Evaluation of different media formulations on spore production and toxicity of *Bacillus thuringiensis* subsp. *aizawai*

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This work is dedicated to my parents, Rosy and Lucas Moloto and my siblings Karabo & Moeder. I thank them for their patience, encouragement and support throughout my entire studies. Thank you for this wonderful opportunity. May God Almighty bless you.

To God be the Glory. "For with men these things are impossible, but with God all things are possible. Luke 1:37"

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PREFACE

The experimental work discussed in this dissertation was conducted during the period of February 2010 to March 2012 in the School of Environmental Sciences and Development, Microbiology, North-West University, Potchefstroom Campus, Potchefstroom, South Africa. The study was conducted under the supervision and co-supervision of Dr. S. Claassens and Dr. J.J. Bezuidenhout. I hereby declare that this is my own original, unaided work and has not been presented for any other degree, examination or research purpose. The reference style used in this dissertation is Harvard style.

Perseverance Sehaole

March 2012

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SUMMARY

One of the major constrains in beekeeping is damage caused by the greater wax moth (*Galleria mellonella*). Due to problems and concerns regarding the use of chemical insecticides, biopesticides have been introduced to manage problematic insects. For the biological control of greater wax moth, *Bacillus thuringiensis* subsp. *aizawai* (*B.t.a)* has been widely researched and used. Large scale production of *B.t.a* as a biopesticide is expensive because of the high cost of commercial media formulations. In this study, three raw materials, horse manure, soy meal and maize chops, were selected for evaluation as raw materials for media production based on their economical feasibility and availability and compared to two commercial media (Tryptone soy broth and bactopeptone). Sixty three raw material formulations and two commercial media were evaluated in terms of spore yield and toxicity towards *G. mellonella*. For each formulation the spore and vegetative cell yield was determined using direct microscopy. From the results obtained from the 63 formulations, the 12 highest spore yielding formulations were selected and re-evaluated for confirmation of the results. Spores obtained from the 12 highest spore yielding formulations were used in a bioassay to determine their toxicity against *G. mellonella* larvae and the protein concentration were also determined. Spore morphology was investigated for the 12 highest spore yielding formulations. Formulations containing 3.75 g horse manure, 5 g maize chops and with soy meal ranging between 1.25 g and 3.75 g yielded the highest spore yields. In particular the combination of 3.75 g horse manure, 5 g maize chops and 1.25 g soy meal had the highest spore yields but also resulted in 100 % mortality against *G. mellonella* larvae within 3 days. The yields obtained were also significantly higher than those for the commercial media against which they were evaluated. As such it was concluded that the

selected formulation represents a feasible alternative to commercial media for the production of *B.t.a*.

Key words: *Bacillus thuringiensis* subsp. *aizawai*; bioassay; commercial media; endospores; *Galleria mellonella*; raw materials formulations

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
B.t	*Bacillus thuringiensis*
B.t.a	*Bacillus thuringiensis aizawai*
Bacto	Bactopeptone
CPD	Critical Point Dried
Cry	Crystal
Cyt	Cytolytic
G. mellonella	*Galleria mellonella*
K_2HPO_4	Dipotassium hydrogen orthophosphate anhydrous
KH_2PO_4	Potassium di-hydrogen orthophosphate
N/A	Nutrient agar
PCR	Polymerase Chain Reaction
rpm	Rotation per minute
SEM	Scanning Electron Microscope
Subsp	Subspecies
TSB	Tryptone soy broth
US	United states
USDA	United States Department of Agriculture

CHAPTER 1- INTRODUCTION

1.1 Background

Insects have been the cause of great financial loss in agriculture due to destruction of crops (Weeden *et al*., 2007). Thus, insecticides where introduced globally for the control of insect pests. The use of insecticides was considered successful, until it was discovered that they cause environmental pollution and their continuous administration lead to insect resistance (Prabakaran and Balaraman, 2006). Biological pesticides (biopesticides) where then introduced to manage insects and to overcome the problems associated with chemical insecticides. Different biopesticides have high specificity towards different insects. This results in low negative impacts to the environment and minimises the risk of insects developing resistance. *Bacillus thuringiensis* is an example of a valuable biopesticide because it is highly pest specific and generally non-injurious to beneficial insects (Guerrero *et al*., 2007). Other advantages include no appalling odour upon application (Weeden *et al*., 2007), higher degradability and a higher level of safety for non-target organisms (Obeidat, 2008).

1.2 Problem statement

Honey bee pollination in agriculture is of extreme importance to increase the yield of a variety of crops. Therefore, farmers cannot depend on feral honey bees that happen to nest near crop fields. Beekeeping and migratory beekeepers have thus become popular as one of the components of highly productive mixed farming systems (Swamy *et al.*, 2009). Unfortunately, this venture is not without obstacles for the beekeepers and infections of beehives play an important role in the viability of beekeeping.

Larvae of the greater wax moth (*Galleria mellonella*), which belong to the Lepidoptera family, live in and destroy honeycombs when they feed on honey and pollen (Saraswathy and Kumar, 2004). This results in financial losses because the larvae tend to destroy the broodcomb and

eventually the beehives. The female greater wax moth can lay 300-600 eggs in a cluster on the comb and after hatching the larvae start to form tunnels and move from comb to comb (Knoxfield, 2006). These wax moth larvae are harmless to honey bees but rather harmful to the productivity and of the combs.

One of the generally used biocontrol agents in agriculture is *B. thuringiensis.* It is a gram positive, aerobic, spore forming parasporal bacterium, which produces an intracellular proteinaceous crystal that has insecticidal, antitumor and immune response-enhancing properties (Bulla *et al*., 1977; Helassa *et al.,* 2009). Different subspecies are specific in their toxicity to certain insects and studies have shown that the application of *Bacillus thuringiensis* subsp. *aizawai* (*B.t.a*) is effective against *G. mellonella* (Liu and Tzeng, 2001). When wax moth larvae ingest the spores of *B.t.a,* the environment of the midgut promotes crystal solubilisation. This will cause damage to the insect midgut and lead to feeding inhibition, septicaemia and death (Brar *et al.,* 2007). While *B.t.a* is effective as a biocontrol agent, widespread application in this context is often challenged by production and formulation costs associated with culture media and processes. The economic implications of these culturing conditions on a large scale thus prevent the implementation of this biocontrol agent in agriculture. Studies using various raw materials have demonstrated potential to replace costly refined commercial media for the production of spores of this bacterium (Brar *et al*., 2006; Devi *et al*., 2005), but question remains about the effectiveness of using these materials.

In this study, media formulations using three different raw materials were evaluated as possible cheaper alternatives to commercially available refined media. When choosing an alternative medium it is important that it should not only be effective in producing a high spore yield, but that the toxicity of the spores should be at least comparable to those cultured on refined commercial media and should retain toxicity to the target insect.

1.3 Aims and objectives

The aim of this study was to evaluate media formulations of raw materials which can be used as alternatives to current refined commercial formulations for the production of spores of *B.t.a.*

Specific objectives included:

- Using different combinations of soybean meal, horse manure and maize chops to create media formulations that can be used to produce spores from *B.t.a.*
- Evaluating the spore yield obtained for each of the alternative media formulations against the refined commercial media formulations, TSB and bactopeptone.
- Comparing the spore toxicity of the spores cultured on the alternative media formulations to those cultured on the refined commercial media formulations, TSB and bactopeptone, using *G. mellonella* larvae.
- Characterising and comparing spores by describing spore position and parasporal structure of spores obtained from the various alternative and refined commercial media.

1.4 Chapter layout

1. Introduction
2. Literature review
3. Materials and methods
4. Results and discussion
5. Conclusions and recommendations
6. References

CHAPTER 2 - LITERATURE REVIEW

2.1 The role of honey bees in agriculture & industry

African honey bees (*Apis mellifera scutellata*) are native to sub-Saharan Africa. They are about the same size, shape and colour as European honey bees (*Apis mellifera ligustica*) (Hodgson and Roe, 2009). Currently, honey bees are largely domesticated and are not only used to produce hive products, such as honey, wax and royal jelly, but are primary species used for pollination of agricultural crops globally (Parker *et al.*, 2010). Two types of worker honey bees are found in the hives: those which stay in the hive to do the necessary work and those which go out regularly to collect food and water (May, 1969).

These honey bees produce wax which is rich in nutrients, pollen and honey (Knoxfield, 2006). Wax is one of the most useful products of honey bees. It is used in the pharmaceutical industry to manufacture antibiotics, dentistry, cosmetics and in moisturisers as a source of vitamin E. It is also used to make oil colours, wax papers, cards, wood polish and shoe polish. The final product of the bees known as honey, has been used as an antiseptic agent, as a sensitive skin moisturiser, to ease stomach ache, to treat ulcers and to cure seasonal allergies (Jafari *et al*., 2010).

An important aspect in the agricultural industry for which bees are used, is pollination (Knoxfield, 1994). Pollination is one of the most important factors in fruit production and it is carried out with the use of pollen (Attridge, 1917). Pollen is transferred from one place to the other by clinging to the hairs on the bee's body and is carried in this way to various flowers, on which some of the pollen sticks. Many types of commonly grown fruit require pollination in order to bear satisfactory marketable crops. It is an essential requisite for the stone and pome fruit industries (May, 1969).

2.2 The wax moth (*Galleria mellonella*)

G. mellonella, also known as the wax moth, is classified into the order Lepidoptera and is a major pest in the beekeeping industry (Laridon, 2006). Among the several natural enemies of bees, the wax moth causes some of the greatest losses to the beekeeping industry (Swamy *et al.*, 2009). It is one of the most devastating and economically challenging pests (Jafari *et al*., 2010). The larvae of the wax moth live in and destroy honeycombs when they feed on honey and pollen (Saraswathy and Kumar, 2004). Since they attack the wax, they form tunnels in it and cause wax destruction and contamination of the honey (Knoxfield, 2006).

2.3 *Galleria mellonella* life cycle

The wax moth life cycle is made up of four stages: eggs, larvae consisting of several instars, pupae, and the adult moth. Adult moths are greyish to purplish brown, have dark markings and lead-coloured tips on the forewings, pale brownish or yellowish hind wings and a wingspan of approximately 3.18 cm. The wings are held over the back when at rest (Jafari *et al*., 2010). Male and female wax moths mate and the female will lay 300-600 eggs in a cluster on the comb within four to five days (Knoxfield, 2006)**.** To lay eggs, the adult female moths fly at night and deposit masses of eggs on unprotected honeycombs and in the crevices of the honeycomb. Larvae hatch within seven days, crawl onto the comb, and begin their feeding activity on the honey, pollen and wax produced by honeybees (Shimanuki, 1980). The larval stage may only take 20 days when food and temperature are ideal (Somerville, 2007). When the larva reaches maturity it starts to pupate in a coarser silk with which it makes a cocoon that is papery in texture and very strong. The colour of the cocoon is normally white. Many cocoons may be clustered together and after pupation the adult moth emerges to complete the life cycle (Bronskill, 1961).

2.4 The effect of the wax moth on honeycomb

The wax moth affects honeycombs by feeding on them and form tunnels from one honeycomb to the other. Newly-hatched larvae feed on honey and pollen and then burrow into pollen storage cells, later extending their tunnels to the midrib of the comb as they grow (Somerville, 2007). They feed on the wax of the honeycombs in the larval stage and cause severe damage. These honey bee enemies weaken the colony, decreasing its value for honey production (Swamy *et al.*, 2009). Combs which are subject to attack are those in storage areas, those which are weak and have dead colonies. Strong and vigorous colonies do not usually suffer severe damage but there are two critical periods during the year when damage caused by larval activity is often severe. The first period is mid to late spring when colonies weakened by the stress of winter are susceptible to moth destruction. The second period is late summer, after the spring honey flow (Warren and Huddleston, 1962). To control this problem of the wax moth, biopesticides such as *B. thuringiensis* have been used.

2.5 *Bacillus thuringiensis* as a biopesticide

B. thuringiensis is a Gram positive, aerobic, entomopathogenic, spore forming parasporal bacterium, which produces an intracellular proteinaceous crystal that has insecticidal, antitumor and immune response-enhancing properties (Bulla *et al*., 1977; Helassa *et al.,* 2009). It occurs naturally in various ecological niches such as soil, plant surfaces and dust from stored products and insects (De Maagd *et al*., 2003). It forms asexual reproductive spores, which enables it to survive in adverse conditions (Swadener, 1994) and produces different insecticidal toxic proteins in parasporal crystals during the stationary phase of its growth cycle.

B. thuringiensis produces crystalline inclusion (parasporal crystal) bodies during the sporulation of its growth cycle. These parasporal crystals consist of proteins, which exhibit highly toxic insecticidal activity (Çetinkaya, 2002). These proteins are toxic to a wide variety of insects

which attack economically important crops (Baig and Mehnaz, 2009). These bacteria have a tendency of utilising a large variety of protein toxins to help them invade, infect, and finally kill their hosts, through their action on the insect midgut (De Maagd *et al*., 2003). Spores are the dormant stage of the bacterial life cycle during unfavourable environmental conditions (Obeidat, 2008). Unlike vegetative cells, they have higher resistance to external factors such as mechanical force, temperature, starvation, ionizing radiation, chemical solvents, detergents, hydrolytic enzymes, pH extremes, antibiotics and dehydration. Spore structure is different from the structure of normal vegetative bacterial cells (De Vries, 2006; Errington, 2003). The longevity that spore products can have is a direct consequence of their high resistance towards external factors (Wolken *et al*., 2003). Spore structure is constituted of different and unique parts which serve different functions (Figure 2.1). The core consists of cytoplasm and cytoplasmic matter. Around the core is the inner membrane where germination receptors are located. The inner membrane is surrounded by the cortex which has a thick cell wall and prevents core dehydration. This is followed by the coat which helps the spore to resist a variety of chemicals and ultraviolet rays and the exosporium which covers the spore coat (De Vries, 2006).

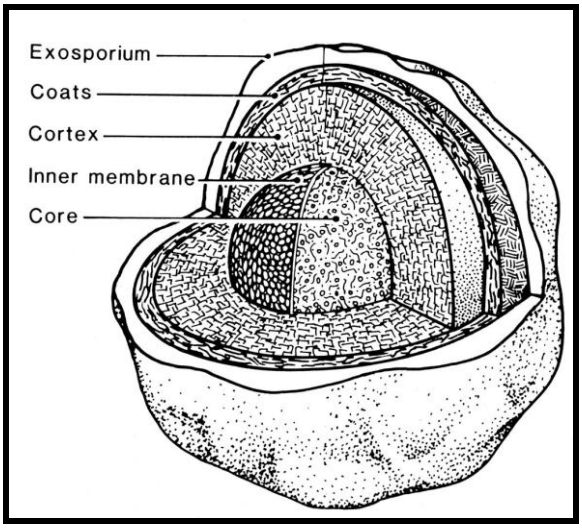

Figure 2.1: The internal structure of a bacterial spore (De Vries, 2006).

Spore forming bacteria have a spore enhancement system for sporulation. The main stimulus for sporulation is starvation (Errington, 2003). The sporulation process takes place when a

spore forming bacteria produces large crystalline inclusions (Liu and Tzeng, 2001). Since sporulation and germination in bacilli are dependent on the nutritional status of the organism, a study of the nutritional requirement of *B. thuringiensis* is important for defining the control mechanisms which regulate spore and parasporal crystal formation. Sporulation involves a unique process of asymmetric cell division, followed by engulfment of the smaller cell and eventually leads to the sacrifice of the original bacterial cell for the production of a single spore (De Vries, 2006; Errington, 2003). Certain amino acids support growth, sporulation and crystal formation of *B. thuringiensis.* A lower concentration of cystine or cysteine promotes growth, sporulation and crystal formation (Rajalakshmi and Shethna, 1980). During the sporulation process protein synthesis takes place whereby protease is included in the process. If sufficient intracellular proteases are not produced it will result in formation and release of immature spores. Furthermore, it could have an impact on the formation of crystal proteins (Brar *et al*., 2007).

The sporulation process occurs concurrently with the parasporal crystal formation. The parasporal crystal formation is composed of seven stages (Figure 2.2) which includes axial filament formation, forespore septum formation, engulfment of forespore, spore wall development, transformation of spore nucleoid and spore maturation (Bechtel and Bulla, Jr. 1976). There are two types of toxins produced during parasporal crystal formation. These are referred to as parasporal inclusions, namely the Cry (crystal) and Cyt (cytolic) toxins. Cry toxin exhibit a toxic effect to a target organism and Cyt toxin exhibits hemolytic activity (Brar *et al.,* 2006).

