3	95.7	95.3	96.0	95.8	98.4	94.9													
B. cohnii DSM 6307^T	94.5	94.0	93.9	94.0	94.0	94.0	94.2	94.7	94.2	94.4	94.7	95.9	94.8												
B. pseudomycoides DSM 12442^T	98.4	93.4	93.4	93.5	93.4	93.3	94.0	93.3	93.7	93.7	94.2	93.3	94.9	93.8											
B. halmapalus DSM 8723^T	95.1	94.2	94.1	94.2	94.3	94.2	94.2	94.6	94.4	94.4	95.2	96.0	95.3	98.1	93.8										
B. luciferensis LMG 18422^T	95.6	94.3	94.3	94.4	94.4	94.6	94.7	94.6	95.0	94.8	94.9	95.2	95.2	95.7	94.5	96.0									
B. funiculus DSM 15141^T	95.0	92.8	92.9	92.9	92.8	92.9	93.2	94.7	93.4	93.4	94.3	94.7	94.1	95.0	94.1	94.8	95.4								
B. mycoides DSM 2048^T	99.5	94.3	94.3	94.4	94.3	94.4	94.7	94.5	94.8	94.6	95.0	94.2	95.5	94.6	98.0	94.8	95.3	94.9							
B. weihenstephanensis DSM 11821^T	99.5	94.3	94.3	94.4	94.3	94.4	94.7	94.5	94.8	94.6	95.0	94.2	95.5	94.6	98.0	94.8	95.3	94.9	100.0						
B. cereus IAM 12605^T (D16266)	100.0	94.6	94.6	94.7	94.6	94.5	95.0	94.6	94.9	94.7	95.1	94.5	95.8	94.5	98.4	95.1	95.6	95.0	99.5	99.5					
B. thuringiensis ATCC 10792^T	100.0	94.6	94.6	94.7	94.6	94.5	95.0	94.6	94.9	94.7	95.1	94.5	95.8	94.5	98.4	95.1	95.6	95.0	99.5	99.5	100.0				
B. endophyticus CIP 106778^T	93.7	94.3	94.5	94.4	94.3	94.5	94.1	93.4	94.8	94.8	95.7	94.7	95.2	94.7	93.0	94.1	94.5	93.6	93.8	93.8	93.7	93.7			
B. aeolius DSM 15084^T	93.6	93.0	93.2	93.3	93.0	93.2	93.5	93.2	93.9	93.9	95.0	93.8	94.5	93.6	92.9	92.7	93.5	93.5	93.7	93.7	93.6	93.6	94.9		
B. smithii DSM 4216^T	93.2	92.8	93.0	93.1	92.8	93.1	93.0	92.7	93.5	93.7	94.4	93.8	94.4	93.7	92.5	92.7	93.2	93.5	93.5	93.5	93.2	93.2	94.9	96.4	

* B = *Bacillus;* See figure 4.1 for the NCBI Accession numbers for the reference strains.

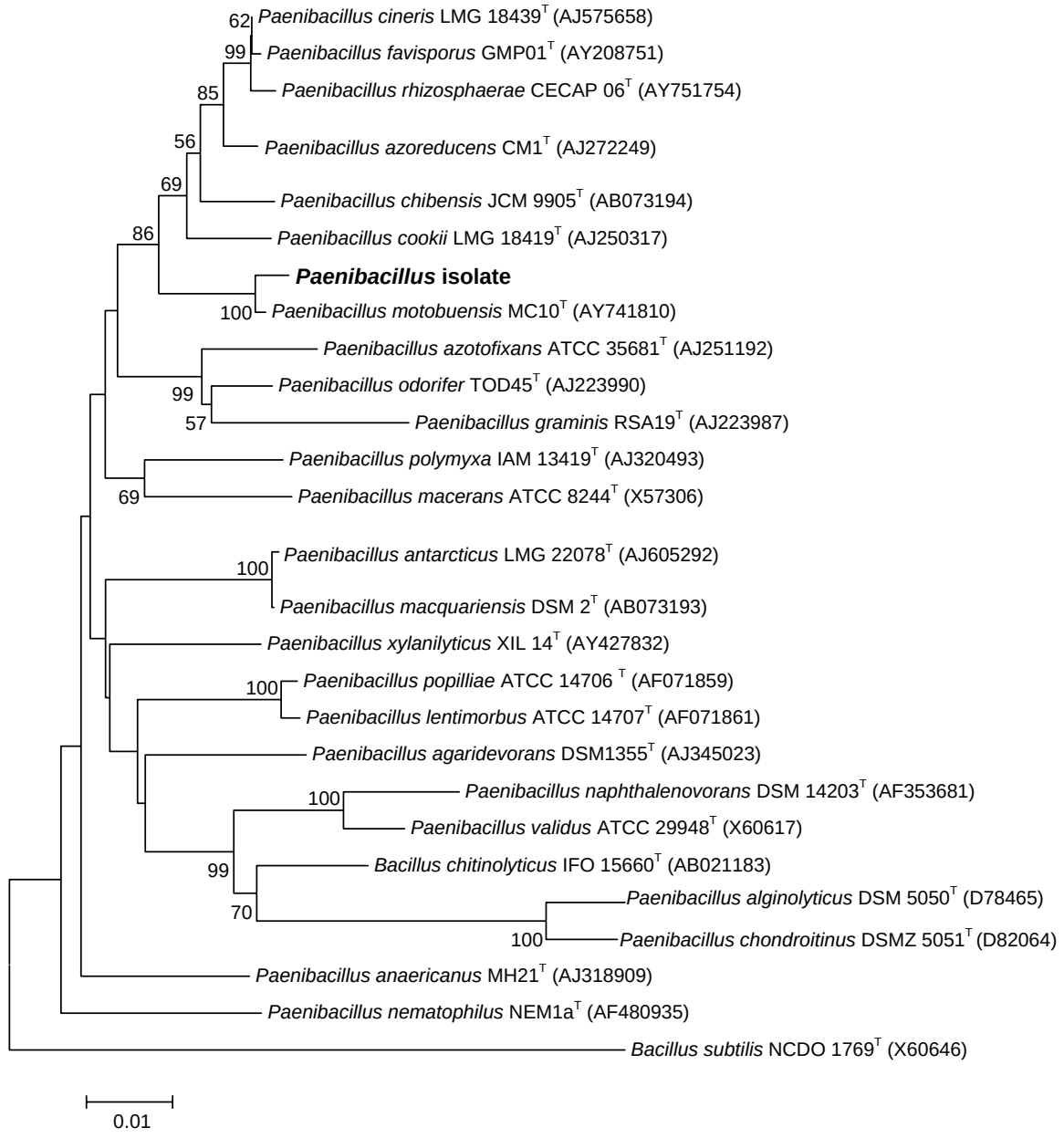


Figure 4.2 Neighbour-joining tree showing the phylogenetic position of the *Paenibacillus* isolate to other *Paenibacillus* type strains based on 16S rDNA gene sequence comparison. *Bacillus subtilis* NCDO 1769^T (X60646) was used to root the tree (Dasman et al., 2002). Bootstrap values greater than 50 % are shown at the nodes (1000 replications). Scale bar indicates substitutions per nucleotide position.

The phylogenetic tree constructed using the 16S rDNA sequences in Figure 4.2 shows that the *Paenibacillus* isolate clusters closely with *Paenibacillus motobuensis*. The sequence similarity of the *Paenibacillus* isolate has a 99.5 % (Table 4.7) sequence similarity to *Paenibacillus motobuensis*

Table 4.7 Calculated sequence similarity (%) of the 16S rDNA gene sequence of the *Paenibacillus* isolate and type strains of *Paenibacillus*.