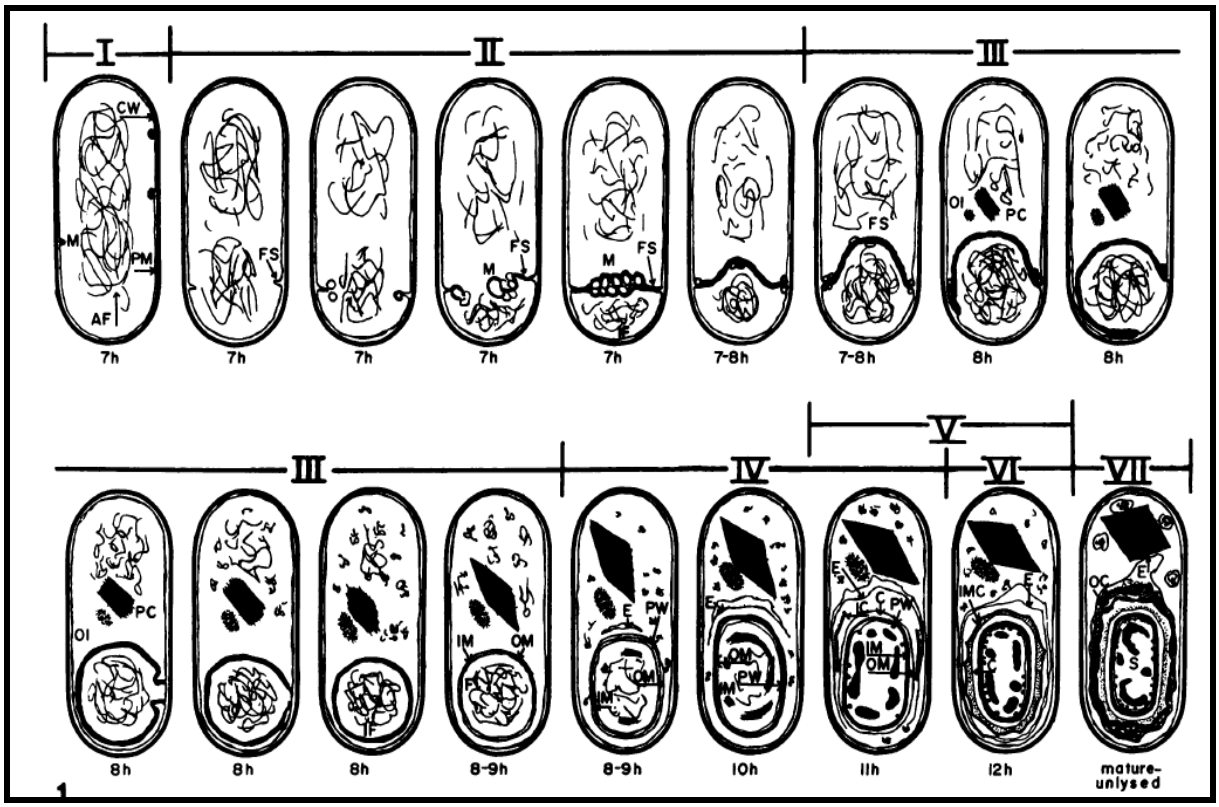

Figure 2.2: Stage I, axial filament formation; stage II (7 to 8 h), forespore septum formation involving mesosomes; stage III (8 to 9 h), engulfment with mesosome involvement; stages IV to VI (9 to 12 h), formation of exosporium, primordial cell wall, cortex, and spore coats accompanied by transformation of the spore nucleoid; stage VII (post-12 h), spore maturation (Bechtel and Bulla, Jr. 1976).

According to Lambert and Peferoen, Berliner conducted a research were he noticed the specificity of *B. thuringiensis* when he found that the bacterium was toxic to Mediterranean flour moth larvae (*Anagasta kuehniella*) but not to mealworm larvae (*Tenebrio molitor*). “In 1928, a pioneering project was started in Europe with spore-crystal preparations of *B. thuringiensis* produced in small-scale laboratory fermenters for control of the European corn borer (*Ostrinia nubilalis*), a severe corn pest. The earliest commercial production of *B. thuringiensis* began in France in approximately 1938, under the trade name Sporeine. During the 1960s, several industrial formulations of *B. thuringiensis* were manufactured in the United States (US), Soviet Union, France and Germany and used with various degrees of commercial success. A major advance occurred when the first collection of *B. thuringiensis* strains was assembled by Howard Dulmage and Clayton Beegle of the United States Department of Agriculture (USDA). By the end of the 1970s, the two US producers of *B. thuringiensis* were Abbott Laboratories and

Sandoz, Inc. Various improved formulations of a limited number of strains have been developed by these companies. Until the 1970s, it was generally believed that *B. thuringiensis* was only active against Lepidoptera. In 1977, Goldberg and Margalit isolated *B. thuringiensis* subsp. *israelensis*, from a mosquito breeding pond in the Negev desert (Israel) which is highly toxic to mosquito and black fly larvae. In 1983, Krieg and co-workers isolated another *B. thuringiensis* subsp*. tenebrionis,* from dead mealworm pupae. This subspecies is highly toxic to elm leaf beetle (*Agelastica alni)* and Colorado potato beetle (*Leptinotarsad ecemlineata*) larvae. These two findings stimulated the organisation of screening programmes in search of *B. thuringiensis* with activities against other economically important insects and during the 1980s the foundation of new biotechnology companies whose core business consisted of the development of novel *B. thuringiensis* based insecticides (Lambert and Peferoen, 1992)."

In 1995, *B. thuringiensis* was used in the potato industry and *B.t*-potato became the first *B.t*-crop to be commercialised. These potatoes were engineered to express the Cry3A protein for protection against Colorado potato beetles (*Leptinotarsa decemlineata*). In 1996, *B.t*-cotton was released to protect cotton against tobacco budworm, *Heliothis virescens* (Fabricius), and pink bollworm (*Gelechia gossypiella*). In 1996 *B.t*-maize was developed and commercialised, containing Cry1Ab protein (De Maagd *et al.*, 1999).

Currently, there is worldwide interest in environmentally friendly, non-toxic and biodegradable bioinsecticides produced by the soil bacterium *B. thuringiensis* for use in agricultural crops (corn, soybean, wheat, cotton, rice, vegetables, fruits, etc.), forestry, floriculture, and disease vector control (Rowe and Margaritis, 2003). The major advantage of this biopesticide is that it is essentially non-toxic to humans, domestic animals and wildlife (Cranshaw, 2008).

While *B. thuringiensis* can be used in agriculture, its effectiveness is affected and reduced by a number of factors. It can be reduced by environmental conditions, the narrow host range, wash-

off by rain and dew that dilutes the microbe dose on the plant (Navon, 2000). *B. thuringiensis* based products tend to have a shorter shelf life than others (Cranshaw, 2008). Furthermore, the range of non-target species that have been found to be susceptible to sunlight degradation and direct toxic action of *B. thuringiensis* has decreased (Zhou *et al.,* 2005; Hunsberger, 2000). Since *B. thuringiensis* does not kill rapidly, users may incorrectly assume that it is ineffective a day or two after treatment. If production of *B. thuringiensis* exceeds consumption by insect larvae and degradation by the soil microbiota, the toxin could accumulate to concentrations that may (1) constitute a hazard to non-target organisms and (2) result in the selection and enrichment of toxin-resistant target insects (Zhou *et al.,* 2005).

For *B. thuringiensis* to be effective it must be ingested by insects during their feeding stage of development (Figure 2.3) and this will lead to death. The crystal dissolves in the intestine of susceptible insect larvae to become activated toxins (Hofte and Whiteley, 1989). When insect larvae ingest the spores, the environment of the midgut promotes crystal solubilisation and enzymatic cleavage to yield an active toxin which is processed by midgut proteases (Aronson *et al.,* 2001; Zhou *et al*., 2005). The major proteases of the lepidopteran insect midgut are trypsin-like or chymotrypsin-like (Schnepf *et al*., 1998). For several *B. thuringiensis* toxins, specific high-affinity binding sites have been demonstrated to exist on the midgut epithelium of susceptible insects. This explains the extreme specificity of these proteins (Hofte and Whiteley, 1989). When the toxin binds to the epithelium it disturbs the ion pump which will cause pore formation. The pore forming capacity of the delta-endotoxin is affected by pH and is directly correlated with toxin potency (Saraswathy and Kumar, 2004). These pores allow ions and water to pass freely into the cells, resulting in swelling, lysis and midgut damage and eventually death due to starvation (Pigott and Ellar, 2007). The larval membrane consists of phosphatidylcholine, sphingomyelin and phosphatidyl-ethanolainine and the crystal toxin released by *B. thuringiensis* binds to the cell membrane components, therefore this leads to loss of membrane integrity and cytolysis (Liu and Tzeng, 2001).

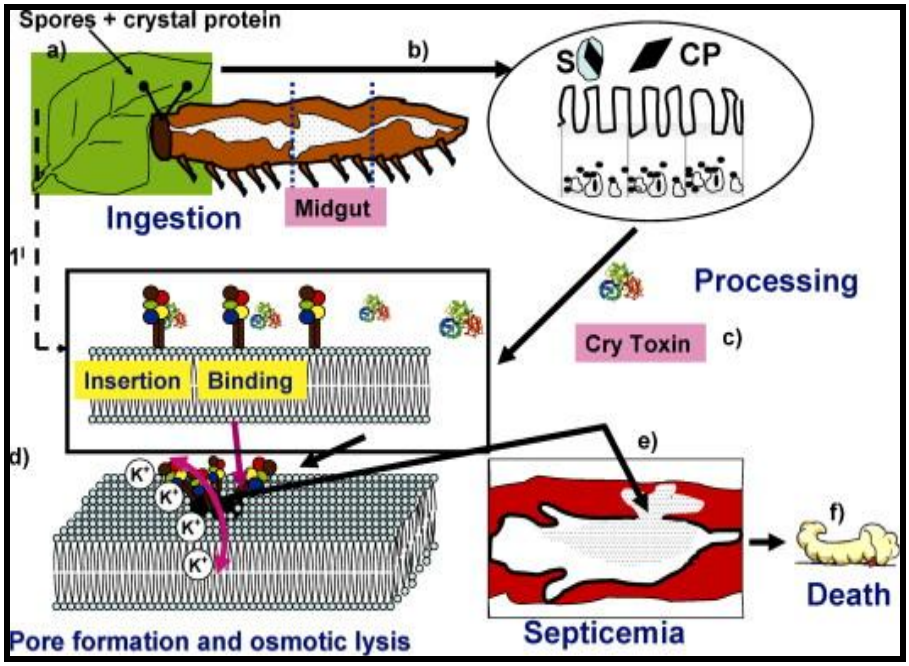

Figure 2.3: Mode of action for *Bacillus thuringiensis:* (a) ingestion of spores and crystal toxin by larvae; (b) crystal protein and spores exert force on the larval midgut; (c) proteolytic processing of *Cry* protoxins into active toxin, toxins bind to specific receptors on the epithelium and further insert into cell membrane; (d) *Cry* toxins disturb ionic pump, cause pore formation resulting in osmotic lysis; and (e) midgut damage leading to feeding inhibition, septicemia by spores (f) the larvae eventually die because of starvation (Brar *et al.,* 2007)

2.6 Specificity of *Bacillus thuringiensis* towards the wax moth

The toxic action of biopesticides is often specific to a single group or species of insects, and this specificity means that most microbial biopesticides do not directly affect beneficial insects (including predators or parasites of pests) in treated areas (Liu and Tzeng, 2001). *B. thuringiensis* have different strains and subspecies and several isolates of *B. thuringiensis* have been used as biopesticides against different insect orders such as Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera and Acari (Baig and Mehnaz, 2009). Different subspecies are specific in their toxicity to certain insects and studies have shown that the application of *B.t.a* is an effective measure against *G. mellonella* (Liu and Tzeng, 2001). When wax moth larvae ingest the spores of *B.t.a,* the environment of the midgut promotes crystal solubilisation. This will cause damage to the insect midgut and lead to feeding inhibition,

septicaemia and death (Brar *et al.,* 2007). The toxicity spectrum of *B. thuringiensis* subspecies is determined by the different crystal genes (cry genes) carried by their strains, and by the encoded proteins (cry proteins) (Quesada-Moraga *et al.*, 2004). There are different primary strains (Table 2.1) which can be used to manage insects.

Table 2.1: Primary strains of *Bacillus thuringiensis* used in managing insects.

Strain	**Target**	**Reference**
Bacillus thuringiensis subsp. *aizawai*	• Targets wax moth larvae/caterpillars • Active against Lepidopteran pests	• Cranshaw, 2008 • Rukmini *et al*., 2000 • Smith *et al*., 1996
Bacillus thuringiensis subsp. *CrylAb delta-endotoxin*	• Targets many Lepidopteran larvae • Targets the larvae of tobacco hornworm	• Cranshaw, 2008 • Francis and Bulla, 1997
Bacillus thuringiensis subsp. *Cry3Bb1 variant*	• Targets corn rootworm larvae	• Cranshaw, 2008 • Duan *et al*., 2002
Bacillus thuringiensis subsp. *israelensis*	• Used against larvae of mosquitoes, black flies and fungus gnats	• Donovan *et al*., 1988 • Held *et al*., 1990 • Ibarra and Federici, 1956
Bacillus thuringiensis subsp. *japonensis*	• Targets larvae of scarab beetles	• Cranshaw, 2008 • Mashtoly *et al*., 2010 • Suzuki *et al*., 1992
Bacillus thuringiensis subsp. *kurstaki*	• Kills only leaf and needle feeding caterpillars • Targets moths and is used commercially to protect cabbages	• Cranshaw, 2008 • Donovan *et al*., 1988 • Swadener, 1994
Bacillus thuringiensis subsp. *kyushuensis*	• Active against mosquitoes larvae	• Held *et al*., 1990 • Knowles *et al.,* 1992 • Yu *et al*., 1991
Bacillus thuringiensis subsp. *medellin cyt1Ab1*	• Active against mosquitoes larvae	• Escobar *et al.,* 2000 • Thiéry *et al*., 1998 • Wirth *et al*., 2001
Bacillus thuringiensis subsp. morrisoni	• Causes disease in moth & butterfly caterpillars	• Swadener, 1994 • Yu et al., 1991
Bacillus thuringiensis subsp. *sphaericus*	• Active against mosquito larvae of the genus *Culex*	• Thanabalu *et al*., 1992 • Thiéry *et al*., 1998 • Trisrisook *et al*., 1990

Strain	Target	Reference
Bacillus thuringiensis subsp. *tenebrionis*	• Targets larvae of leaf beetles • Active against Coleoptera	• Crecchio and Stotzky, 1998 • Tapp and Stotzky, 1995 • Thomas *et al*., 2000
Bacillus thuringiensis subsp. *tolworthi*	• Used in maize crop protection	• Capalbo *et al*., 2001 • Herbert *et al*., 1971 • Huber-Lukac *et al*., 1986

2.7 Culturing and enumeration of *Bacillus thuringiensis* spores

For the sporulation process to yield abundant numbers of spores, mass culturing is recommended. The cost of *B. thuringiensis* production through existing fermentation technology and commercial media formulations is high. The use of *B. thuringiensis* based biopesticides is limited due to these high production costs. It was observed that it may become feasible if affordable ways for mass production were developed (Valicente and Mouráo, 2008). A less expensive medium for culturing of *B. thuringiensis* can be facilitated by using cost effective raw materials to develop an appropriate growth medium (Prabakaran and Balaraman 2006). This medium should be feasible for use in a large-scale production, it should be effective in producing a high spore yield and the toxicity of the spores should be at least comparable to those cultured on refined commercial media and should retain toxicity to the target insect. Recently, studies using various raw materials have demonstrated potential to replace costly refined commercial media for the production of spores of *B. thuringiensis* (Brar *et al*., 2006).

For an increase in spore yield, media formulations have to be rich in nutrients, thus in studies of various media standardised for production of *B. thuringiensis* showed that sources of carbon and nitrogen, basal salts such as magnesium, sodium, calcium, and good aeration are the primary growth requirements. Various agricultural and industrial by-products, such as maize glucose, soybean flour, peanuts, cane molasses and liquid swine manure are rich in carbon and nitrogen and may be used as raw materials in biopesticide production (Devi *et al*., 2005).

With mass culturing of *B. thuringiensis,* different raw materials, commercial media and techniques are implemented. For example, culturing has been carried out using a submerged process to explore the feasible production of endotoxin. In semi-solid fermentation processes, solid materials are used to grow microorganisms. This technique is inexpensive for the production of *B. thuringiensis* spore on a large scale (Capalbo, 1994).

Different techniques are used to study bacteria. Staining techniques can be used to assist in the characterisation and identification of bacteria. Gram staining is one of the techniques which are widely employed in bacteriological identification. It is a differential staining procedure because it divides bacteria into two classes; Gram negative and Gram positive. Gram positive bacteria retain the crystal violet and stain purple; whereas after lipid interaction with ethanol, Gram negative bacteria lose their crystal violet and stain pink (Prescott *et al.*, 2005). Another staining procedure that can be used is endospore staining. It is a structural stain used to determine whether the bacteria contain endospores. Bacteria in the genera of Bacillus form an exceptionally resistant structure capable of surviving for long periods in an unfavourable environment. This dormant structure is called an endospore since it develops within the cell. Endospores can be situated in three different positions; centrally, terminally and sub-terminally. The endospores always stain green and vegetative cells are pink (Prescott *et al.*, 2005).