	***Paenibacillus* isolate**	*P. popilliae* ATCC 14706^T	*P. lentimorbus* ATCC 14707^T	*P. azotofixans* ATCC 35681^T	*P. naphthalenovorans* DSM 14203^T	*P. odorifer* TOD45^T	*P. graminis* RSA19^T	*P. motobuensis* MC10^T	*P. agaridevorans* DSM 1355^T	*Bacillus subtilis* NCDO 1769^T	*P. alginolyticus* DSM 5050^T	*P. chondroitinus* DSMZ 5051^T	*B. chitinolyticus* IFO 15660^T	*P. polymyxa* IAM 13419^T	*P. validus* ATCC 29948^T	*P. cookii* LMG 18419^T	*P. azoreducens* CM1^T	*P. cineris* LMG18439^T	*P. macquariensis* DSM 2^T	*P. antarcticus* LMG 22078^T	*P. chibensis* JCM 9905^T	*P. azoreducens* CM1^T	*P. nematophilus* NEM1a^T	*P. favisporus* GMP01^T	*P. anaericanus* MH21^T	*P. rhizosphaerae* CECAP 06^T	*P. xylanilyticus* XIL 14^T	*P. macerans* ATCC 8244^T
***Paenibacillus* isolate**																												
P. popilliae ATCC 14706^T	95.2																											
P. lentimorbus ATCC 14707^T	95.2	99.6																										
P. azotofixans ATCC 35681^T	95.6	95.3	95.1																									
P. naphthalenovorans DSM 14203^T	93.4	94.2	94.2	93.1																								
P. odorifer TOD45^T	96.5	95.1	94.9	97.9	93.2																							
P. graminis RSA19^T	95.0	93.9	93.7	96.2	92.0	97.0																						
P. motobuensis MC10^T	99.5	95.4	95.4	95.9	94.0	96.8	94.9																					
P. agaridevorans DSM 1355^T	95.1	96.3	96.2	95.2	94.2	95.4	93.8	95.3																				
Bacillus subtilis NCDO 1769^T	89.6	89.4	89.4	89.4	88.4	89.4	88.2	89.9	89.6																			
P. alginolyticus DSM 5050^T	91.2	92.8	92.7	90.9	93.1	91.2	90.2	91.8	92.3	86.5																		
P. chondroitinus DSMZ 5051^T	91.4	92.7	92.6	91.5	93.1	91.8	90.3	92.0	92.8	87.2	98.3																	
B. chitinolyticus IFO 15660^T	94.8	95.7	95.6	94.1	95.6	94.3	93.0	95.2	95.6	89.0	94.4	94.5																
P. polymyxa IAM 13419^T	96.3	95.4	95.4	95.7	94.2	95.6	94.4	96.5	95.7	89.1	91.2	91.2	95.0															
P. validus ATCC 29948^T	93.6	94.8	94.8	93.7	97.9	94.0	93.0	94.1	95.4	88.9	93.7	93.6	96.4	94.4														
P. cookii LMG 18419^T	96.9	95.4	95.4	95.9	93.3	97.1	94.9	97.2	96.5	89.6	92.3	92.6	94.7	95.3	94.7													
P. azoreducens CM1^T	97.9	95.1	95.1	95.5	93.9	96.2	94.8	98.1	96.4	90.1	92.0	92.0	95.3	96.3	94.9	98.2												
P. cineris LMG18439^T	97.7	95.3	95.3	95.7	93.6	96.6	95.0	97.9	96.4	89.7	91.9	91.9	95.4	96.7	94.9	98.1	99.3											
P. macquariensis DSM 2^T	95.1	96.2	96.4	95.3	93.6	95.9	93.8	95.4	95.5	90.2	91.9	91.8	94.1	94.9	94.1	95.7	95.4	95.4										
P. antarcticus LMG 22078^T	95.2	96.1	96.3	95.2	93.4	95.8	93.9	95.3	95.4	90.1	91.8	91.7	94.0	94.8	94.0	95.8	95.5	95.3	99.9									
P. chibensis JCM 9905^T	97.2	94.9	94.9	95.4	93.1	96.6	94.1	97.6	95.4	90.4	92.1	92.3	95.0	95.3	93.8	98.2	98.4	98.7	96.2	96.1								
P. azoreducens CM1^T	97.9	95.1	95.1	95.5	93.9	96.2	94.8	98.1	96.4	90.1	92.0	92.0	95.3	96.3	94.9	98.2	100.0	99.3	95.4	95.5	98.4							
P. nematophilus NEM1a^T	94.4	95.4	95.4	94.4	93.3	94.7	93.3	94.9	94.4	89.9	91.2	91.0	93.2	95.3	92.9	95.2	95.5	95.3	95.6	95.5	95.5	95.5						
P. favisporus GMP01^T	97.6	95.2	95.2	95.6	93.4	96.5	94.9	97.8	96.3	89.6	91.8	91.8	95.3	96.6	94.8	97.9	99.2	99.9	95.3	95.2	98.6	99.2	95.2					
P. anaericanus MH21^T	96.1	95.2	95.2	95.2	93.3	96.3	94.5	96.6	95.1	89.9	92.6	92.6	94.3	95.6	93.8	96.2	95.8	96.2	95.9	95.8	96.4	95.8	95.6	96.1				
P. rhizosphaerae CECAP 06^T	97.4	95.1	95.1	95.4	93.3	96.3	94.7	97.6	96.2	89.4	91.5	91.5	95.1	96.5	94.5	97.7	99.0	99.7	95.1	95.0	98.4	99.0	95.1	99.6	95.8			
P. xylanilyticus XIL 14^T	95.8	95.7	95.8	95.4	94.9	95.7	94.3	96.2	96.3	89.8	92.0	91.8	95.2	96.5	95.7	95.7	96.6	97.0	96.3	96.2	95.7	96.6	94.8	96.9	96.1	96.8		
P. macerans ATCC 8244^T	95.6	95.5	95.5	95.9	94.5	95.7	94.4	95.8	94.9	89.4	91.0	91.0	94.0	96.7	93.9	95.6	95.4	95.4	95.6	95.6	95.4	95.4	95.5	95.3	95.5	95.2	95.6	

* P = *Paenibacillus;* See figure 4.2 for the NCBI Accession numbers for the reference strains.

pH of the samples

The pH of the samples remained relatively constant throughout storage (Table 4.6). The pH of the UF and XG samples were close to neutral, whereas the IS suspension had an initial pH of 3.77, which decreased slightly on accelerated storage, but did not differ significantly ($P < 0.05$) over the 12 week period.

Table 4.8 pH of suspension formulations stored at 35 °C for 12 weeks.

Sample	Storage time (weeks)			
	0	**4**	**8**	**12**
IS	3.77 ± 0.24^{a}	3.57 ± 0.03^{a}	3.99 ± 0.06^{a}	3.11 ± 0.17^{a}
UF	5.39 ± 0.23^{b}	7.64 ± 0.04^{c}	7.91 ± 0.08^{c}	6.92 ± 0.22^{c}
XG	5.47 ± 0.52^{b}	7.19 ± 0.10^{c}	6.85 ± 0.36^{c}	6.60 ± 0.30^{c}

IS, Instant Starch; UF, Unformulated; XG, Xanthan Gum. Means ± standard deviation. Means followed by the same letter are not significantly different ($F_{2, 33} = 14.94$).

4.5 DISCUSSION

The storage stability of a biopesticide is an important aspect for the commercialization of the product (Tamez-Guerra et al., 2002). Since it is expensive to produce purified baculoviruses on an industrial scale, they are often not highly purified and therefore microbial contaminants may influence the stability of the biopesticide on storage. Formulation ingredients can help to overcome this problem (Jones and Burges, 1997; Lasa et al., 2008).

The low temperature storage evaluation showed that the XG and IS additives used in the suspension formulations produced a suspension with enough viscosity to keep the active ingredient in suspension at 4 °C for seven days compared to the Unformulated (UF) suspension formulation which settled out. Not much settling is expected to occur beyond seven days of refrigerated storage. Although the UF sample re-suspended after being shaken, the formulated suspensions form a more aesthetically pleasing product.

The accelerated storage study gave an indication of the stability of the suspensions over a two year period if stored at room temperature. The IS suspension completely lost its insecticidal activity and the UF suspension maintained the lowest median lethal dose on storage with a three-fold reduction, compared to a 4.8-fold reduction for the XG suspension. A loss in insecticidal activity also occurred in a study conducted by Lasa et al. (2008) with a formulation of a multiple nucleocapsid nucleopolyhedrovirus (*Spodoptera exigua*), although a much larger decrease in the insecticidal activity of 16 times over an 18 month storage period at 25 °C was reported.

A decrease in the insecticidal activity would compromise the consistency of the product when used in the field, where the concentration required to control the insect population will need to be higher after storage. Measures to counteract this such as refrigeration could be considered.

The total aerobic plate counts showed a decrease in the microbial load on accelerated storage. The UF suspension contained a higher microbial load throughout the accelerated storage period than the formulated samples. Although the formulated samples may contain more nutrients for contaminating bacteria, the free water content in the UF suspension may be higher, whereas the formulated suspensions would have contained molecularly bound water (Burges and Jones, 1998a). Microorganisms rely on a water activity of above 0.6 in their environment to reproduce (Esse et al., 2004), and although the water activity of the suspensions was not measured, it is expected to be lower in the formulated suspensions and this could be the reason for the higher microbial load in the UF suspension.

Two bacterial isolates were found to be associated with these suspensions, namely *Bacillus* and *Paenibacillus*. *Bacillus* species are gram positive, facultatively anaerobic spore-forming bacteria. Phylogenetic analysis based on 16S rDNA was done to confirm the identification of the colonies that grew on the media, and determine which type strains they cluster with. Based on this analysis, the

Bacillus isolate had a 100 % sequence similarity to *Bacillus cereus* and *Bacillus thuringiensis*. Since *B. cereus* is a facultative pathogen, further characterization and analysis using the full 16S rDNA sequence will need to be carried out to identify the isolate to species level. Members of the *Bacillus* species have been found to be associated with other lepidopterans (Broderick et al., 2004; Grzywacz et al., 1997; Lasa et al., 2008; Podgwaite et al., 1983). These bacteria are ubiquitous in the environment and are commonly isolated from soil, plants and insect habitats (Gao et al., 2008; Goepfert, 1976; Reva and Smirnov, 2002)

Paenibacillus are also gram positive, facultatively anaerobic spore-forming bacteria. They contain their endospores in swollen sporangia and sometimes stain as gram-negative. Bacteria from the *Paenibacillus* genus were previously classified as the genus *Bacillus,* but were separated into their own genus in the early 1990's (Chorin et al., 1997; Lida et al., 2005). The *Paenibacillus* isolate was found to have a 99.5 % sequence similarity to *Paenibacillus motobuensis.* *Paenibacillus* species commonly occur in the environment such as *Paenibacillus motobuensis*, which was first isolated from a compost sample (Lida et al., 2005). This fact could explain the presence of this species in our sample, since the wheatgerm purchased for the insect diet came from an organic farmer who uses compost as a fertilizer.

Since both bacteria isolated from these samples are ubiquitous in the environment, they may have been introduced from ingredients used to prepare the wheatgerm diet for *Helicoverpa armigera*, and since the virus was propagated *in vivo*, was carried through to the virus suspensions.

Low concentrations of fungi were detected in the samples and it is thought that these contaminants were introduced from the environment. No human pathogens such as coliforms and *Staphylococcus* species were detected in the samples. The only cause for concern is that the *B. cereus* type strain has a 100 % sequence similarity to the *Bacillus* isolate however, this could also be *B. thuringiensis*, which is commonly associated with the environment (Gao et al., 2008; Goepfert,

1976; Reva and Smirnov, 2002). Further analysis would be required to identify the *Bacillus* isolate to species level. The presence of *Salmonella* and *Shigella* were screened for in a preliminary study and not detected.

The decrease in the insecticidal activity of the suspensions could be due to several factors including the microbial load from the natural flora of the insects when producing the virus *in vivo*. Since it is not practical to purify the virus when producing it on a large scale, the microorganisms in the insects could be carried through to the final product. Impurities are an important factor in the stability of a virus suspension since they may have adverse effects on the virus (Burges and Jones, 1998a).

The microbial load can affect the pH of the suspension due to the accumulation of waste products (Jones and Burges, 1998), and it has been recommended that the pH of a suspension containing virus should be near neutral (Ignoffo and Garcia, 1966). A study found that a baculovirus was inactivated after exposure to a pH of 3 for 30 minutes (LeBlanc and Overstreet, 1991). This could explain the loss in activity of the IS suspension. Although the IS suspension had lower concentrations of contaminating bacteria, the additive may have resulted in the low pH values. Since the IS suspension's pH decreased to 3.57 after four weeks of accelerated storage and continued to decrease to a pH of 3.11 after 12 weeks of storage at accelerated conditions, this could have inactivated the baculovirus.

The accelerated storage conditions provided a useful tool for determining the storage stability of the suspensions in a short time period. This study indicates that XG is the most promising additive of the two examined here for a baculovirus biopesticide in terms of the attributes evaluated, because it remained suspended upon low temperature storage, contained a microbial load low enough to conform to the registration requirement for biopesticides according to the Environmental Protection Agency in the USA, preserved its insecticidal activity and maintained a pH near neutral.

CHAPTER 5

The development of a spray-dried baculovirus biopesticide using a multi-factorial optimization strategy

5.1 ABSTRACT

Response Surface methodology was used to optimize spray-drying conditions to produce a baculovirus wettable powder. *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus (HearSNPV) was spray-dried with two carriers, namely Polysaccharide-WM and Polysaccharide-MS using inlet temperature, air speed and feed flow rate as the model input factors. The response factors monitored were powder yield (mg/ml), active ingredient yield of the occlusion bodies (OBs/mg) and moisture content (%). The effect of spray-drying on the microbial load of the samples was also examined. The optimal conditions for Polysaccharide-WM were determined to be an inlet temperature of 130 °C, air speed dial setting of 45 and feed flow rate of 9 ml/minute. According to the predictive models obtained, this would give a powder yield of 337.5 mg/ml, active ingredient yield of 8.0×10^5 OBs/mg and moisture content of 4.8 %. The optimal conditions for Polysaccharide-MS were determined to be an inlet temperature of 130 °C, air speed dial setting of 45 and feed flow rate of 5.4 ml/minute. According to the predictive models obtained, this would give a powder yield of 110.9 mg/ml, active ingredient yield of 1.0×10^6 OBs/mg and moisture content of 4 %. These conditions will give suitable outlet temperatures of between 63 °C – 76 °C. Spray-drying reduced the microbial load of the sample four-fold. This study provides the foundation for the commercial production of a baculovirus biopesticide wettable powder produced by spray-drying.

5.2 INTRODUCTION

Biopesticides have limitations when it comes to the commercialization of the product, which include shelf-life stability and residual activity after field application. These can be overcome by formulating the product in various ways and by using additives. Four basic functions of formulation have been identified, namely: to stabilize the microorganism during production, distribution and storage; to assist handling and application of the microorganism; to protect the microorganism from environmental factors that may inactivate it; and to enhance the activity of the microorganism at the target site (Jones and Burges, 1998). The challenge is to optimize the formulation to address all four of the above points, as well as be practical and economically viable (Behle et al., 2003; Jones and Burges, 1998; Tamez-Guerra et al., 2002).

Baculoviruses are used to control lepidopteran pests on crops because they are highly specific and persistent in the environment (Hails, 1997). Wettable powders and granules of baculoviruses can be prepared by freeze-drying, spray-drying or air-drying. Dried products have the advantage of easy and stable storage, but they are often dusty which makes them an inhalation risk (Seaman, 1990). A good product would remain viable on the shelf for a few years, however it may be acceptable for a product to have a shorter storage stability provided the expiration date is indicated on the pack (Tamez-Guerra et al., 2002).