In order for stained bacteria to be identified, light microscopes are used to determine endospore count and vegetative cell count. A direct microscopy count method can be used to count the number of endospores and vegetative cells directly from the microscope. In this technique one can clearly distinguish between endospores and vegetative cells. To detect the appearance of spores and crystals, the phase contrast microscope is applied. A rapid and simple method also used, is to stain the crystal protein with Coomassie brilliant blue before observation. In such morphological studies, differences between crystals, spores and vegetative cells are easy to observe (Sharif and Alaeddinoglu, 1988).

Another method of enumeration is the use of plate counts. A disadvantage of this technique is that only the number of bacterial colonies can be counted directly. The technique allows the counting of endospores or vegetative cells, thus it is always advisable to perform microscopy counts after staining pure colonies.

2.8 Spore yield and toxicity

Media formulations affect spore yield and toxicity. Therefore, not all raw materials will give equal results. Raw materials have been used in previous studies to investigate their ability to produce spores and toxin. In a study by Prabakaran and Balaraman (2006), raw materials which were used included soybean flour, ground cake flour and wheat bran. Defatted groundnut cake was used to evaluate spore yield and toxicity production of *B. thuringiensis*. Defatted groundnut cake was used as the first nitrogen source and grain flour, soybean, and defatted milk powder as the second nitrogen source for the bulk production (Prabakaran and Balaraman, 2006). The study found that raw materials could provide a very useful substrate for the production of spores of *B. thuringiensis* subsp*. israelensis.*

Coconut water was used in another study were it was incorporated in various fermentation media for *B. thuringiensis* production. It was found to be unsuitable for both growth as well as sporulation. This shows that not all raw materials can enhance sporulation (Fernandez *et al*., 1974) and are therefore not suitable to be used as media formulation.

Sludge from wastewater treatment plants has also been used for *B. thuringiensis* growth, sporulation and toxicity production. The toxicity level was found to be very low. In some instances the sludge yielded slow growth of *B. thuringiensis*, but this was advantageous because it will give the spores enough time to mature properly (Vidyarthi *et al*., 2002).

In a study (Devi *et al*., 2005) conducted on wheat bran as a raw material for alternative media, it was found that the wheat bran was a good source of fibre, vitamins and minerals with a low level of saturated fat. Although the carbohydrates and proteins present in wheat bran have the potential to serve as carbon and nitrogen sources, they are not readily available and thus need to be supplemented to permit initial bacterial growth. Thereafter, bacteria can break down wheat bran to utilise available carbon, nitrogen, and essential salts. Wheat bran and molasses where used in a cost-effective semi-solid fermentation for mass production of *B. thuringiensis*, and also to standardise the method of aeration to support optimal growth.

To determine the toxicity of *B. thuringiensis* spores produced on media formulation from raw materials, bioassays can be conducted on the target organism. In various studies (Devi *et al*., 2005; Yadav *et al*., 2001; Zhuang *et al*., 2011), larvae of the target organism was exposed to spore produced from raw material formulations. After exposing the larvae to the toxin, larval mortality was scored up to 5 days of exposure (Valicente and Mouráo, 2008).

CHAPTER 3 – MATERIALS AND METHODS

3.1 Organism

Bacillus thuringiensis subsp. *aizawai (B.t.a)* was used in this study for spore production. The strain which was used was 6100, obtained from DSMZ (Deutsche Sammlung van Mikroorganismen und Zellkulturen GmbH) in Germany. This strain is effective as a biopesticide against *G. mellonella* (Cranshaw, 2008).

3.2 Resuscitation and staining of organism

For the resuscitation of *B.t.a,* tryptone soy broth (TSB) was used and for *B.t.a* maintenance nutrient agar (N/A) was used. TSB was prepared in 1000 ml distilled water then dispensed into two 50 ml Erlenmeyer flasks. The mouth of the flask was covered with a cotton plug and foil for autoclaving at 121˚C for 15 minutes. After sterilisation, TSB was used to resuscitate *B.t.a.* For purification of colonies, N/A was prepared in 1000 ml distilled water and autoclaved at 121˚C for 15 minutes. It was then poured into sterile petri dishes and kept at 4˚C until further use.

Resuscitation of *B.t.a* was accomplished by inoculating a lyophilised pellet into an Erlenmeyer flask containing TSB. The culture was incubated overnight at 37˚C on a rotary shaker at 150 rpm to resuscitate the bacteria. Streak plates were performed on N/A plates and incubated at 37˚C for 24 hours to confirm purity of colonies. Gram staining was performed to confirm purity and identify contamination of *B.t.a* (Bergey and Holt, 1994). Endospore staining was performed to determine the presence of endospores (Mormak and Casida, 1985).

3.3 Media preparation and screening of media formulations

Media formulations of raw materials were evaluated against two commercial media formulations TSB and bactopeptone. These commercial media were used because of their ability to culture *Bacillus* and yield high endospore production. Each medium was prepared in 1000 ml of distilled water and dispensed in Erlenmeyer flasks, then sealed with a cotton plug and foil for sterilisation. After sterilisation, a sterile loop of *B.t.a* was inoculated in these commercial media formulations.

Three types of raw materials in varying combinations were evaluated for production of *B.t.a*. The raw materials included maize chops, soy meal and horse manure. Maize chops are chopped maize kernels without a germ and provides source of nitrogen. In this study, soy meal is a soy based formulation which is high in vitamins (Vit C, D, E, B_3, B_6, B_2, B_1, biotin and pantothenic acid) and minerals such as calcium, iodine, iron, magnesium, selenium, choline and zinc. The soy meal also serves as a source of protein and carbohydrates. The horse manure was obtained from horses which followed a diet of teff and lucerne, which are high in fibre. Teff is rich in bran and germ which contain high amounts of calcium, protein, carbohydrates and fibre (Vaughan and Geissier, 1997). It also contains phosphorous, iron, copper, aluminium, barium, and thiamine. Lucerne is high in protein contents and digestible fibre (Katić *et al.,* 2009).

In terms of the media formulations, horse manure provided a primary source of nitrogen and carbon, while soy meal and maize chops provided nitrogen as a secondary source as well as proteins and amino acids (Woodworth *et al.,* 2000 and Kim *et al.,* 2010). All the raw materials are rich in carbon and nitrogen.

In total, 63 media formulations made up of varying combinations of the three raw materials were evaluated (Table 3.1). Initial combination contained varying amounts of raw materials in the following amounts: 10 g; 2.5 g; and 1.25 g. after optimisation through evaluation and comparison of spore yields obtained on raw material formulations versus commercial media, the following amounts were used in the different combination: for soy meal 0.0, 1.25; 2.5 and 5.0 g; for horse manure and maize chops 0.0; 2.5; 3.75 and 5 g. Each formulation also contained different amounts of potassium di-hydrogen orthophosphate (KH_2PO_4) and dipotassium hydrogen orthophosphate anhydrous (K_2HPO_4) which served as buffers.

All raw material formulations were soaked in distilled water in an Erlenmeyer flask and filtered through a muslin cloth to remove all the insoluble solid particles present (Prabakaran and Balaraman, 2006). Every flask was autoclaved at 15 psi for 25 min (Devi *et al*., 2005).

Nutrient broth was prepared and used to resuscitate *B.t.a* by inoculating a loop full organisms and incubating for 48 hours. Resuscitated *B.t.a* culture (0.1 ml) was inoculated in each raw material formulation and commercial media formulation then incubated for one hour (Endospore development was started in Nutrient broth with resuscitation, then further development was carried out in different raw materials) and 50 ml of each media formulation was centrifuged in a falcon tube for 5 min at 2000 rpm. From the falcon tube 0.01 ml of each raw material formulation and commercial media were used to perform endospore staining. Vegetative cells and endospores were enumerated using a direct microscopy count method (Prabakaran and Balaraman, 2006). All experiments were conducted in triplicate.

Table 3.1: Summary of raw material combinations to make up 63 formulations for media preparation

Formulation	Maize chops (grams)	Horse Manure (grams)	Soy meal (grams)	Formulation	Maize chops (grams)	Horse Manure (grams)	Soy Meal (grams)	Formulation	Maize chops (grams)	Horse Manure (grams)	Soy Meal (grams)
1	0.00	0.00	1.25	23	2.50	2.50	5.00	45	5.00	3.75	1.25
2	0.00	0.00	2.50	24	3.75	2.50	0.00	46	5.00	3.75	2.50
3	0.00	0.00	5.00	25	3.75	2.50	1.25	47	5.00	3.75	5.00
4	2.50	0.00	0.00	26	3.75	2.50	2.50	48	0.00	5.00	0.00
5	2.50	0.00	1.25	27	3.75	2.50	5.00	49	0.00	5.00	1.25
6	2.50	0.00	2.50	28	5.00	2.50	0.00	50	0.00	5.00	2.50
7	2.50	0.00	5.00	29	5.00	2.50	1.25	51	0.00	5.00	5.00
8	3.75	0.00	0.00	30	5.00	2.50	2.50	52	2.50	5.00	0.00
9	3.75	0.00	1.25	31	5.00	2.50	5.00	53	2.50	5.00	1.25
10	3.75	0.00	2.50	32	0.00	3.75	0.00	54	2.50	5.00	2.50
11	3.75	0.00	5.00	33	0.00	3.75	1.25	55	2.50	5.00	5.00
12	5.00	0.00	0.00	34	0.00	3.75	2.50	56	3.75	5.00	0.00
13	5.00	0.00	1.25	35	0.00	3.75	5.00	57	3.75	5.00	1.25
14	5.00	0.00	2.50	36	2.50	3.75	0.00	58	3.75	5.00	2.50
15	5.00	0.00	5.00	37	2.50	3.75	1.25	59	3.75	5.00	5.00
16	0.00	2.50	0.00	38	2.50	3.75	2.50	60	5.00	5.00	0.00
17	0.00	2.50	1.25	39	2.50	3.75	5.00	61	5.00	5.00	1.25
18	0.00	2.50	2.50	40	3.75	3.75	0.00	62	5.00	5.00	2.50
19	0.00	2.50	5.00	41	3.75	3.75	1.25	63	5.00	5.00	5.00
20	2.50	2.50	0.00	42	3.75	3.75	2.50	64	Tryptone soy broth (TSB)		
21	2.50	2.50	1.25	43	3.75	3.75	5.00	65	Bactopeptone		
22	2.50	2.50	2.50	44	5.00	3.75	0.00				

Note: Formulation 64 and 65 serves as control (commercial media formulations)

Total number of raw material media formulations which were tested was 63. From the 63 media formulations, 12 with the highest spore yield were re-evaluated to confirm spore yield (Table 3.2) and conduct toxicity bioassay on *G. mellonella* larvae.

Raw material formulations where filtered through muslin cloth to remove the insoluble particles and residues where pressed down for complete extraction (Devi *et al.,* 2005). Endospores and vegetative cells where enumerated as previously described. After direct microscopy count, the supernatant in the falcon tube was poured out and re-centrifuged to remove excess water from the pellet. The pellet was lyophilised before use for the toxicity test.

Table 3.2: Twelve highest spore yielding formulations

Formulation	Maize chops (grams)	Horse manure (grams)	Soy meal (grams)
16	0.00	2.50	0.00
25	3.75	2.50	1.25
32	0.00	3.75	0.00
38	2.50	3.75	2.50
41	3.75	3.75	1.25
45	5.00	3.75	1.25
46	5.00	3.75	2.50
47	5.00	3.75	5.00
52	2.50	5.00	0.00
53	2.50	5.00	1.25
56	3.75	5.00	0.00
61	5.00	5.00	1.25

3.4 Bioassay for determination of toxicity

A spore toxicity test was conducted to determine the toxicity of spores derived from various media formulations against the larvae of *G. mellonella*. The spore crystal complex produced in each of the 12 media formulations (Table 3.2) derived from different formulation of raw materials were assayed against laboratory-reared *G. mellonella*. Fifth instar larvae were fed an artificial diet. The food source was composed of 200 ml glycerine, 200 ml honey and 500

grams of fine (stage 1) Nestlé baby cereal (Sammataro and Avitabile, 1996). The honey and glycerine were warmed to 35º C and then mixed together, followed by 500 grams of fine Nestlé baby cereal, covered and left to stand overnight in the fridge. The food source was fed to the larvae by placing at the bottom of a container with the larvae.

For evaluation of the toxicity of the various formulations against *G. mellonella* larvae, lyophilized spore powder was suspended in 50 ml of a 0.02 % Tween-80 solution.

$$1 \text{ ml Tween-80} + 9.9 \text{ ml distilled water} = 1\ \%$$

$$\frac{0.02\ \% + 100 \text{ ml distilled water}}{1\ \%} = 2 \text{ ml}$$

Therefore 2 ml of the solution was added into 100 ml of distilled water and used to suspend spore powder.

The solution was allowed to soak for 15 minutes after which the suspension was vortexed and filtered through muslin cloth to remove residues (Devi *et al.,* 2005). Correction for the amount of spores applied was not done for each formulation; rather the total yield per formulation was applied during the toxicity assay.

Suspensions with *B.t.a* were sprayed on the food source and larval mortality was scored. Different formulations contained different amounts of spores (Table 3.3) and 5 ml of each spore suspension was sprayed on the food source. The food source was placed in a petri dish with holes in the lid for ventilation. Containers with water were placed around the petri dishes in a closed but ventilated wooden box to humidify the air. Each petri dish included three larvae and the experiment was conducted in triplicate (n=9). Larval mortality was scored up to five days after larval infection by *B.t.a* (Valicente and Mouráo, 2008).

Table 3.3: Amount of spores sprayed on the food source per ml and 5 ml

Formulation	Spores/ml	Spores/5ml
16	558.37	2791.84
25	1021.24	5106.22
32	1428.42	7142.08
38	900.55	4502.76
41	1599.51	8050.54
45	2454.96	12274.82
46	935.04	4675.18
47	598.16	2990.79
52	230.77	1153.87
53	856.78	4283.92
56	831.05	4155.27
61	904.53	4522.64

3.5 Toxin estimation

This is the estimation of toxin in the crystal proteins. Toxin estimation was performed to determine the amount of spores applied on the larvae (Table 3.3). The correlation of protein concentration was analysed by linear regression (appendix B) using concentrations of crystal proteins versus protein absorbance. The protein concentration assay was conducted in triplicate.

For extraction of the crystal protein, 0.1 g of lyophilised powder containing *B. thuringiensis* spores was placed in a 50 ml centrifuge tube. To this, 10 ml of 1 M sodium chloride solution was added to remove extracellular and cell-associated metalloproteases. The suspension was mixed thoroughly and centrifuged at 1000 rpm for 10 min. The supernatant was discarded and the pellet was washed twice with sterile distilled water. To this pellet, 10 ml of 100 mM sodium chloride solution was added (Lowry *et al*., 1951). The tubes were incubated for 2 hours at room temperature (25˚C) at 100 rpm. The suspension was then centrifuged and the supernatant used for estimation of the crystal protein employing the method of Lowry *et al*. (1951).

For the standard curve a series of tubes containing 0.125; 0.25; 0.5; 0.75; 1.0; 1.5 and 2.0 mg/ml stock solution were prepared. From the raw material formulation extract, 5 µl was added to 495 µl distilled water and 500 µl Bradford stock solution, mixed together and allowed to stand for 5 min. Analysis of toxin was determined by spectrophotometry at 595 nm.

3.6 Morphology

Morphology describes the structure of bacteria, spores and crystals. A morphology investigation was performed to determine whether the different media formulations affected spore and crystal structure. First, a *B.t.a* stain was performed to determine the presence of bacteria in the raw material formulation. The stain was performed using toluidine blue. A bacterial smear was prepared on a clean slide. Then the slide was dipped into toluidine blue for 1 min, rinsed with distilled water and dabbed dry with a paper towel. The stained slide was observed under a phase contrast microscope and photographs were taken (Prescott *et al.*, 2005).

Spore and crystal morphologies of the *B.t.a* strain were investigated by Scanning Electron Microscope (SEM) and Coomassie brilliant blue staining. Three raw material formulations (16, 45 and 53) were chosen as representatives of the 12 highest spore yielding formulations, based on their formulation composition. The aim is to have different formulations with different raw materials. Commercial media formulation (TSB) was also used for morphological analysis. After sterilisation of the media, *B.t.a* culture was inoculated in the raw material formulations and commercial media formulation and incubated for one

hour. After incubation, 50 ml was centrifuged in a falcon tube for 5 min at 2000 rpm and the supernatant was used for the SEM.