Spray-drying has been used successfully to produce powders of nucleopolyhedroviruses, granuloviruses and *Bacillus thuringiensis* (Arthurs and Lacey, 2004; Behle et al., 2003; Tamez-Guerra, 2000a; Tamez-Guerra et al., 2000b; Tamez-Guerra et al., 2002; Tamez-Guerra et al., 1996). Baculoviruses can withstand high temperatures for short time periods. The baculovirus occlusion bodies (OBs) are protected by a polyhedrin coat, which makes them highly stable in the environment (Funk et al., 1997). *Heliothis* NPV for example has been shown to withstand a temperature of 70 °C for 10 minutes (LeBlanc and Overstreet, 1991), and this enables them to withstand spray-drying conditions.

Optimal spray-drying conditions vary between facilities, and depend on the pathogen, spray-dryer type and encapsulating agent. Thus the spray-drying conditions need to be optimized according to these factors (Tamez-Guerra, 2000a). The conditions are regulated by adjusting the inlet temperature, feed flow rate, air speed and deblocker of the spray-dryer. The combination of these settings and the nature of the product being spray-dried determines the outlet temperature which is an important factor when spray-drying since this is the temperature that the encapsulated agents will be exposed to (Tamez-Guerra, 2000a).

Studies that successfully prepared powders containing baculoviruses used inlet temperatures ranging from 100-125 °C combined with a feed flow rate of 8 ml/min (Arthurs and Lacey, 2004; Behle et al., 2003; Tamez-Guerra, 2000a; Tamez-Guerra et al., 2000b; Tamez-Guerra et al., 2002). These conditions resulted in outlet temperatures ranging from 60 to 75 °C, thus enabling the virus to retain its insecticidal activity. These high temperatures may have the added advantage of reducing contaminating organisms in the powders (Jones and Burges, 1997).

Additives which enhance the active ingredient, such as flour, oil and sugar often act as phagostimulants and have other enhancing properties such as facilitating adhesion of the virus to plants. These can be used as carriers or encapsulating agents to spray-dry the active ingredients (Tamez-Guerra et al., 2002). Lignin, pregelatinised corn flour, and sugar have been used successfully to encapsulate viral occlusion bodies. These formulated baculoviruses were found to have significantly higher insecticidal activity in field trials than unformulated ones (Behle et al., 2003).

The objective of this study was to determine the optimum spray-drying conditions to produce a baculovirus wettable powder with a good yield, high concentration of the active ingredient and an acceptable moisture content using two commercially available polysaccharides manufactured for spray-drying. A muti-factorial

approach to optimizing the spray-drying process was used, because a combination of factors affect the spray-drying conditions and quality of the final product.

5.3 MATERIALS AND METHODS

5.3.1 HearSNPV propagation

Third instar larvae (5 day-old) were inoculated with a lethal dose of 6 x 10^5 OBs/larva by allowing larvae to feed on diet plugs of the artificial wheatgerm diet that had been surface-contaminated with the virus (Evans and Shapiro, 1997). Cadavers of diseased insects were harvested upon death and stored at -20 °C.

5.3.2 HearSNPV extract preparation

An extract of HearSNPV was prepared by thawing cadavers and homogenizing them in sterile distilled water using a tissue homogenizer for 6 minutes. The homogenate was filtered through one layer of miracloth with a pore size of 22 – 25 μm (Calbiochem, catalogue number 475855) to remove large insect debris. The crude extract was stored at 4 °C until use.

5.3.3 Spray-drying suspension preparation

Two modified maize starch polysaccharides were used in a multi-factorial design, namely Polysaccharide-WM and Polysaccharide-MS.

Each suspension contained a final concentration of 5 x 10^8 OBs/ml and the suspension to be spray-dried was prepared by slowly adding the polysaccharide (32 %, w/w for Polysaccharide-WM and 36 %, w/w for Polysaccharide-MS) to the crude extract in sterile distilled water whilst agitating with a mechanical mixer at 7000 rpm for 6 minutes. The suspension was then left to settle to allow the foam to dissipate. The Polysaccharide-WM suspension took 1 hour to settle, while the Polysaccharide-MS suspension took 3 hours to settle.

5.3.4 Multi-factorial design and analysis

Response Surface methodology and a central composite design with three numeric variables (factors), namely inlet temperature, air speed, and feed flow rate was

used to predict the effect of the individual factors as well as the interaction of the three factors on the spray-dried powder. The upper and lower limit of each factor was determined in preliminary trials. Each numeric factor was varied over five levels; plus and minus alpha (axial points), plus and minus one (factorial point) and the centre point. To avoid infeasible extremes, factor highs and lows were entered in terms of alpha. The full design was analysed as a single block and is shown in Table 5.1. Three responses were monitored, namely powder yield (mg/ml), active ingredient yield (OBs/mg) and moisture content. Design Expert version 6.0.10 was used to set-up and analyse the multi-factorial design.

Table 5.1 Multi-factorial experimental design for each suspension.

Run	Inlet Temperature (Degrees Celcius)	Air Speed (Dial setting)	Feed Flow Rate (ml/min)
1	139.9	34	12.6
2	110.1	46	5.4
3	110.1	46	12.6
4	100.0	40	9.0
5	139.9	46	12.6
6	139.9	46	5.4
7	125.0	40	9.0
8	125.0	40	9.0
9	110.1	34	5.4
10	125.0	40	9.0
11	110.1	34	12.6
12	150.0	40	9.0
13	125.0	30	9.0
14	125.0	40	3.0
15	125.0	40	9.0
16	125.0	50	9.0
17	125.0	40	9.0
18	125.0	40	9.0
19	125.0	40	15.0
20	139.9	34	5.4

5.3.5 Spray-drying

The spray-drying suspensions were spray-dried using a Labplant Laboratory Spray-dryer (SD06) using the spray-drying conditions shown in Table 5.1. The temperature of the suspension to be spray-dried was recorded at the start of each run to ensure they were all similar since this could affect the inlet temperature of the sample and therefore affect the encapsulating conditions. Three factors were

used to optimize the spray-drying process namely inlet temperature (A), air speed (B) and feed flow rate (C). The deblocker was not changed and was set at the medium setting for each run. The outlet temperature was monitored throughout each run and the collection of powder on the main chamber and cyclone bottle was noted. The suspension was continually agitated using a magnetic stirrer during spray-drying to ensure that the active ingredient remained evenly suspended. 180 ml of suspension with a concentration of 5 x 10^8 OBs/ml was spray-dried per run.

5.3.6 Evaluation of spray-dried powders

Powder yield (mg/ml): the yield of powder formed per milliliter of suspension fed into the spray-dryer.

Active ingredient yield (OBs/mg): The concentration of OBs per milligram of powder produced was determined by suspending 100 mg of the powder in 1000 µl of sterile distilled water and quantifying using a haemocytometer. Five replicates were counted per sample.

Moisture content: The moisture content was determined by drying a sample of the powder at 90 °C for 24 hours. The percentage of weight loss after drying was used as the measure of the moisture content. These were carried out in duplicate for all samples.

Microbial load: Total aerobic plate counts were determined using Plate Count Agar (Merck). Chromogenic UTI agar (Oxoid) was used to detect and enumerate *Bacillus* species, *Enterococcus* species, *E. coli*, coliforms and *Proteus* species. Spore counts were determined by heating a suspension of the powder in sterile distilled water at 80 °C for 30 minutes to kill the vegetative cells, with agitation at 10 minute intervals and then quantifying the spores using plate count agar. DRBC agar was used to detect moulds and yeasts.

The microbial load was determined for the initial baculovirus extract as well as for each polysaccharide before being spray-dried. The microbial load of each spray-dried wettable powder was determined after spray-drying. The powders were

analyzed by suspending 100 mg of the powder in sterile distilled water, and serial dilutions in phosphate buffered saline were plated onto the media discussed above.

5.3.7 Statistical analysis

Analysis of variance (ANOVA) was used to determine whether the spray-drying conditions affected the microbial load of the powders. The means were compared using the Bonferonni post hoc test at the 95 % confidence level. SAS Enterprise Guide 3.0.1.396 (SAS Institute Inc., Cary, NC, USA) was used to perform the statistical analysis.

5.4 RESULTS

5.4.1 Measured outlet temperature

The temperature of the suspension being fed into the spray-dryer was ambient for all the samples and the outlet temperatures ranged from 52 °C to 85 °C, depending on the combination of variable settings used.

5.4.2 Optimization of spray-drying with Polysaccharide-MS

Powder yield

The predictive model for the effect of the three factors ($F_{2, 19} = 3.36$, $P < 0.05$) on powder yield (mg/ml) only had a 4.49 % chance of occurring due to noise, and can be explained by the Response Surface linear model:

$$\text{Powder yield (mg/ml)} = -14.42 + 0.56\,(A) + 1.56\,(B) - 3.36\,(C) \quad \text{(Equation 1)}$$

where (A) is the inlet temperature, (B) the air speed and (C) the feed flow rate. The only model term that significantly affected powder yield ($F_{2, 19} = 4.81$, $P < 0.05$) was the feed flow rate (C). This model is illustrated by the cuboidal graph (Figure 5.1), which shows the effects of the three model terms on powder yield (mg/ml). The high and low values for feed flow rate are represented by C+ and C-, and similarly the high and low values for inlet temperature and air speed are shown. The highest powder yield (118.91 mg/ml) is obtained at the model

term combination A+, B+ and C-, indicating that a lower feed flow rate, higher inlet temperature and higher air speed gives a higher powder yield.

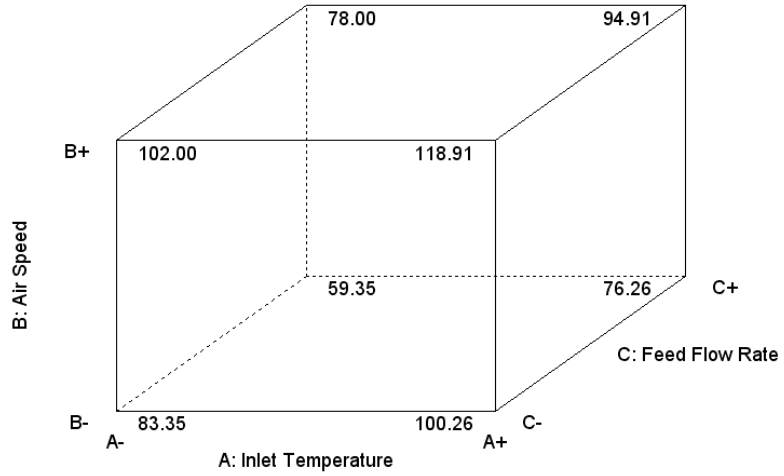


Figure 5.1 Cuboidal graph of the model showing the effect of the three factors on powder yield (mg/ml) obtained with Polysaccharide-MS.

Active ingredient yield

No significant model was obtained for the active ingredient yield ($F_{2, 19} = 1.13$, $P < 0.05$) therefore it was not significantly affected by any of the model terms.

Moisture content

The overall model for the effect of the three factors ($F_{2,(19} = 4.40$, $P < 0.05$) on the moisture content (%) of the powders can be explained by the Response Surface linear model:

$$\text{Moisture content (\%)} = 11.34 - 0.06\,(A) - 0.02\,(B) + 0.27\,(C)$$

(Equation 2)

The inlet temperature ($F_{2, 19} = 5.43$, $P < 0.05$) and feed flow rate ($F_{2, 19} = 7.65$, $P < 0.05$) rate significantly affected the moisture content of the powders. The chance that the model F-value of 4.40 occurred due to noise was 1.94 %. The cuboidal graph (Figure 5.2) represents the high and low inlet temperatures as A+ and A- respectively, and similarly the high and low feed flow rate as C+ and C-. From this cuboidal graph, it can be seen that the lowest moisture content value of

4.16 % occurs at a combination of the statistically significant model terms of a high inlet temperature, high air speed and low feed flow rate.

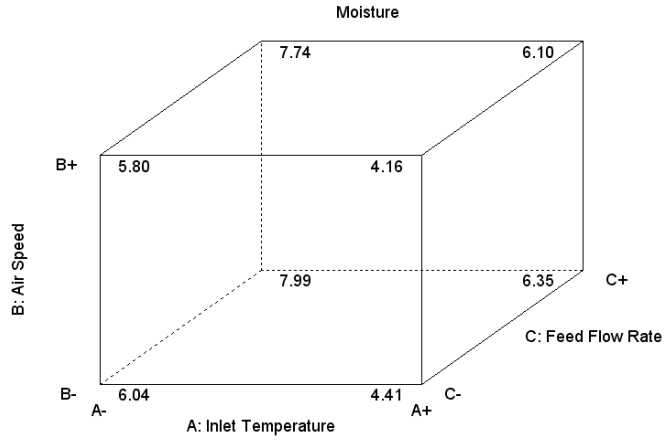


Figure 5.2 Cuboidal graph of the model describing the effects of the three factors on the moisture content of powders produced with Polysaccharide-MS.

5.4.3 Optimization of spray-drying with Polysaccharide-WM

Powder yield (mg/ml)

The overall model ($F_{2, 19} = 25.10$, $P < 0.05$) of the effect of the three factors can be explained by the Response Surface quadratic model:

$$\text{Powder yield (mg/ml)} = 68.75 + 1.55\,(A) - 5.24\,(B) - 1.98\,(C) + 0.08\,(B^2) + 0.5\,(C^2) - 0.10\,(AC) \quad \text{(Equation 3)}$$

The model terms inlet temperature (A) ($F_{2, 19} = 25.77$, $P < 0.05$), air speed (B) ($F_{2, 19} = 8.14$, $P < 0.05$), feed flow rate (C) ($F_{2, 19} = 98.32$, $P < 0.05$), feed flow rate squared (C^2) ($F_{2, 19} = 12.48$, $P < 0.05$) and the interaction between inlet temperature and feed flow rate (AC) ($F_{2, 19} = 4.56$, $P < 0.05$) were found to significantly affect the yield of the powder. The model terms AB, BC, A^2 and B^2 were removed because they were not significant. Figure 5.3 illustrates that the highest yield of powder (135.81 mg/ml) was obtained at the model term combination of A+, B+ and C-. The F-value of 25.10 of this model has a 0.01 % chance of occurring due to noise. According to the cuboidal graph, an increase in

inlet temperature or air speed and a decrease in feed flow rate results in an increased powder yield (mg/ml).

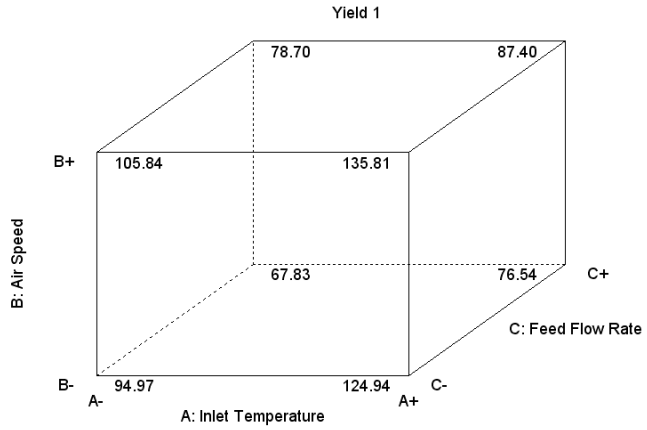


Figure 5.3 Cuboidal graph of the model showing the effect of the three factors on powder yield (mg/ml) for powders produced with Polysaccharide-WM.