Bacteria produced from TSB and different raw material formulations were filtered through a 0.4 µm (13 mm) nuclepore filter with the use of a syringe. The bacteria were fixed with 70 % ethanol overnight, dehydrated in an ethanol series of 80 %, 90 % and 2X 100 % for 15-30 min. The samples were critical point dried (CPD) to replace ethanol with carbon dioxide. The samples were mounted on SEM stubs with double-sided carbon tape and they were coated with 20 nm gold/palladium (66/33 %). FEI quanta 200 Electronic SEM with high vacuum mode (10 KV) was used to analyse the results (Apaydin *et al*., 2008).

For Coomassie brilliant blue staining, smear was prepared on a clean slide. Then the slide was stained with Coomassie brilliant blue for 3 min, rinsed with distilled water and dabbed dry with a paper towel. The stained slide was observed under a phase contrast microscope and photographs were taken (Prescott *et al.*, 2005).

3.7 Statistical Analyses

Statistical analyses were performed and graphs were generated with the use of Statistica 10 (StatSoft Inc ©, 2011). A one-way breakdown analysis of variance, ANOVA, was performed, after which a Tukey's Honest significance test was performed to determine statistical significance between the various raw material formulations and commercial media formulations.

CHAPTER 4 – RESULTS AND DISCUSSION

4.1 Gram staining

The Gram staining technique was used to verify the purity of the *B.t.a* culture (Figure 4.1)

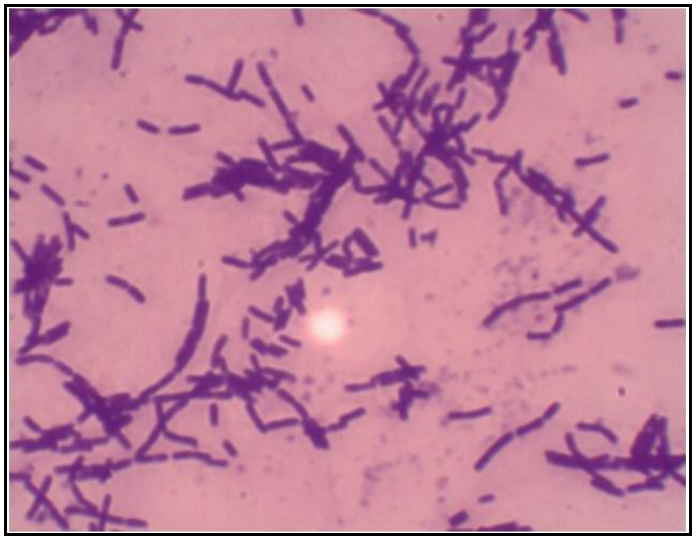

Figure 4.1: Photograph of a Gram staining taken at 1000x magnification with oil immersion to determine whether the cells were Gram positive or negative. *Bacillus thuringiensis* tested positive for the Gram stain because bacterial cells appeared to be dark purple and were confirmed as rod shaped bacilli.

The *B.t.a* culture tested positive for the Gram stain. Cells were rod shaped bacilli and the culture was not contaminated.

4.2 Endospore staining

During endospore staining, endospores stain green and vegetative cells pink (Figure 4.2). The *B.t.a* pure culture was stained to determine the presence of endospores. Endospores and vegetative cells were observed

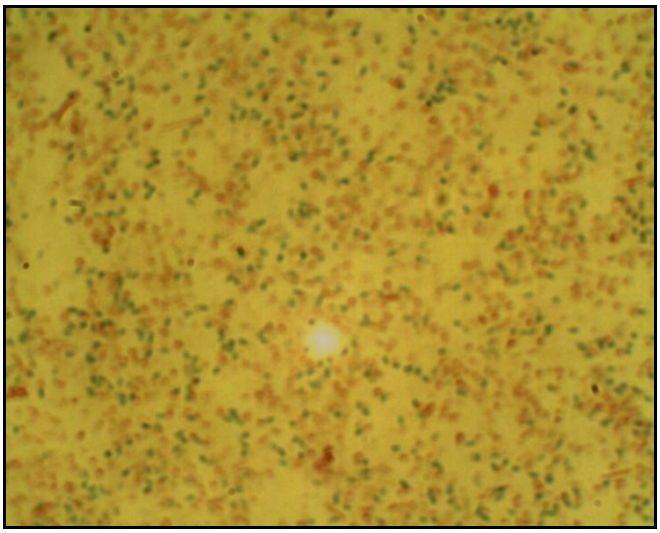

Figure 4.2: Photograph of an endospore stain taken at 1000x magnification with oil immersion to detect the presence of endospores. Green cells indicate endospores which were stained with malachite green and the pink/red cells are vegetative tissue of bacterial cells stained by Safranin.

4.3 Endospore count

After Gram staining and endospore staining were performed, endospore and vegetative cell enumeration was done and from this the percentage spore count was determined by means of direct microscopy count. Raw material formulations and commercial media formulations were compared to observe differences or similarities in the yields of endospores. Figure 4.3 shows the number of endospores obtained for 63 raw materials formulations as well as the two commercial media formulations (TSB and bactopeptone). Experiments were conducted in triplicate. Table 4.1 shows the homogenous groupings of the 12 highest spore yielding raw material formulations and commercial media formulations.

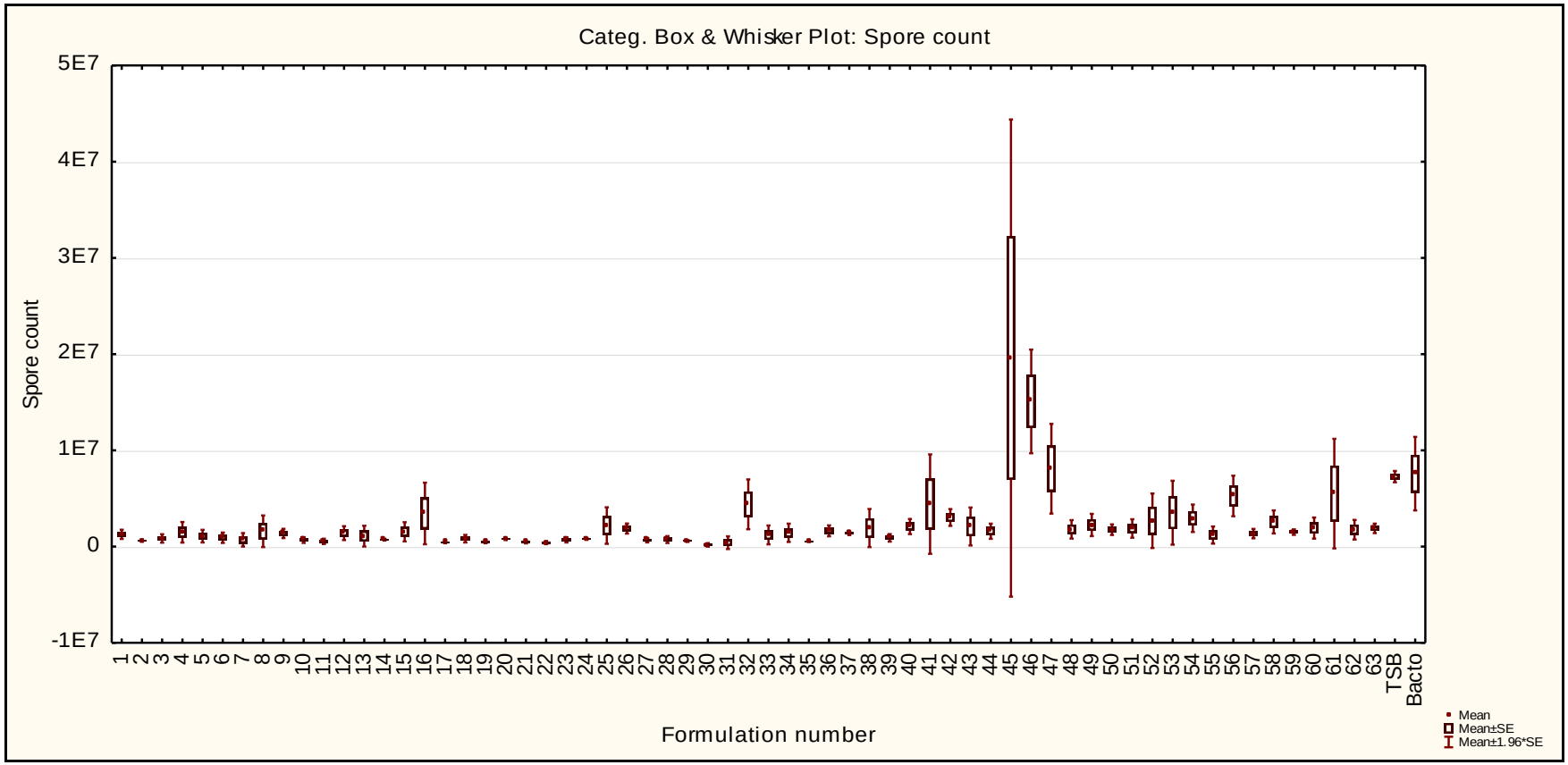

Figure 4.3: Box and whisker plot of endospore counts for the various raw material formulations and commercial media formulations (TSB and Bactopeptone) which were tested.

The highest spore count was observed for formulation 45 (3.75 g horse manure; 5 g maize chops and 1.25 g soy meal), followed by formulation 46 (3.75 g horse manure; 5 g maize chops and 2.5 g soy meal) and formulation 47 (3.75 g horse manure; 5 g maize chops and 5 g soy meal). When these are compared to the commercial media, it is clear that raw material formulations yielded higher spore counts. The results also indicated that media formulation plays an important role in spore yield. Commercial media formulations also yielded relatively high amounts of endospores. The mean maximum spore count differed significantly between media. The standard error of formulation 45 (3.75 g horse manure; 5 g maize chops and 1.25 g soy meal) was very high; this could have resulted from experimental error and therefore this formulation was included in the 12 formulations that were re-evaluated to confirm spore yield results.

Other authors showed variability with respect to *B.t* production using raw materials. Prabakaran and Balaraman (2006) found that soybean flour; groundnut cake powder and wheat bran performed better than commercial media formulations in terms of spore yield. For large scale production of *Bacillus thuringiensis* subsp*. israelensis* ground cake powder was used as first nitrogen source and soybean flour and wheat bran as second nitrogen source. The latter medium containing soy bean flour showed the highest spore count and toxicity. According to their study soy bean flour yielded the best results. Devi *et al.* (2005) used wheat bran and supplemented it with molasses and yeast extract. The combination was enriched with carbon and nitrogen sources which mainly came from the molasses and yeast extract and *B. thuringiensis* utilised these sources to sporulate. The spore count varied significantly and increased with the addition of molasses. However, spore counts were higher in the combination where both carbon and nitrogen sources were provided as molasses and yeast extract. In this study, those formulations which yielded the high spore counts were those with horse manure in them. The bran in the teff might have contributed to the higher spore yield

because bran is a source of carbon and nitrogen (Vaughan and Geissier, 1997). The amounts of soy meal and maize chops also have an influence on spore production because they are the nitrogen source (Hembry *et al*., 1973).

4.4 Vegetative cell count

Vegetative cell counts were performed to determine the number of vegetative cells produced in comparison to the endospores. Figure 4.4 shows the number of vegetative cells for the 63 raw material formulations as well as the commercial media formulations.

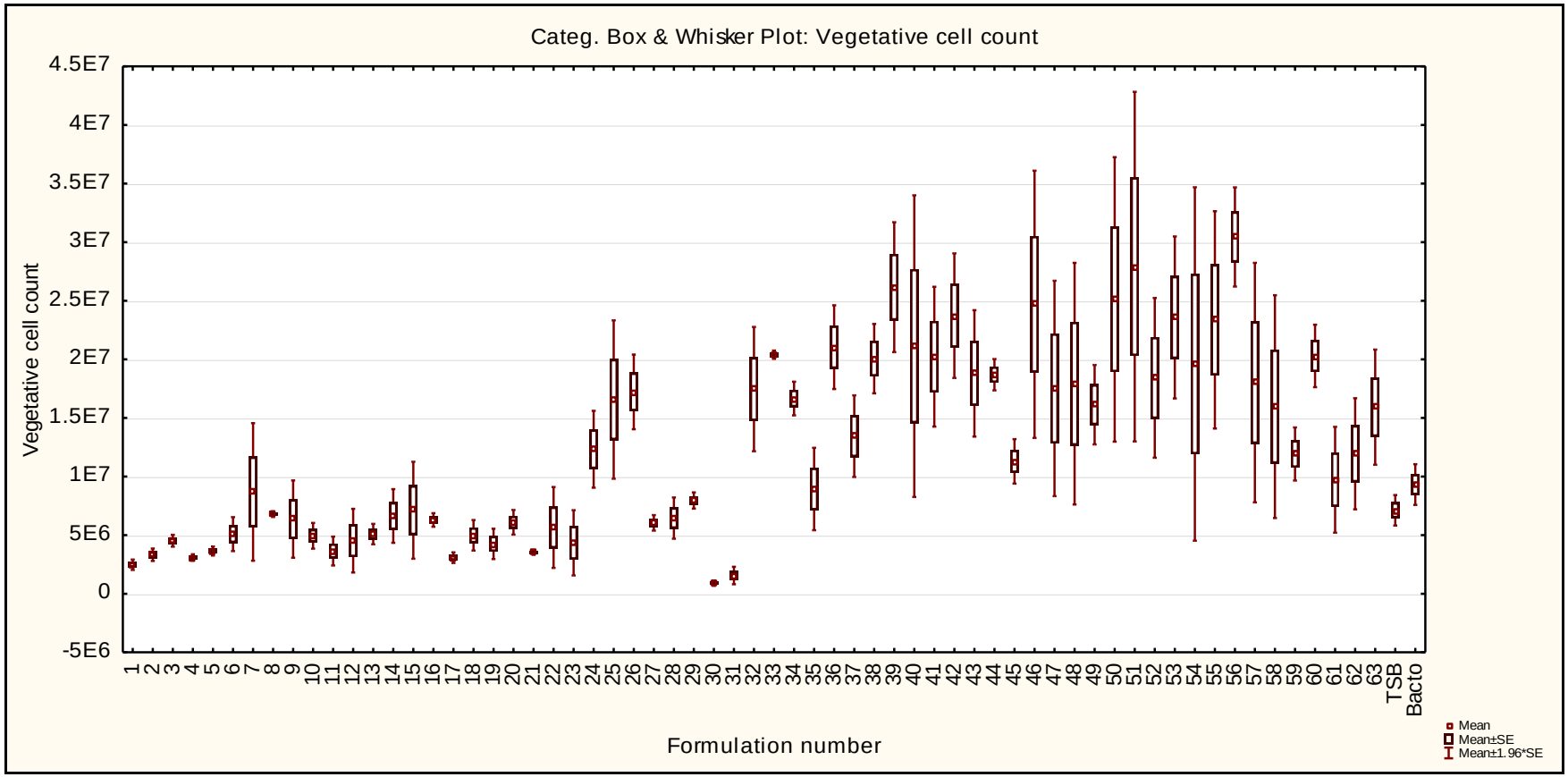

Figure 4.4: Box and whisker plot of vegetative cell count for the various raw material formulations and commercial media formulations (TSB and Bactopeptone) which were tested.

Raw material formulations which yielded high vegetative cell counts were those with 3.75 g and 5 g of horse manure, whereas formulations with 0 g and 2.5 g horse manure yielded lower amount of vegetative cells. Commercial media formulations also yielded the lower amount of vegetative cells when compared to formulations with 3.75 g and 5 g of horse manure, thus formulations with 0 g and 2.5 g horse manure performed the same as the commercial media. Formulation composition can influence the amount of vegetative cells produced.

From the results it is clear that 3.75 g and 5 g of horse manure produced the best results in terms of vegetative cell counts. The results also show that different raw material formulations had a variable influence on the amount of vegetative cells observed.

4.5 Spore percentage

A determination of the total count of spores and vegetative cells enables the calculation of spore percentage which is the ratio of spores to vegetative cells. Spore percentage allows a quantitative comparison of the ability of spores and vegetative cells and the effect that different raw materials or commercial media have on spores and vegetative cells (Cook and Lund, 1962). Figure 4.5 shows spore percentage obtained for 63 raw material formulations and two commercial media formulations. Results can be influenced because of media composition on spore yield.

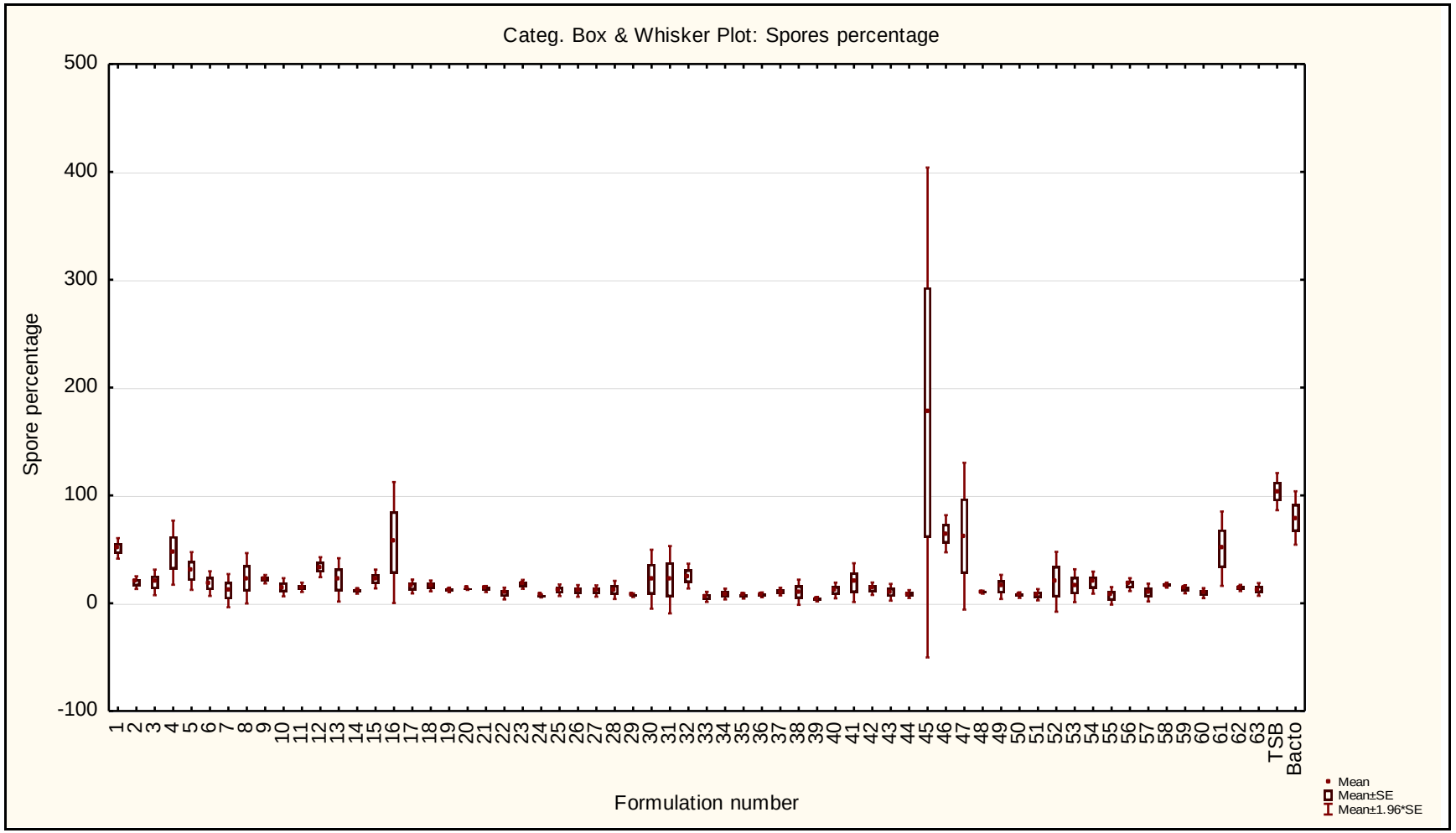

Figure 4.5: Box and whisker plot of spore percentages for the various raw material formulations and commercial media formulations (TSB and Bactopeptone) which were tested.

Formulation 45 (3.75 g horse manure; 5 g maize chops and 1.25 g soy meal), formulation 46 (3.75 g horse manure; 5 g maize chops and 2.5 g soy meal) and formulation 47 (3.75 g horse manure; 5 g maize chops and 5 g soy meal) yielded high spore percentages. When comparing these results to that of commercial media formulations, it shows that raw material formulations yielded higher percentages when compared to the commercial media. Statistical Significant differences can be observed also on Table 4.1.

Most formulations yielded lower spore percentages than TSB and bactopeptone. In the study of Cook and Lund (1962), they found that when the spore percentage is low it may be because (a) a proportion of spores are not viable, (b) they may be viable but fail to germinate in the growth conditions supplied, or (c) germination may be initiated, but the environment may fail to support outgrowth and multiplication (Cook and Lund, 1962).

Table 4.1: Statistical mean ± standard error of the 63 raw material formulation and the commercial media formulations (Superscript lettering represents statistically significant differences in the data derived from ANOVA) (95% confidence interval).

Form	Spore yield	Vegetative cells	Spore percentage	Form	Spore yield	Vegetative cells	Spore percentage
1	1.28E+06±2.46E+05 [a]	2.47E+06±2.28E+05 [a,b,c]	5.09E+014.84E+00 [a,b,]	34	1.21E+06±4.84E+05 [a]	1.67E+07±7.32E+05 [a,b,c,d,e,f,g,h,I,j,k,l,m,n]	8.49E+00±2.58E+00 [a,b,]
2	6.20E+05±5.78E+04 [a]	3.32E+06±2.69E+05 [a,b,c,d]	1.92E+01±3.00E+00 [a,b,]	35	1.44E+06±4.56E+04 [a]	8.94E+06±1.80E+06 [a,b,c,d,e,f,g,h,I,j,k]	6.83E+00±1.26E+00 [a,b,]
3	8.46E+05±2.26E+05 [a]	4.52E+06±2.59E+05 [a,b,c,d,f]	1.93E+01±6.09E+00 [a,b,]	36	5.67E+05±2.92E+05 [a]	2.10E+07±1.83E+06 [g,h,I,j,k,l,m,n]	7.77E+00±1.12E+00 [a,b,]
4	1.49E+06±5.46E+05 [a]	3.09E+06±1.37E+05 [a,b,c,d]	4.69E+01±1.52E+01 [a,b,]	37	1.64E+06±9.56E+04 [a]	1.34E+07±1.78E+06 [a,b,c,d,e,f,g,h,I,j,k,l,m]	1.07E+01±1.82E+00 [a,b,]
5	1.08E+06±3.36E+05 [a]	3.64E+06±1.96E+05 [a,b,c,d,e]	3.00E+01±8.94E+00 [a,b,]	38	1.38E+06±1.01E+06 [a]	2.01E+07±1.51E+06 [f,g,h,I,j,k,l,m,n]	1.02E+01±5.94E+00 [a,b,]
6	9.22E+05±2.77E+05 [a]	5.09E+06±7.42E+05 [a,b,c,d,f,g]	1.83E+01±5.84E+00 [a,b,]	39	1.93E+06±1.98E+05 [a]	2.62E+07±2.83E+06 [I,j,k,l,m,n]	3.71E+00±1.10E+00 [a]
7	7.00E+05±3.57E+05 [a]	8.68E+06±2.99E+06 [a,b,c,d,e,f,g,h,I,j]	1.17E+01±7.84E+00 [a,b,]	40	9.09E+05±4.01E+05 [a]	2.11E+07±6.57E+06 [g,h,I,j,k,l,m,n]	1.18E+01±3.72E+00 [a,b,]
8	1.59E+06±8.38E+05 [a]	6.79E+06±1.25E+05 [a,b,c,d,e,f,g]	2.31E+01±2.01E+00 [a,b,]	41	2.09E+06±2.64E+06 [a]	2.02E+07±3.04E+06 [f,g,h,I,j,k,l,m,n]	1.91E+01±9.20E+00 [a,b,]
9	1.37E+06±2.45E+05 [a]	6.37E+06±1.68E+06 [a,b,c,d,e,f,g]	2.23E+01±4.30E+00 [a,b,]	42	4.42E+06±4.46E+05 [a]	2.37E+07±2.71E+06 [I,j,k,l,m,n]	1.34E+01±2.96E+00 [a,b,]
10	6.83E+05±1.50E+05 [a]	4.94E+06±5.60E+05 [a,b,c,d,e,f,g]	1.48E+01±1.19E+01 [a,b,]	43	3.03E+06±1.01E+06 [a]	1.88E+07±2.75E+06 [d,e,f,g,h,I,j,k,l,m,n]	1.02E+01±3.99E+00 [a,b,]
11	5.44E+05±1.42E+05 [a]	3.64E+06±6.27E+05 [a,b,c,d,e]	1.47E+01±2.22E+00 [a,b,]	44	2.09E+06±4.04E+05 [a]	1.87E+07±6.82E+05 [c,d,e,f,g,h,I,j,k,l,m,n]	8.47E+00±1.87E+00 [a,b,]
12	1.40E+06±3.69E+05 [a]	4.53E+06±1.39E+06 [a,b,c,d,e,f]	3.35E+01±4.73E+00 [a,b,]	45	1.96E+06±2.75E+06[c]	2.47E+07±5.82E+06 [,j,k,l,m,n]	6.45E+01±8.78E+00 [a,b,]
13	1.09E+06±5.54E+05 [a]	5.09E+06±4.45E+05 [a,b,c,d,e,f,g]	2.17E+01±1.03E+01 [a,b,]	46	1.60E+06±1.26E+07 [a]	1.13E+07±9.66E+05 [a,b,c,d,e,f,g,h,I]	1.77E+02±1.16E+02[c]
14	7.26E+05±4.56E+04 [a]	6.63E+06±1.17E+06 [a,b,c,d,e,f,g]	1.14E+01±1.28E+00 [a,b,]	47	1.51E+06±2.38E+06[b,c]	1.75E+07±4.69E+06 [b,c,d,e,f,g,h,I,j,k,l,m,n]	6.22E+01±3.47E+01 [a,b,]
15	1.54E+06±5.09E+05 [a]	7.13E+06±2.11E+06 [a,b,c,d,e,f,g,h]	2.25E+01±4.46E+00 [a,b,]	48	8.10E+06±4.93E+05[a,b]	1.79E+07±5.26E+06 [c,d,e,f,g,h,I,j,k,l,m,n]	1.02E+01±6.80E-01 [a,b,]
16	3.45E+06±1.63E+06 [a]	6.29E+06±2.95E+05 [a,b,c,d,e,f,g]	5.63E+01±2.86E+01 [a,b,]	49	1.79E+06±5.90E+05 [a]	1.61E+07±1.73E+06 [a,b,c,d,e,f,g,h,I,j,k,l,m,n]	1.51E+01±5.73E+00 [a,b,]
17	4.64E+05±6.09E+04 [a]	3.07E+06±2.30E+05 [a,b,c,d]	1.56E+01±3.30E+00 [a,b,]	50	2.25E+06±2.79E+05 [a]	2.51E+07±6.19E+06 [k,l,m,n]	7.52E+00±1.29E+00 [a,b,]
18	8.26E+05±2.04E+05 [a]	4.99E+06±6.65E+05 [a,b,c,d,e,f,g]	1.60E+01±2.56E+00 [a,b,]	51	1.76E+06±4.92E+05 [a]	2.79E+07±7.61E+06 [m,n]	7.87E+00±2.65E+00 [a,b]
19	5.24E+05±8.98E+04 [a]	4.25E+06±6.59E+05 [a,b,c,d,e,f]	1.24E+01±1.08E+00 [a,b,]	52	1.88E+06±1.44E+06 [a]	1.84E+07±3.48E+06 [c,d,e,f,g,h,I,j,k,l,m,n]	1.99E+01±1.42E+01 [a,b,]
20	7.86E+05±5.48E+04 [a]	6.09E+06±5.36E+05 [a,b,c,d,e,f,g]	1.29E+01±2.60E-01 [a,b,]	53	2.69E+06±1.69E+06 [a]	2.36E+07±3.53E+06 [I,j,k,l,m,n]	1.63E+01±7.77E+00 [a,b,]
21	4.68E+05±6.62E+04 [a]	3.52E+06±1.03E+05 [a,b,c,d,e]	1.32E+01±1.46E+00 [a,b,]	54	3.52E+06±7.27E+05 [a]	1.96E+07±7.69E+06 [e,f,g,h,I,j,k,l,m,n]	1.91E+01±5.23E+00 [a,b,]
22	4.21E+05±8.29E+04 [a]	5.65E+06±1.76E+06 [a,b,c,d,e,f,g]	8.97E+00±2.80E+00 [a,b,]	55	2.94E+06±4.55E+05 [a]	2.34E+07±4.73E+06 [h,I,j,k,l,m,n]	6.82E+00±4.14E+00 [a,b,]
23	7.00E+05±1.38E+05 [a]	4.35E+06±1.42E+06 [a,b,c,d,e,f]	1.75E+01±2.13E+00 [a,b,]	56	1.20E+06±1.07E+06 [a]	3.04E+07±2.16E+06[n]	1.72E+01±3.07E+00 [a,b,]
24	8.02E+05±5.21E+04 [a]	1.23E+07±1.67E+06 [a,b,c,d,e,f,g,h,I,j,k,l,m]	6.67E+00±6.70E-01 [a]	57	5.26E+06±2.50E+05[c]	1.80E+07±5.21E+06 [c,d,e,f,g,h,I,j,k,l,m,n]	9.88E+00±4.22E+00 [a,b,]
25	2.19E+06±9.66E+05 [a]	1.66E+07±3.45E+06 [a,b,c,d,e,f,g,h,I,j,k,l,m]	1.21E+01±2.76E+00 [a,b,]	58	1.35E+06±6.10E+05 [a]	1.60E+07±4.85E+06 [a,b,c,d,e,f,g,h,I,j,k,l,m,n]	1.68E+01±1.20E+00 [a,b,]
26	1.88E+06±2.64E+05 [a]	1.72E+07±1.63E+06 [a,b,c,d,e,f,g,h,I,j,k,l,m,n]	1.14E+01±2.76E+00 [a,b,]	59	2.56E+06±1.44E+05 [a]	1.19E+07±1.16E+06 [a,b,c,d,e,f,g,h,I,j,k,l,m]	1.29E+01±1.93E+00 [a,b,]
27	6.70E+05±1.18E+05 [a]	6.04E+06±3.39E+05 [a,b,c,d,e,f,g]	1.14E+01±2.69E+00 [a,b,]	60	1.51E+06±5.57E+05 [a]	2.03E+07±1.36E+06 [f,g,h,I,j,k,l,m,n]	9.38E+00±2.35E+00 [a,b,]
28	7.29E+05±1.84E+05 [a]	6.46E+06±8.97E+05 [a,b,c,d,e,f,g]	1.23E+01±4.30E+00 [a,b,]	61	1.92E+06±2.91E+06 [a]	9.73E+06±2.30E+06 [a,b,c,d,e,f,g,h,I,j,k]	5.06E+01±1.76E+01 [a,b,]
29	5.77E+05±4.56E+04 [a]	7.95E+06±3.53E+05 [a,b,c,d,e,f,g,h,I]	7.33E+00±8.70E-01 [a,b,]	62	5.51E+06±5.19E+05[b]	1.19E+07±2.42E+06 [a,b,c,d,e,f,g,h,I,j,k,l,m]	1.41E+01±1.47E+00 [a,b,]
30	5.77E+05±9.57E+04 [a]	9.00E+05±1.14E+05 [a]	2.23E+01±1.40E+01 [a,b,]	63	1.76E+06±2.50E+05 [a]	1.59E+07±2.51E+06 [a,b,c,d,e,f,g,h,I,j,k,l,m,n]	1.28E+01±3.00E+00 [a,b,]
31	1.78E+05±3.40E+05 [a]	1.55E+06±3.79E+05 [a,b]	2.18E+01±1.59E+01 [a,b,]	TSB	1.90E+06±3.02E+05 [a,b]	7.12E+06±6.62E+05 [a,b,c,d,e,f,g,h]	1.04E+02±8.83E+00 [a,b,]
32	4.05E+05±1.32E+06 [a]	1.75E+07±2.70E+06 [b,c,d,e,f,g,h,I,j,k,l,m,n]	2.51E+01±5.88E+00 [a,b,]	Bacto	7.58E+06±1.95E+06 [a,b]	9.31E+06±8.86E+05 [a,b,c,d,e,f,g,h,I,j,k]	7.90E+01±1.26E+01 [a,b,]
33	4.39E+06±4.99E+05 [a]	2.04E+07±1.81E+05 [f,g,h,I,j,k,l,m,n]	5.92E+00±2.40E+00 [a]				

4.6 Re-evaluation of 12 raw material formulations

From the 63 raw material formulations results, 12 with the highest spore yield were chosen to be re-evaluated. Formulations 16, 25, 32, 38, 41, 45, 46, 47, 52, 53, 56 and 61 were re-evaluated to confirm spore yield results obtained.

4.6.1 Endospore count

A total of 12 formulations out of 63 formulations had the highest endospore yield. These 12 formulations (Table 3.2) where re-evaluated to confirm spore yield results obtained. Figure 4.6 shows endospore counts obtained for the 12 highest spore yielding raw material formulations and commercial media formulations. Table 4.2 shows the homogenous groupings of the 12 highest spore yielding raw material formulations and commercial media formulations.