Active ingredient yield

The Response Surface reduced quadratic model ($F_{2, 19} = 7.71$, $P < 0.05$) for the active ingredient yield (OBs/mg) explains the interaction of the model terms as follows:

$$\text{Active ingredient yield (OBs/ml)} = 5.56 \text{ X } 10^6 - 15103.88\,(A) - 1.81 \text{ X } 10^5\,(B) + 58849.60\,(C) - 172.98\,(A^2) - 4311.00\,(C^2) + 1423.00\,(AB) \quad \text{(Equation 4)}$$

where feed flow rate (C) ($F_{2, 19} = 10.96$, $P < 0.05$), feed flow rate squared (C^2) ($F_{2, 19} = 7.86$, $P < 0.05$) and the interaction between inlet temperature and air speed (AB) ($F_{2, 19} = 22.92$, $P < 0.05$) significantly affected the yield of active ingredient in the powder. Figure 5.4 shows that the highest yield of active ingredient (1.155 x 10^6 OBs/mg) is produced at the model term combination A-, B- and C-. However, the interactions of the model terms in this respect are not linear. Figure 5.5 illustrates that this yield decreases at a combination of a high inlet temperature and low air speed and vice versa, at a constant feed flow rate of 9 ml/min.

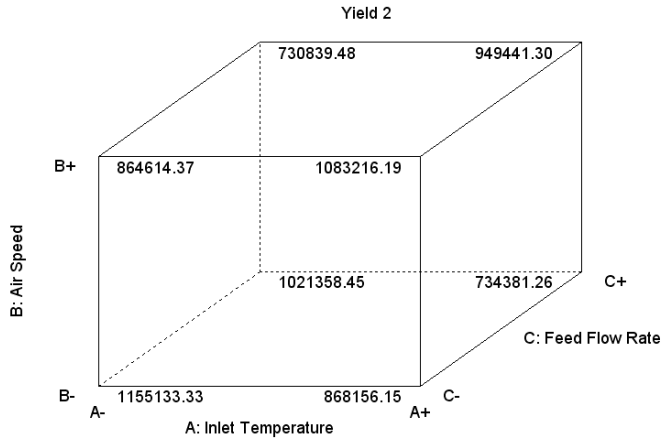


Figure 5.4 Cuboidal graph of the model describing the effects of the three factors on the active ingredient yield of powders produced with Polysaccharide-WM.

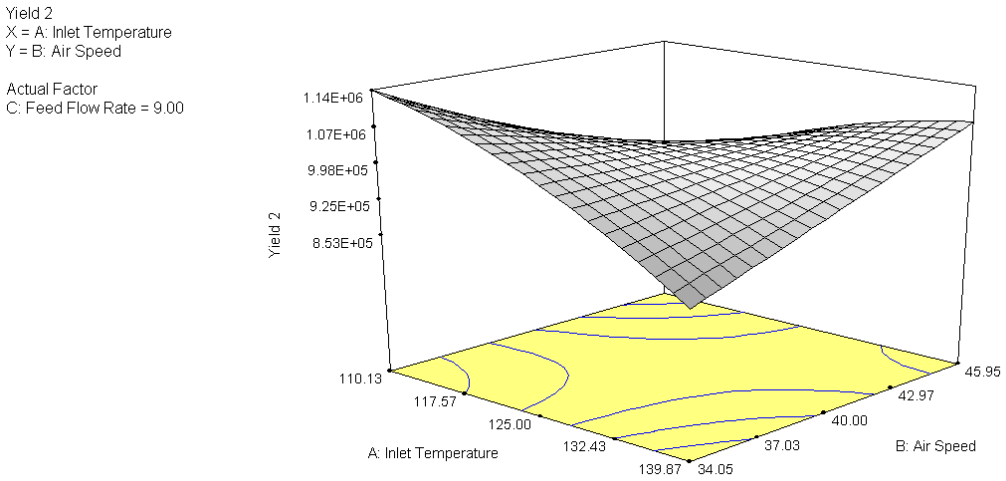


Figure 5.5 The interaction of inlet temperature and air speed at a constant feed flow rate of 9 ml/min on the active ingredient yield (OBs/ml) of powders produced with Polysaccharide-WM.

Moisture content

The overall model ($F_{\alpha(0.05),\ (2),\ (19)} = 4.12$) for the moisture content of powders prepared using Polysaccharide-WM as a carrier can be defined by the following Response Surface reduced quadratic equation:

Moisture content (%) =

$$89.10 - 1.06\,(A) - 1.07\,(B) + 1.01\,(C) + 4.17 \times 10^{-3}\,(A^2) + 0.01\,(B^2) - 0.04(C^2)$$

(Equation 5)

The model terms in this model that significantly affect the moisture content of the powder are feed flow rate (C) ($F_{2,\ 19} = 11.34$, $P < 0.05$) and the square of inlet temperature (A^2) ($F_{2,\ 19} = 6.97$, $P < 0.05$). Air speed (B) did not significantly affect the moisture content ($F_{2,\ 19} = 1.46$, $P < 0.05$). The chance of the F-value of 4.12 occurring due to noise is 1.54 %.

Equation 5 and the Cuboidal graph (Figure 5.6) show that the moisture content of powders prepared using Polysaccharide-WM as a carrier is lowest (3.23 %) at the model term combination A+, B+, C-. However, this cuboidal graph only illustrates the effect of high and low values of the model terms. Figure 5.7 gives a better factor range of the interaction between air speed (B) and inlet temperature (A) on moisture content (%). According to Figure 5.7, at a constant feed flow rate of 9 ml/min, the highest moisture content is produced at a combination of a low inlet temperature and low air speed. However, the centre of the contour graph illustrates that a low moisture content is produced at the midpoints of air speed and inlet temperature, and the interaction between a high inlet temperature and low air speed, or vice versa increases the moisture content.

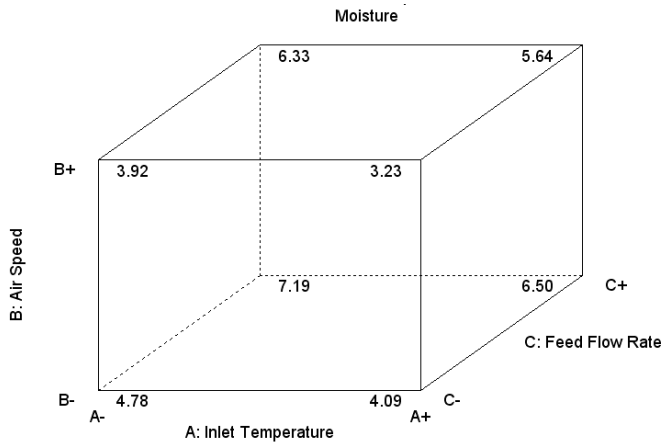


Figure 5.6 Cuboidal graph of the effect of the three factors on the moisture content of the powders formed with Polysaccharide-WM.

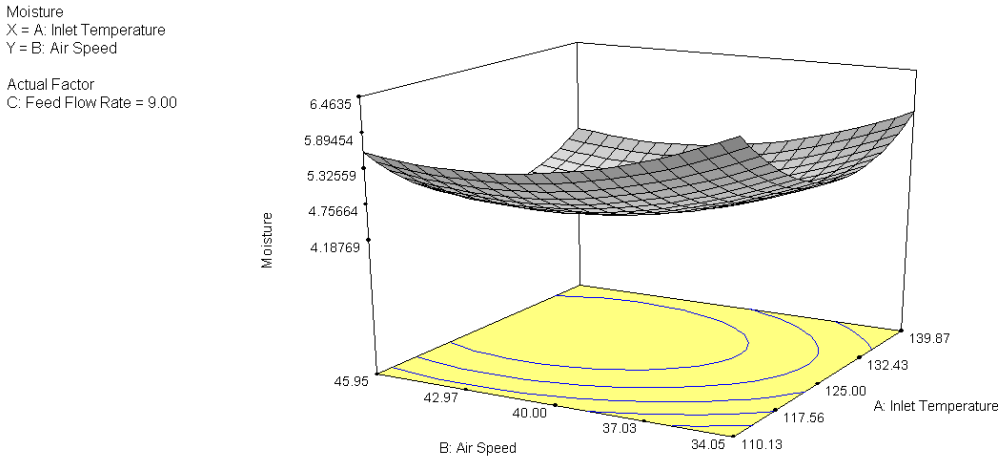


Figure 5.7 The effect of air speed and inlet temperature on the moisture content of powders produced with Polysaccharide-WM at a feed flow rate of 9 ml/min.

5.4.4 Microbial load of the powders

The initial microbial load of the crude suspension that was spray-dried with the polysaccharides contained a mean total aerobic count of 8.51 log cfu/ml. The bacterial species detected in this crude extract were *Bacillus* (log 6.07 cfu/ml), *Enterococcus* (log 8.4 log cfu/ml) and mould (3.26 log cfu/ml).

The polysaccharide-type was the only variable that significantly affected the total aerobic counts of the powders ($F_{1, 39} = 70.96$, $P < 0.05$). The Polysaccharide-MS powders contained a total aerobic count of 2.3 log cfu/ml and the Polysaccharide-WM powders 2.5 log cfu/ml. Only *Bacillus* species were detected in the wettable powders after spray-drying.

5.5 DISCUSSION

Spray-drying Polysaccharide-WM displayed more effects of the interactions between the model factors than Polysaccharide-MS with the same set of model terms. Cuboidal graphs were used to illustrate the effect of the three factors on the three responses, namely powder yield (mg/ml); active ingredient yield

(OBs/mg); and moisture content (% of moisture in the powder). Each plane of the cube represents high and low values of a particular variable and from this, the effect of the combination of factors on the response can be determined.

When Polysaccharide-MS was used as a carrier, the greatest powder yield (mg/ml) was obtained at a high inlet temperature, high air speed and low feed flow rate. This corresponds to a study which found that an increase in temperature increases the encapsulation yield and encapsulation efficiency of lycopene (Shu et al., 2006). An increase in inlet temperature was also found to increase the powder yield of a spray-dried fruit from Amazonia (açai) and was said to occur due to the increased heat energy, leading to a more efficient mass transfer process (Tonon et al., 2008).

An increase in the outlet temperature was found to occur with higher inlet temperatures and lower feed flow rates. This was also found in a study on the effect that spray-drying conditions have on spray-dried insulin, and was attributed to the fact that more heat energy is supplied at these settings (Ståhl et al., 2002).

The general trend in biopesticide development is to obtain a powder with the highest possible concentration of active ingredient without compromising the insecticidal activity (Burges and Jones, 1998b). The Response Surface linear model showed that none of the three model terms significantly affected the active ingredient yield (OBs/mg) when Polysaccharide-MS was used as a carrier. Since all three factors did not significantly affect the active ingredient yield in this case, the conditions for the highest powder yield should be considered to produce an optimal powder.

The percentage of moisture in the Polysaccharide-MS powders ranged from 3.51 to 8.66 %. Powders with a low moisture content are formed when the temperature gradient between the atomized feed and the drying air is greater, which results in a greater driving force for water evaporation (Tonon et al., 2008). It has been reported that NPV powders containing a moisture content of below 10 % retained

their insecticidal activity much better than those with a moisture content above 10 % (Tamez-Guerra et al., 2002). Burges and Jones (1998a) recommend the moisture content of a dry powder to be 5 % or less for good storage of the product. It is also known that some organisms die if they become too dry (Burges and Jones, 1998b).

On the other hand, a powder that contains too much moisture could result in a reduction in the powder yield (mg/ml) because it will stick to the walls of the spray-dryer glassware, therefore not falling into the collection bottle. Drying aids can be added to suspensions to reduce the amount of powder sticking to the walls of the spray-dry chamber (Langrish et al., 2007), which may result in higher powder yield. The optimal conditions for powder yield for the Polysaccharide-MS powder would give a powder containing a moisture content of approximately 4 %, which is satisfactory in terms of both Tamez-Guerra et al. (2002) and Burges and Jones, (1998a).

The models for the Polysaccharide-WM powders show more of an interaction between the combinations of the factors because the responses are described with quadratic equations rather than linear ones as is the case for the Polysaccharide-MS powders. The cuboidal graphs for the Polysaccharide-WM powders show that a high powder yield (mg/ml) was obtained at a high inlet temperature, high air speed and low feed flow rate. The optimal conditions for the active ingredient yield (OBs/mg) did not follow a linear response and the greatest active ingredient yields can be obtained at either a combination of low inlet temperature and air speed or a high inlet temperature and air speed. The moisture content for all runs was between 1.28 and 7.52 %.

The cuboidal graphs for both polysaccharides showed that the highest powder yield (mg/ml) occurred at the factor combination A+, B+, C-. This factor combination was the same for the lowest moisture content of the powders. This effect was also found in a study of spray-dried ß-galactosidase, where powders

with the highest yield, were also the powders with the lowest moisture content (Broadhead et al., 1994).

The spray drying process decreased the microbial load of the suspensions that were fed into the spray-dryer four-fold for both polysaccharides. The Polysaccharide-MS powders contained a statistically significant lower total aerobic count than that for Polysaccharide-WM powders. No moulds were detected in the spray-dried powders, except for one sample, which indicates that moulds contaminating the suspension will more than likely be eliminated during the spray-drying process. The same applies to the *Enterococcus* species isolated in the crude HearSNPV extract, which were not detected in the spray-dried powders. However, the *Bacillus* species survived the spray-drying process, possibly due to their spores. Although the inlet temperature of the spray-dryer is high (between 100 and 150 °C), the time that the sample is exposed to this temperature is extremely short. The effect of the outlet temperature on the microorganisms being spray-dried is more important to consider than the inlet temperature because the encapsulated particle centre will have the same temperature as the outlet temperature (Tamez-Guerra, 2000a). The outlet temperature for the spray-dry runs ranged from 52 to 85 °C for both polysaccharide carriers. Spores of *Bacillus* species can survive 80 °C for 30 minutes as indicated by the procedure to determine the spore count of a sample, thus spores are expected to survive the spray-drying process.

The outlet temperatures of previous studies have been reported to be between 60 – 75 °C for spray-dried baculovirus biopesticides (Arthurs and Lacey, 2004; Behle et al., 2003; Tamez-Guerra et al., 2000b; Tamez-Guerra et al., 2002). Thus a combination of the model factors that result in an outlet temperature in this range should give a viable active ingredient. According to the results, the optimum spray-drying conditions for Polysaccharide-WM that resulted in the highest powder and active ingredient yield as well as an outlet temperature between 63 and 76 °C was an inlet temperature of 130 °C, an air speed dial setting of 45 and a feed flow rate of 9 ml/minute. According to the predictive models obtained, these

settings would give a powder with a moisture content of 4.8 %, a powder yield of 337.5 mg/ml and an active ingredient yield of 8.0 x 10^5 OBs/mg. The optimum spray-drying conditions for Polysaccharide-MS were similar to those for Polysaccharide-WM with an inlet temperature of 130 °C, an air speed dial setting of 45 and a feed flow rate of 5.4 ml/minute. According to the predictive models obtained for Polysaccharide-MS, these settings would give a powder with a moisture content of 4 %, a powder yield of 110.9 mg/ml and an active ingredient yield of 1.0 x 10^6 OBs/mg.

These results are similar to the spray-drying conditions used to spray-dry baculoviruses in other studies at an inlet temperature ranging between 100 to 125 °C and a feed flow rate of 8 ml/minute when lower inlet temperatures were used and 18 – 20 ml/min when higher inlet temperatures were used (Arthurs and Lacey, 2004; Behle et al., 2003; Tamez-Guerra et al., 2000b; Tamez-Guerra et al., 2002).

The insecticidal activity of the powders in the current study was not assessed, which is an important aspect in evaluating the design of the spray-drying conditions. However, inactivation of the OBs of the insecticidal agent is not expected because the spray-drying conditions can be set to result in a suitable outlet temperature and the time that the occlusion bodies are exposed to the outlet temperature is extremely short. Bioassays could be carried out in future baculovirus biopesticide product development studies to ensure that the active ingredient did not become inactivated during the spray-drying process.