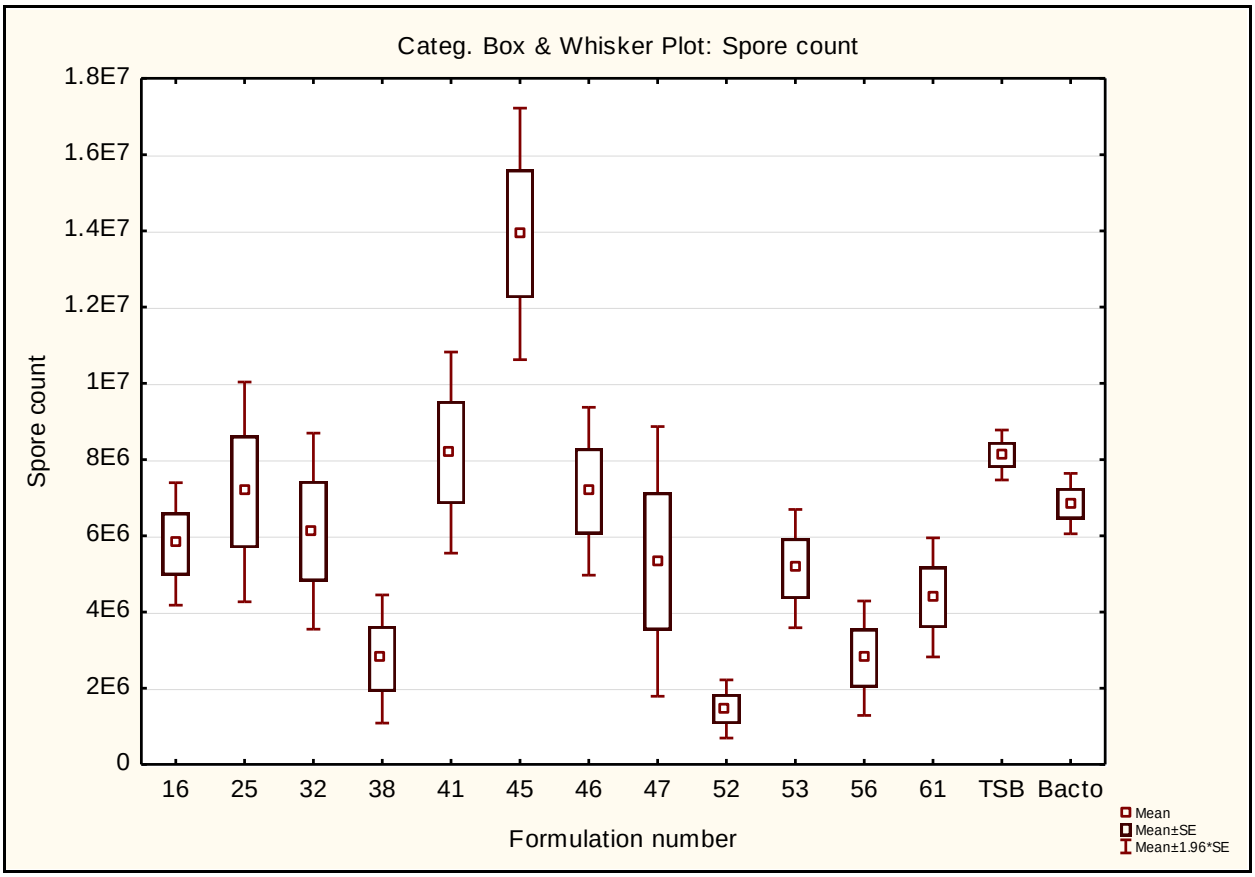

Figure 4.6: Box and whisker plot of endospore counts of the 12 highest spore yielding raw material formulations and commercial media (TSB and Bactopeptone)

Results obtained here were similar to those found in the previous results. Formulation 45 (3.75 g horse manure; 5 g maize chops and 1.25 g soy meal), when evaluated for the first time yielded high endospore count results. For re-evaluation endospore confirmed high yield results. When these 12 highest spore yielding raw material formulations were compared with the previous results, it was observed that they yielded high amounts of spore counts. This is with exception to formulation 53 (5 g horse manure, 2.5 g maize chops and 0 g soy meal) which yielded lower amount of spores. Commercial media yielded high amount of spores, but other raw material formulations yielded better results than these commercial media formulations.

4.6.2 Vegetative cells

Figure 4.7 shows vegetative cell counts obtained for the 12 highest spore yielding raw material formulations and commercial media formulations.

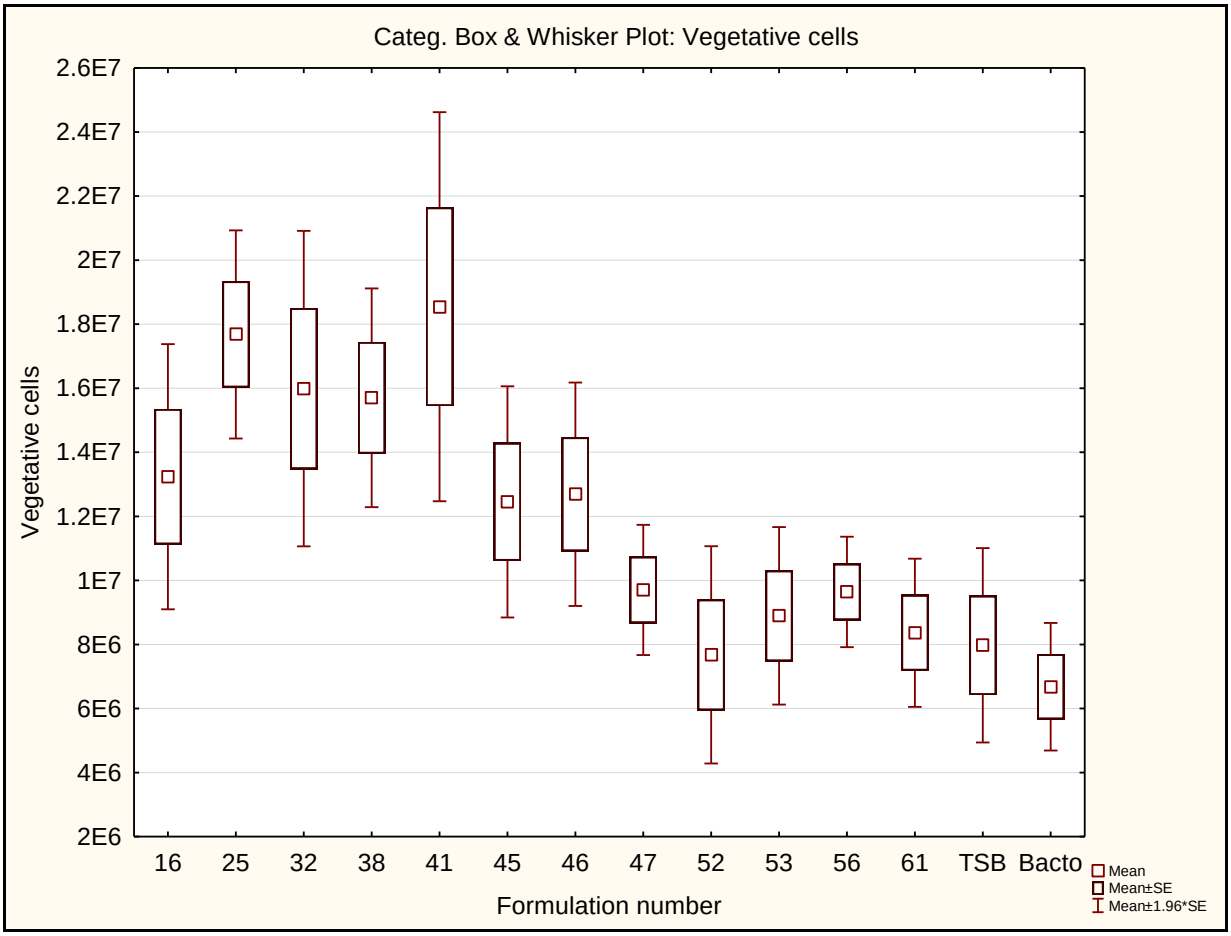

Figure 4.7: Box and whisker plot of vegetative cell counts of the 12 highest spore yielding raw material formulations and commercial media (TSB and Bactopeptone)

When raw material formulations and commercial media formulations were compared, differences were observed between two groups of formulations: (47, 52, 53, 56 and 61) and (16, 25, 32, 38, 41, 45 and 46). Formulation 41 (3.75g horse manure, 3.75g maize chops and 1.25g soy meal) showed the highest vegetative cell count. The formulation that had the lowest vegetative cell count (Formulation 52) was the

one with the combination of 5 g horse manure, 2.5 g maize chops and 0 g soy meal. Commercial media formulations yielded the lowest amount of vegetative cells. When evaluated the first time, commercial media also yielded the lowest amount of vegetative cells (Figure 4.7).

4.6.3 Spore percentage

Spore percentage was calculated for the 12 highest spore yielding formulations. Figure 4.8 shows spore percentages obtained for the 12 highest spore yielding raw material formulations and two commercial media formulations.

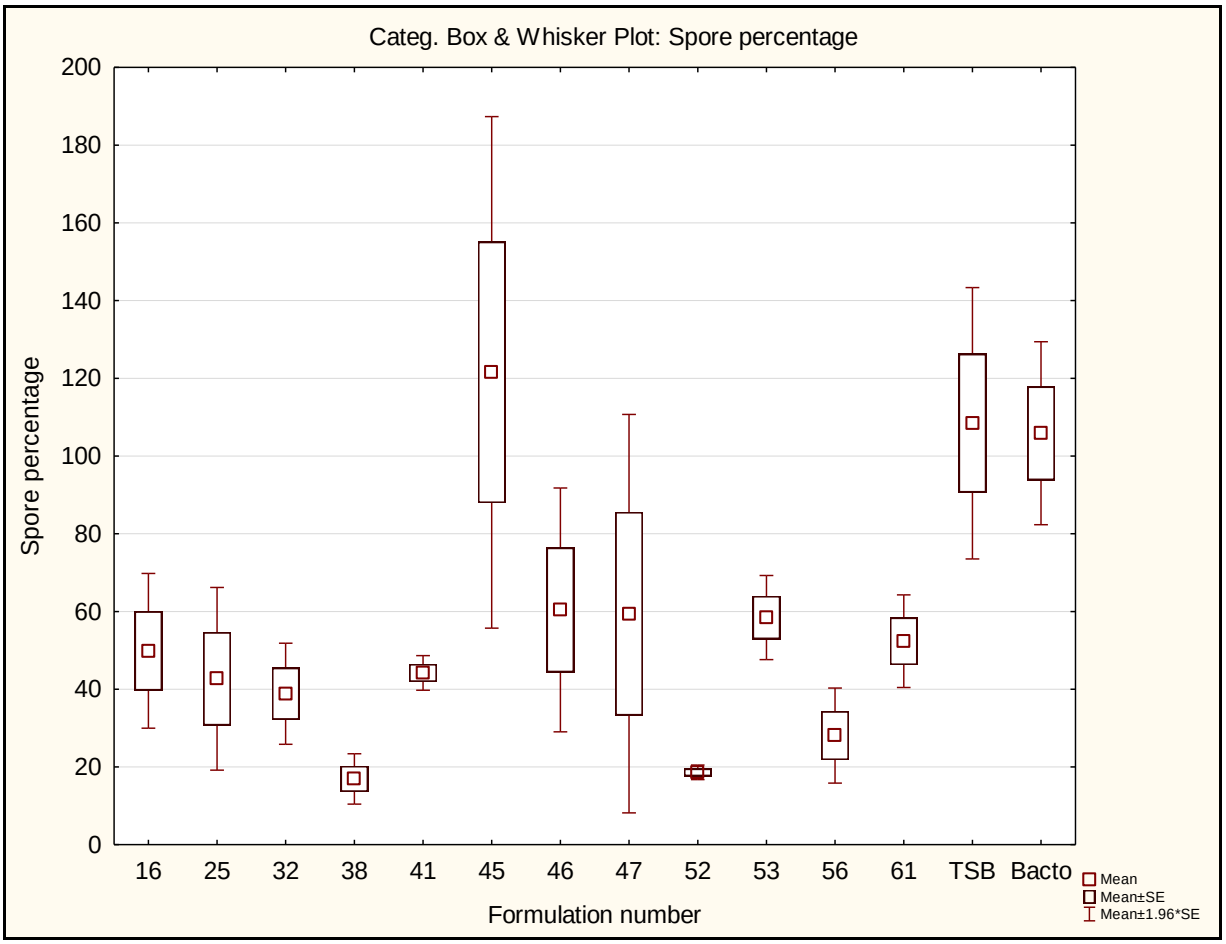

Figure 4.8: Box and whisker plot of spore percentages of the 12 highest spore yielding raw material formulations and commercial media (TSB and Bactopeptone)

Spore percentage was found to be increased in the commercial media. Formulation 45 (3.75 g horse manure; 5 g maize chops and 1.25 g soy meal), formulation 46 (3.75 g horse manure; 5 g maize chops and 2.5 g soy meal) and formulation 47 (3.75 g horse manure; 5 g maize chops and 5 g soy meal) had the highest spore percentage of the 12 raw material formulation. These results are similar to those observed during the first evaluation of the raw materials. Re-evaluation of spore yielding confirmed the raw material formulations that are the most promising in this context.

Table 4.2: 12 highest spore yielding formulations and commercial media formulations (Statistical mean ± standard error and Superscript lettering represents statistically significant differences in the data derived from ANOVA (95% confidence interval).

Formulation	Spore yield	Vegetative cells	Spore percentage
16	5.79E+06± 8.20E+05 ^{a,b}	1.32E+07± 2.11E+06 ^{a,b,c,d}	4.99E+01± 1.02E+01 ^{a,b,c}
25	7.15E+06± 1.47E+06^{b}	1.77E+07± 1.66E+06 ^{c,d}	4.27E+01± 1.20E+01 ^{a,b}
32	6.12E+06± 1.31E+06 ^{a,b}	1.60E+07± 2.51E+06 ^{b,c,d}	3.88E+01± 6.64E+00 ^{a,b}
38	2.77E+06± 8.57E+05 ^{a,b}	1.57E+07± 1.74E+06 ^{a,b,c,d}	1.69E+01± 3.31E+00 ^{a}
41	8.18E+06±1.35E+06 ^{b}	1.85E+07± 3.10E+06 ^{d}	4.42E+01± 2.27E+00 ^{a,b}
45	1.39E+07±1.68E+06 ^{c}	1.25E+07± 1.84E+06 ^{a,b,c,d}	1.22E+02±3.36E+01 ^{c}
46	7.17E+06±1.12E+06 ^{b}	1.27E+07± 1.78E+06 ^{a,b,c,d}	6.04E+01± 1.60E+01 ^{a,b,c}
47	5.33E+06± 1.801E+06 ^{a,b}	9.70E+06 ± 1.04E+07 ^{a,b,c,d}	5.94E+01± 2.62E+01 ^{a,b,c}
52	1.46E+06±3.90E+05 ^{a}	7.68 E+07 ±1.73E+06 ^{a,b}	1.86E+01± 9.60E-01 ^{a}
53	5.14E+06±7.91E+05 ^{a,b}	8.89E+07±1.41E+06 ^{a,b,c}	5.84E+01± 5.52E+00 ^{a,b,c}
56	2.79E+06± 7.66E+05 ^{a,b}	9.64 E+07±8.79E+05 ^{a,b,c,d}	2.81E+01± 6.25E+00 ^{a}
61	4.38E+06± 7.98E+05 ^{a,b}	8.37E+07±1.18E+07 ^{a,b}	5.24E+01± 6.08E+00 ^{a,b,c}
TSB	8.12E+06± 3.35E+06 ^{b}	7.97E+07±1.55E+07 ^{a,b}	1.08E+02± 1.78E+01 ^{b,c}
Bacto	6.84E+06±4.04E+05 ^{a,b}	6.68E+06 ±1.02E+07 ^{a}	1.06E+02± 1.20E+01 ^{b,c}

4.7 Cost analysis

Raw material media formulations are more cost effective than commercial media when produced from readily available raw materials. Table 4.3 shows the cost analysis of the media used in this study. The commercial media formulations are expensive compared to raw material formulations and could hinder the production of biopesticides such as *B.t.a*.

Table 4.3: Comparative cost analysis for producing 1 Liter of commercial media and three raw material media

Medium	Cost per 1 litre
Tryptone soy broth	R 49.50
Bactopeptone	R 51.20
Maize chops	R 0.42
Soybean meal	R 1.17
Horse manure	R 0.37

Thus, the use of raw materials is much more economically feasible for the large scale production of *B.t.a*.

4.8 Bioassay test

B.t.a was evaluated against *G. mellonella* larvae by determining the mortality rate of the larvae. Figure 4.9 shows the results obtained for the 12 highest spore yielding formulations and Table 4.4, the mortality rate of *G. mellonella* after 5 days of treatment.

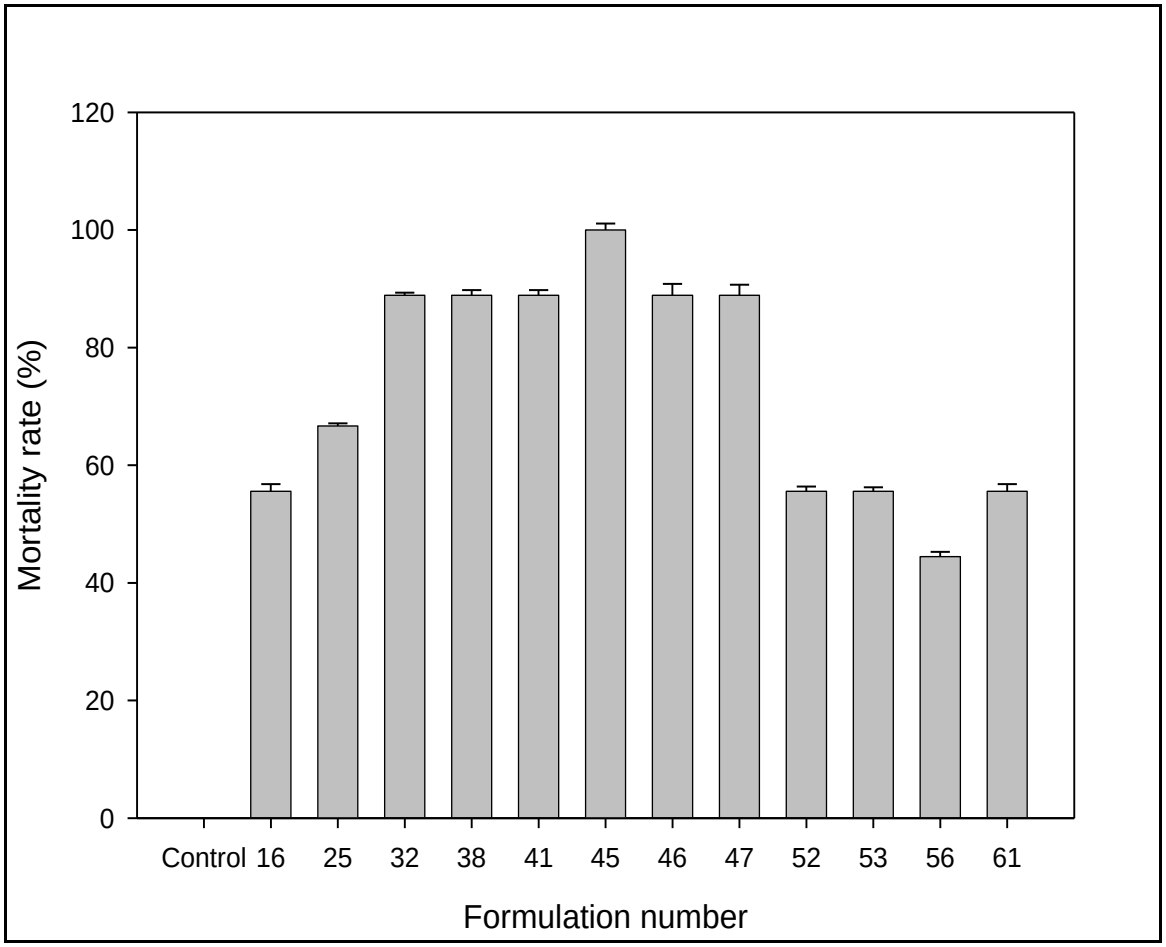

Figure 4.9 Mortality rate of *Galleria mellonella* larvae obtained for the 12 highest spore yielding formulations after 5 days of treatment

Table 4.4: Twelve raw material formulations with highest spore yield and their mortality rate after 5 days treatment

Formulation	Maize chops	Horse manure	Soy meal	Mortality rate
16	0.00	2.50	0.00	55.55%
25	3.75	2.50	1.25	66.66%
32	0.00	3.75	0.00	88.88%
38	2.50	3.75	2.50	88.88%
41	3.75	3.75	1.25	88.88%
45	5.00	3.75	1.25	100%
46	5.00	3.75	2.50	88.88%
47	5.00	3.75	5.00	88.88%
52	2.50	5.00	0.00	55.55%
53	2.50	5.00	1.25	55.55%
56	3.75	5.00	0.00	44.44%
61	5.00	5.00	1.25	55.55%

The bioassay conducted with the *B.t.a* powder obtained through production on the raw material formulations resulted in immediate feeding cessation of the larvae followed by variable death rates after 5 days of treatment. The bioassay results indicated that the *B.t.a* strain at sporulation was toxic against *G. mellonella* 5th instar larvae. Culturing of *B.t.a* on formulation 45 (3.75 g horse manure, 5 g maize chops and 1.25 g of soy meal) was the most effective in terms of spore yield and toxicity when compared to the other formulations. Moreover, this formulation yielded significant cumulative larval mortality of 90% after 3 days and 100% after 4 days of treatment (Table 4.4). From Figure 4.9 it can be observed that with 3.75 grams of horse manure, there is a definite increase in mortality rate. Formulation 38, 41, 45 and 46 the larvae stopped feeding on day 2.

Comparable results were observed in the study of Devi *et al.* (2005) that the media that produced the highest spore counts also showed the highest toxicity. This may be due to an increase in toxin content which is brought on by a high content of carbon and nitrogen in the combination. Mortality rate for the combination (wheat bran, molasses and yeast extract) ranged from 63.33 to 90.00% at 2 days after spraying, with cumulative mortality of 100% at three days. Therefore media formulation in their study influenced mortality rate.

Overall the results from this study clearly demonstrate that the susceptibility of *G. mellonella* larvae varies across different formulations tested (see appendix A). However, *B.t.a* could serve as a powerful biopesticide against the larvae of *G. mellonella* if the correct raw material is used for media formulation. With different formulations, differences in larval mortality were observed and when horse manure was above or below 3.75 g larval mortality was slow. Therefore, 3.75 g horse manure is ideal to produce a high number of spores that are sufficiently toxic to enhance mortality rate.

4.9 Morphology

Different bacteria change their morphology due to environmental changes and nutrients availability (Baranyi and Roberts, 1994). For this study spore and crystal morphology (Figure 4.10 a-i) were evaluated to determined whether spore or crystal morphology change when cultured in raw material formulations and commercial media formulations. Figure 4.10 a-d shows the Coomassie brilliant blue staining of three raw material formulations (16, 45 and 53) and commercial media (TSB). Figure 4.10 e-I show SEM and endospore staining of three raw material formulations (16, 45 and 53) and commercial media (TSB).

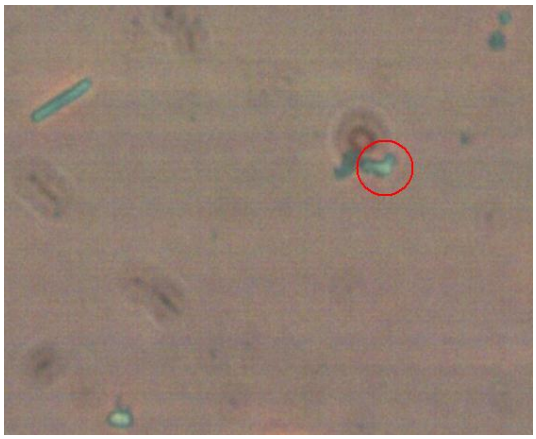

Figure 4.10: (a) Crystal spore of formulation 16

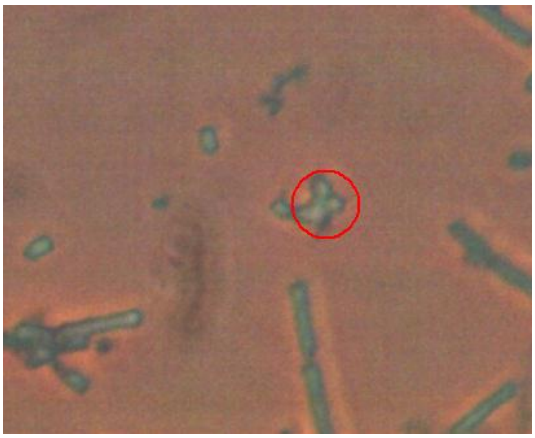

Figure 4.10: (b) Crystal spore of formulation 45

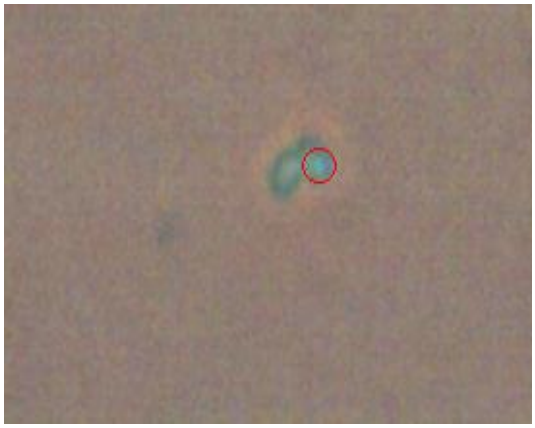

Figure 4.10: (c) Crystal spore of formulation 53

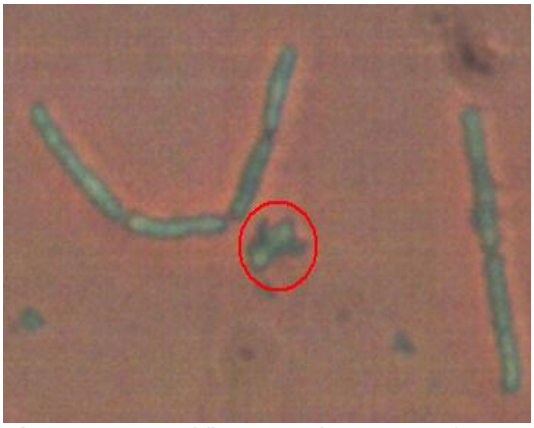

Figure 4.10: (d) Crystal spore of TSB

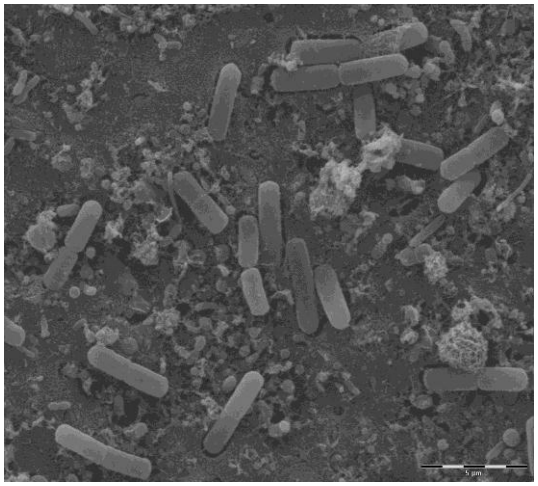

Figure 4.10: (e) SEM results of formulation 16

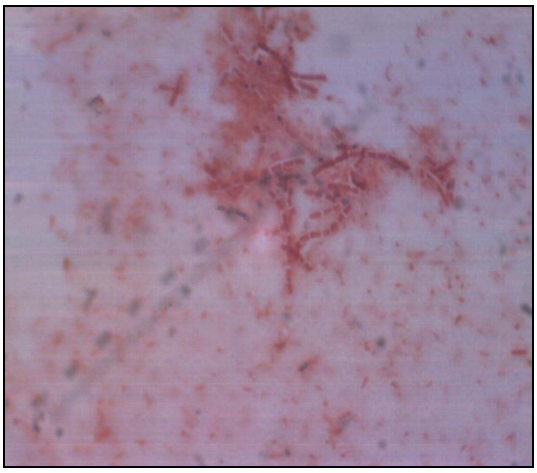

Figure 4.10: (f) *B.t.a* spore staining of formulation 16

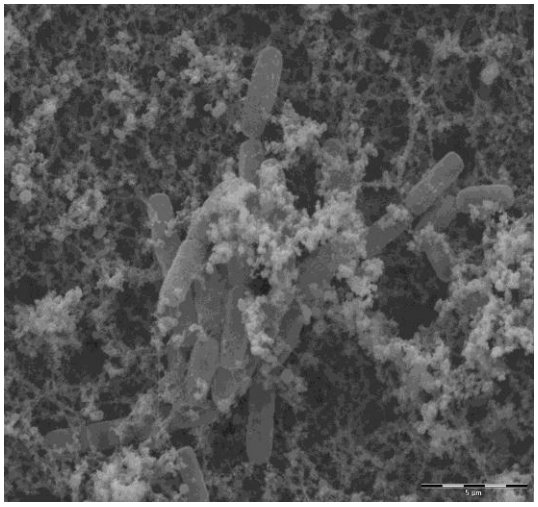

Figure 4.10: (g) SEM results for formulation 45

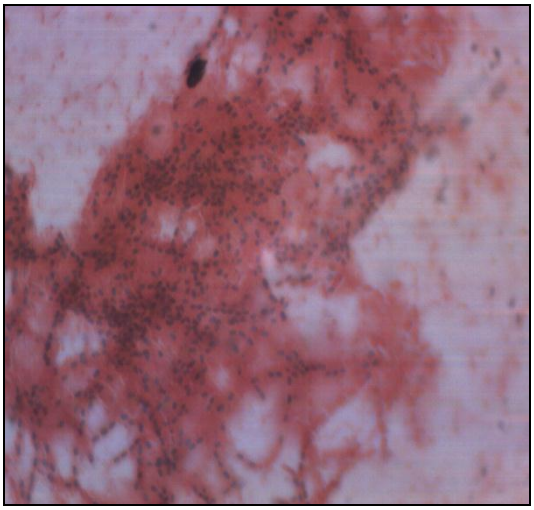

Figure 4.10: (h) *B.t.a* spore staining of formulation 45

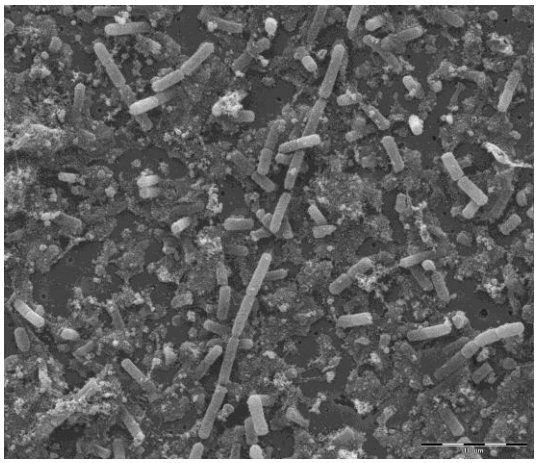

Figure 4.10: (i) SEM results of *B.t.a* in TSB

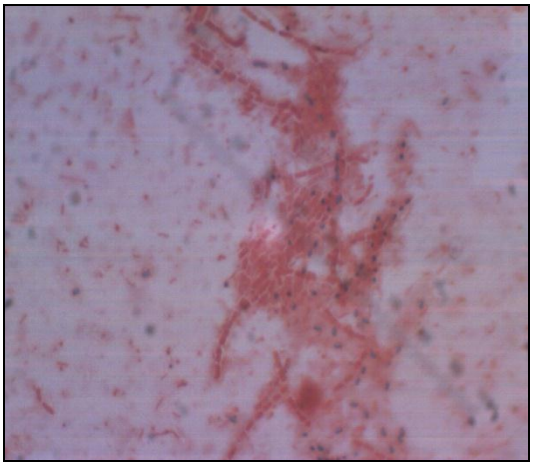

Figure 4.10: (j) *B.t.a* spore staining of TSB

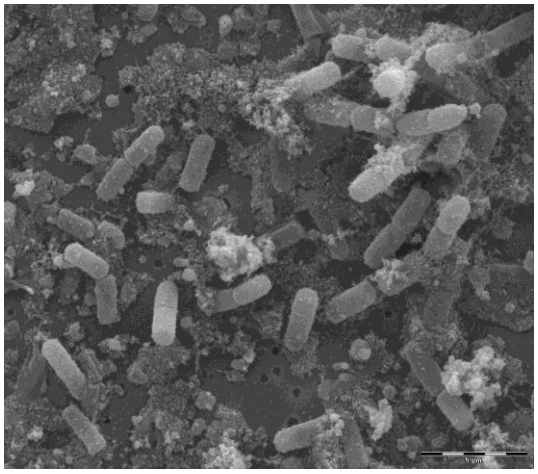

Figure 4.10: (k) SEM results of formulation 53

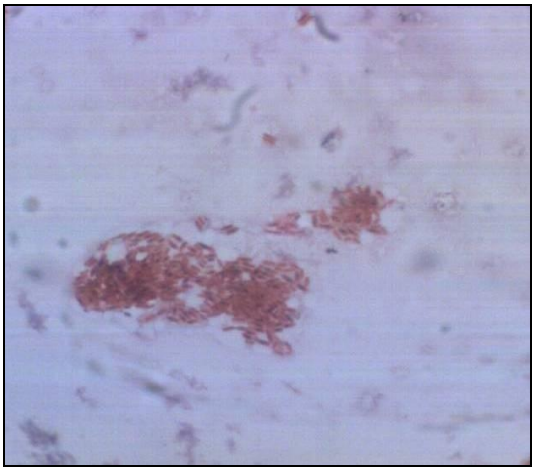

Figure 4.10: (l) *B.t.a* spore staining of formulation 53

Only 3 raw material formulations (16, 45 and 53) were chosen as representatives of the 12 formulations and commercial media (TSB) was also used for morphological analysis. These formulations were chosen because they all represent different raw materials combinations. In figure 4.10 (a-d), crystals were observed in raw material formulations and commercial media and they looked similar. Spore staining was also performed to observe morphology of the spores. Spores and crystals remained the same in the formulations and their structure was

not altered. For SEM, morphology of *B.t.a* was observed as a rod shape and materials around it are debris from different media formulations.