This multi-factorial optimization study was beneficial in terms of establishing the best combination of the numerical factors used to give the optimum spray-drying conditions. This data can be used as the foundation for further optimization of a commercial baculovirus biopesticide formulated as a wettable powder.

CHAPTER 6

Concluding discussion

Since the African cotton bollworm, *Helicoverpa armigera*, is such an important economical pest, and has developed resistance to various chemical insecticides, its biological control is of great importance (Srinivas et al., 2004).

It has been shown that baculoviruses have the ability to provide long term control of lepidopteran insect pests. This is due to their high pathogenicity, narrow host range, persistence in the environment, safety to vertebrates and plants and because they can be applied to crops with conventional spraying equipment (Cory and Hails, 1997; Khetan, 2001; Miller et al., 1983). However, several environmental factors can inactivate baculoviruses namely, temperature, leaf surface exudates, sun, wind and rain (Jones et al., 1997).

Additives can be used to protect baculoviruses from some of these environmental factors, provide storage stability and maximize application efficiency (Jones et al., 1997; Tamez-Guerra et al., 2002). Developing a *H. armigera* single nucleocapsid nucleopolyhedrovirus (HearSNPV) biopesticide that has good storage stability and increased protection from adverse environmental factors, while still retaining its insecticidal activity, may improve its potential as a commercial biopesticide for the control of *H. armigera*. The focus of this project was to develop and evaluate wettable powder and liquid formulations of HearSNPV for the control of *H. armigera.*

Before carrying out formulation work, it was decided to evaluate the baculovirus in its unformulated form in greenhouse trials to determine whether it has potential as a biopesticide. Although formulation ingredients can improve the efficacy of a biopesticide, an unformulated product showing poor activity is not likely to become effective on formulation and hence it was decided to determine the efficacy of the biopesticide in its unformulated form. The results showed a

significant reduction in *H. armigera* infestation per plant seven days post treatment, which illustrated the potential of the biopesticide and suggested that further work using novel formulations was justified. It also provided baseline data of the application rates required to control first instar *H. armigera* larvae on tomato plants.

The most practical way to produce a baculovirus biopesticide is through the *in vivo* propagation of the virus in insect hosts, followed by extraction and purification of the occlusion bodies (OBs) once the insect has died from infection. However, it is too expensive to produce a highly purified product on an industrial scale, thus microbial contaminants in the insects are carried through to the final product (Kelly and Entwistle, 1988).

The effect that the inoculum purity has on the microbial load of the final product is important in biopesticide production, since it could affect the shelf-life of the final product (Burges and Jones, 1998a; Burges and Jones, 1998b). In the current study, the rate zonal centrifugation method produced a highly purified inoculum of the virus and although this method resulted in a low microbial load to OB ratio of 4.85×10^{-6}:1, it is not practical for the production of virus on the large scale required for commercial production (Kelly and Entwistle, 1988).

The effect of a crude HearSNPV inoculum (containing a microbial load to OB ratio of 0.05:1) on the microbial load of insect homogenates was compared to the purified inoculum as well as a sterile distilled water (control) inoculum. Although a higher microbial load was observed for homogenates prepared with the crude inoculum, this was only statistically higher than the sterile distilled water inoculum. Two species of bacteria, namely *Bacillus* and *Enterococcus*, were isolated from these samples. *Enterococcus* is commonly found in insects (Lasa et al., 2008; Xiang et al., 2006) and it is thought that the *Bacillus* isolate was introduced through ingredients used to prepare the insect diet. From this, we concluded that the *in vivo* propagation of the baculovirus could be carried out using a crude inoculum, without significantly affecting the microbial load of the

final product; however, the microbial load increased on storage. This suggests that measures to minimize the microbial load early in production should be taken such as maintaining aseptic techniques throughout the production process. In addition, formulation ingredients as well as refrigeration could be used to inhibit an increase in the microbial load of the biopesticide on storage.

Although the unformulated suspension of HearSNPV was effective in greenhouse trials, the formulation of the product could enhance its storage stability. Tamez-Guerra et al. (2002) state that storage stability is fundamental for commercialization of a biopesticide, thus the HearSNPV needed to be developed further.

Two additives were selected after an initial screening process to prepare liquid suspensions of HearSNPV, namely Xanthan Gum (XG) and Instant Starch (IS). These were compared to an Unformulated (UF) suspension. These additives are commonly used in the food industry as thickening and gelling agents but have not been used in agricultural products. The beneficial properties of these additives, such as the high viscosity formed, added storage stability, lack of syneresis and simple use made them attractive and simple additives for the biopesticide being developed.

The XG additive proved promising. The median lethal dose increased for all the samples tested on storage, and this is consistent with the literature for samples stored at room temperature for 6 months (Lasa et al., 2008). The UF suspension displayed very similar results to the XG suspension, except that it settled upon storage. This settling may be problematic in terms of an aesthetically pleasing product; however, it would be more cost effective to produce. The results obtained from the accelerated storage study demonstrated that the XG suspension displayed the greatest shelf-life in terms of the median lethal dose, microbial load, pH and low temperature storage stability. The suspension formulated with IS was not found to be suitable because its insecticidal activity was completely lost on storage.

The bacteria isolated from the suspension formulations were identified as *Bacillus* and *Paenibacillus* species. These bacteria are different to the ones isolated in the work done on the effect of the inoculum purity on the microbial load of insects used for the *in vivo* propagation of the baculovirus. It is thought that they may have been introduced by new insect diet ingredients obtained just before the suspension formulation study was carried out.

Processing baculoviruses into wettable powders to improve their shelf-life as well as their persistence in the field has been reported by several authors (Arthurs and Lacey, 2004; Behle et al., 2003; Tamez-Guerra et al., 2000b; Tamez-Guerra et al., 2002). Although previous work on spray-dried baculoviruses reported the parameters used, Tamez-Guerra et al. (2000a) noted that optimal spray-drying conditions need to be determined by each facility since they vary according to pathogen type, formulation ingredients and spray-dryer. Due to the large number of factors involved in the spray-drying process, it was decided to determine the best combination of factors for the baculovirus wettable powder using a multi-factorial optimization strategy.

The three responses that were analysed (powder yield, active ingredient yield and moisture content) narrowed down the best combination of factors (inlet temperature, air speed and feed flow rate) that should be used to produce a wettable baculovirus powder in our laboratory. The two polysaccharides tested gave similar results and future spray-drying work with HearSNPV and the polysaccharides evaluated in this study should use an inlet temperature of 130 °C, an air speed dial setting of 45, and a feed flow rate ranging between 5 and 9 ml/minute. The microbial contamination of the powders decreased on spray-drying, which will be beneficial for the shelf-life of the product.

Wettable powder and suspension formulations of a HearSNPV biopesticide were developed in this study. The UF and XG suspensions remained stable under accelerated storage conditions and although their median lethal dose increased over time, they retained insecticidal activity. Spray-drying parameters were

identified for the production of baculovirus powders, and these powders were evaluated according to their physical properties.

Future studies could evaluate methods to reduce the microbial contamination in the biopesticide. Sterilizing insect diet ingredients contaminated with spores, without diminishing the nutritional content, would prevent contaminants being carried through the production process to the final product. The use of preservatives in the biopesticide could also be a measure of minimizing contaminating bacteria.

The use of phagostimulants could be investigated to encourage the larvae to ingest a larger dose of the biopesticide in the field. In addition, further testing of the persistence of the biopesticide in the field, such as adhesion to plants during rainfall and retention of insecticidal activity after exposure to UV could be carried out.

In respect of the wettable powders, optimizing the spray-drying parameters to produce larger granules could enhance the user friendliness of this biopesticide.

The suspensions and wettable powders developed in this project will form the foundation for the development of commercial biopesticides for the control of the African cotton bollworm.

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