4.10 Protein analyses

Protein analysis was performed in order to determine the presence and concentration of crystal proteins in 12 highest spore yielding raw material formulations and commercial media to provide an indication of toxin concentration. Table 4.5 shows 12 highest spore yielding formulations and commercial media formulations and their protein concentration with standard error and superscript lettering which represents statistically significant differences. Figure 4.11 shows protein concentration of the 12 highest spore yielding raw material formulations and commercial media.

Table 4.5: Protein concentration of the 12 highest spore yielding formulations and commercial media formulations (Statistical mean ± standard error and Superscript lettering represents statistically significant differences in the data derived from ANOVA (95% confidence interval).

Formulation numbers	Protein concentration mg/ml
16	2.95 ± 0.097^{b}
25	$2.68\pm0.164^{a,b}$
32	$2.68\pm0.201^{a,b}$
38	$2.68\pm0.073^{a,b}$
41	$2.59\pm0.055^{a,b}$
45	2.79 ± 0.127^{b}
46	$2.90\pm0.043^{a,b}$
47	3.01 ± 0.164^{b}
52	$2.75\pm0.041^{a,b}$
53	$2.76\pm0.081^{a,b}$
56	$2.78\pm0.051^{a,b}$
61	2.94 ± 0.101^{b}
TSB	2.24 ± 0.086^{b}
Bactopeptone	2.30 ± 0.099^{b}

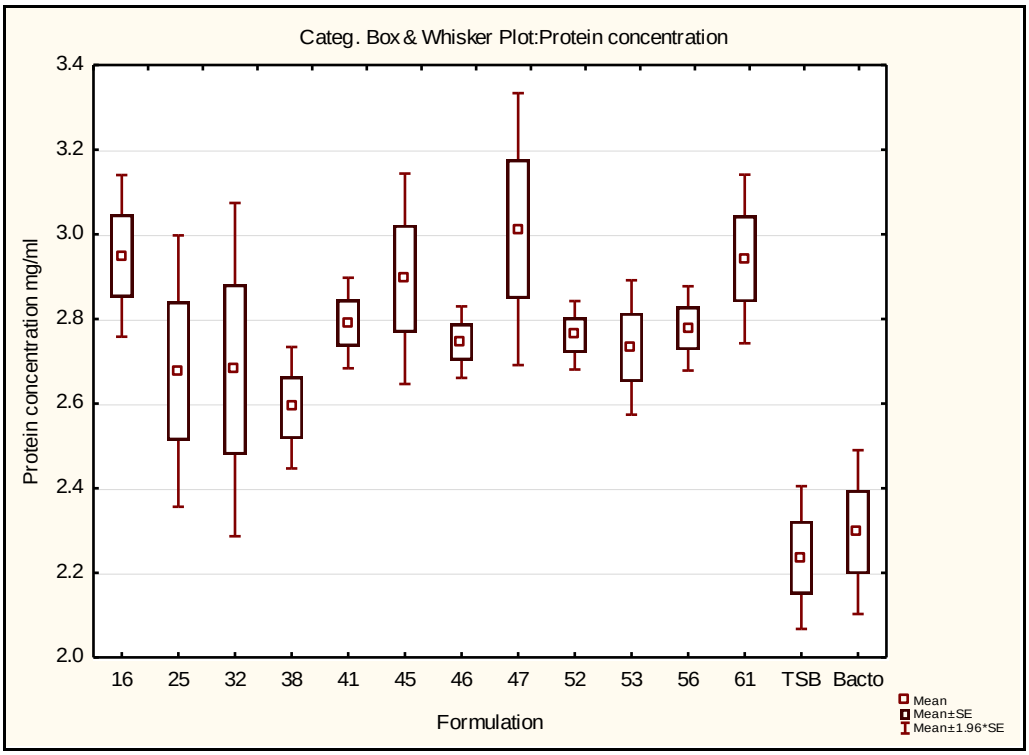

Figure 4.11: Box and whisker plot of protein concentration of the 12 highest spore yielding raw material formulations and commercial media (TSB and Bactopeptone).

It showed that the protein concentration (toxin concentration) differed from one media formulation to the next. Differences were observed in protein content and spore count. Formulation 47 (3.75 g horse manure; 5 g maize chops and 5 g soy meal) yielded the highest protein concentration whereas formulation 38 (3.75 g horse manure; 2.5 g maize chops and 2.5 g soy meal), formulation 41 (3.75 g horse manure; 3.75 g maize chops and 1.25 g soy meal), formulation 32 (3.75 g horse manure; 0 g maize chops and 0 g soy meal) and formulation 25 (2.5g horse manure; 3.75 g maize chops and 1.25 g soy meal) yielded the lowest protein concentrations. Commercial media formulations yielded the lowest toxin concentrations of all formulations. Formulation 45 (3.75 g horse manure; 5 g maize chops and 1.25 g soy meal) resulted in 100 % larval mortality within 3 days and had a protein concentration of 2.79 mg/ml. It can therefore be concluded that protein concentration is not

necessarily an accurate indication of toxin concentration. The formulation with the highest protein concentration (47: 3.75 g horse manure; 5 g maize chops and 5 g soy meal) did not yield 100 % mortality after 5 days. Therefore protein concentration is not equal to toxin concentration. Analyses can be conducted specifically for protein toxin which gives toxin concentration.

CHAPTER 5 – CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

In this study three raw materials were compared to two commercial media formulations for the production of spores of *Bacillus thuringiensis* subsp. *aizawai.* All raw material formulations and commercial media formulations were evaluated for the spore yield and toxicity.

It was concluded that:

- Raw materials can be used to create formulations that can be used as a substitute for commercial media formulations.
- Raw material formulations containing 3.75 g of horse manure showed the highest spore count and highest mortality rate overall.
- Formulations with 3.75 g horse manure; 5 g maize chops and 1.25 g, 2.5 g or 5 g soy meal represents a feasible alternative to commercial media for the production of *B.t.a*.
- Formulation 45 (5 g maize chops, 3.75 g horse manure and 1.25 g of soy meal) was the only formulation that caused 100 % mortality of *G. mellonella* larvae after 5 days.
- Protein concentration is not necessarily an accurate indicator of toxin concentration.
- Sporulation and toxicity were satisfactory after *B.t.a* was produced in these three raw material formulations when compared to commercial media.
- The structure of the spores and crystals were not altered by different raw material formulations used in this study, but the spore yield was different in each formulation.
- Therefore, raw material formulations were considered an economically feasible alternative for large scale production of *B.t.a.*

Because the problem of insects damaging crops persists, and beekeepers spend a fortune on chemical insecticides, biopesticides produced from raw materials can benefit the beekeeping industry without contaminating honey. This study shows that the use of the correct raw materials in the production of *B.t.a.* has much potential to combat *G. mellonella*.

5.2 Recommendations

Recommendations for further investigations include:

Other raw materials can be investigated and their effect on cry proteins. Because protein concentration is not equal to toxin concentration, analyses can be conducted specifically for protein toxin which gives toxin concentration. An increase in scale production can be also be implemented.

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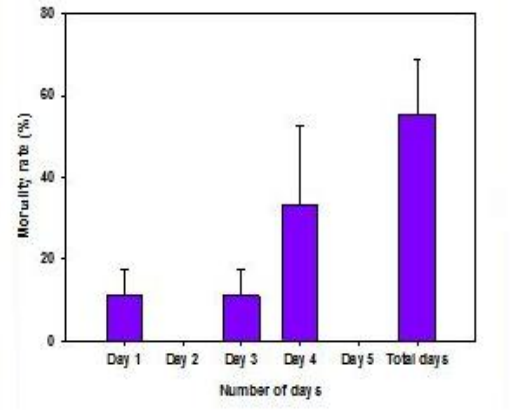
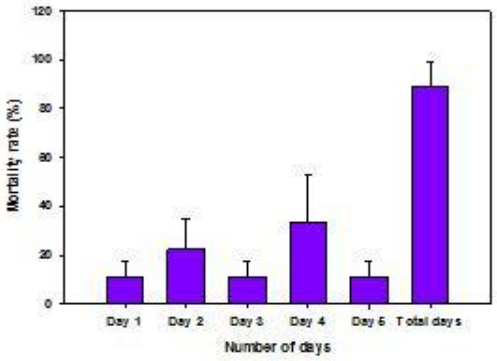
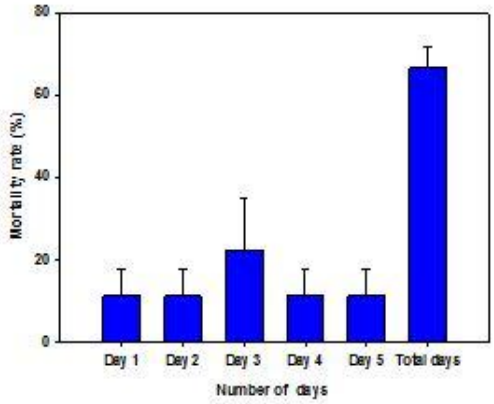
APPENDIX A - Mortality rate graphs for *Galleria mellonella* using different raw material formulations



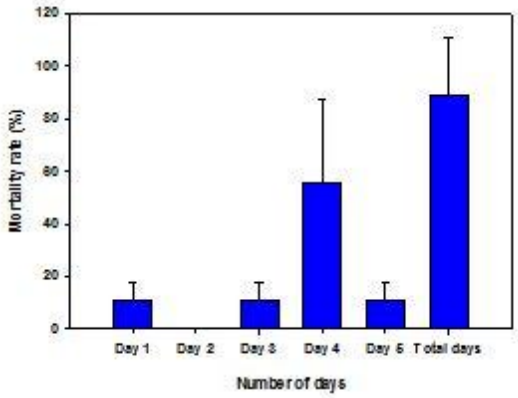
(a) Mortality rate of *Galleria mellonella* for formulation 16



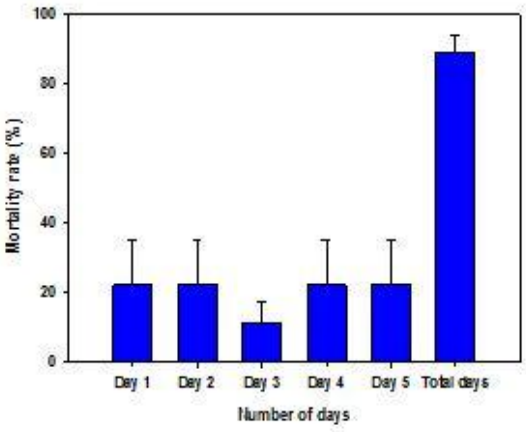
(b) Mortality rate of *Galleria mellonella* for formulation 41



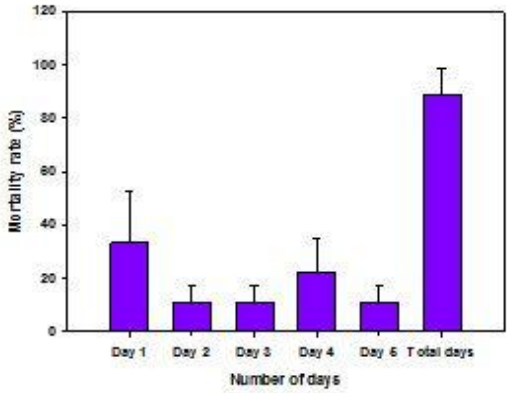
(c) Mortality rate of *Galleria mellonella* for formulation 25



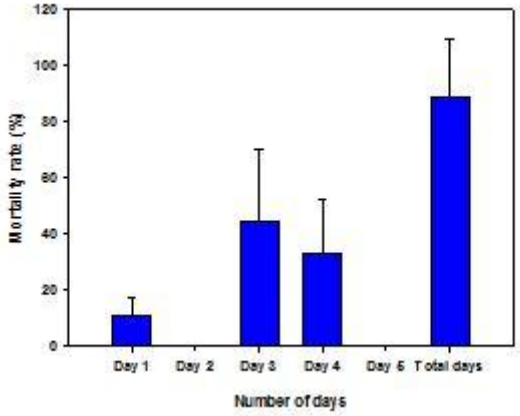
(d) Mortality rate of *Galleria mellonella* for formulation 46



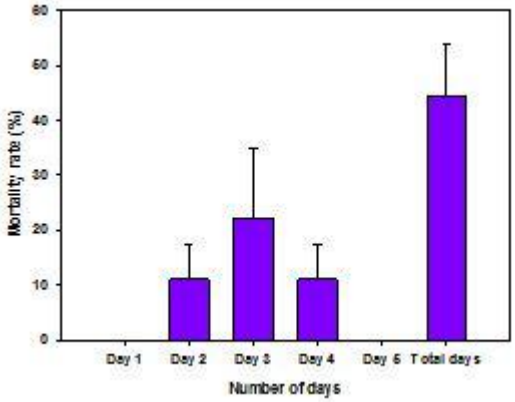
(e) Mortality rate of *Galleria mellonella* for formulation 32



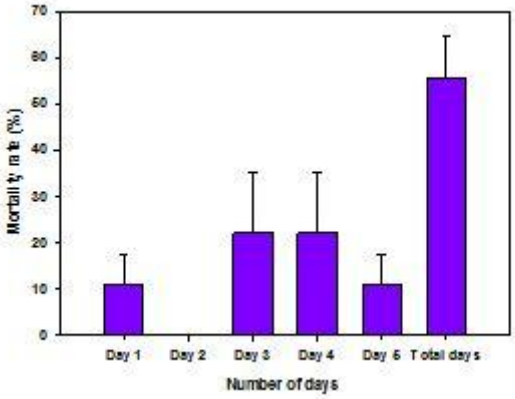
(f) Mortality rate of *Galleria mellonella* for formulation 48



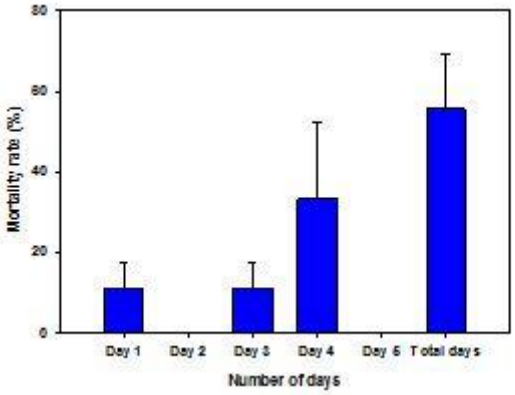
(g) Mortality rate of *Galleria mellonella* for formulation 47



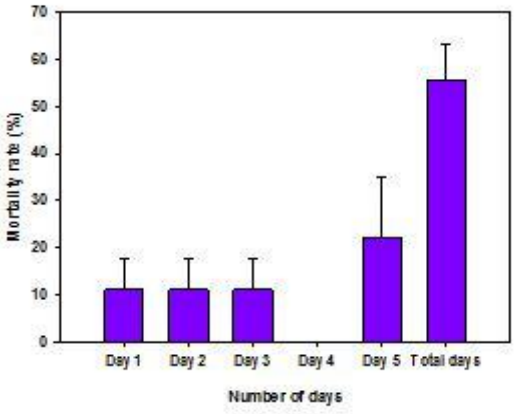
(h) Mortality rate of *Galleria mellonella* for formulation 56



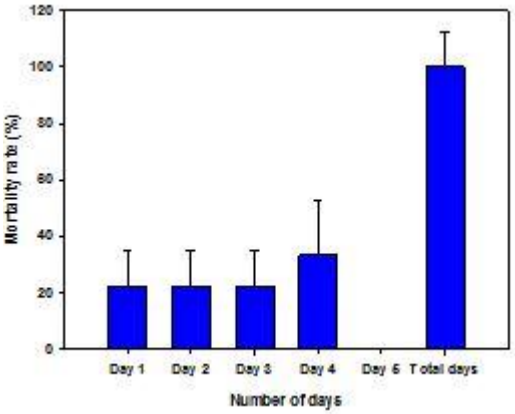
(i) Mortality rate of *Galleria mellonella* for formulation 52



(j) Mortality rate of *Galleria mellonella* for formulation 61

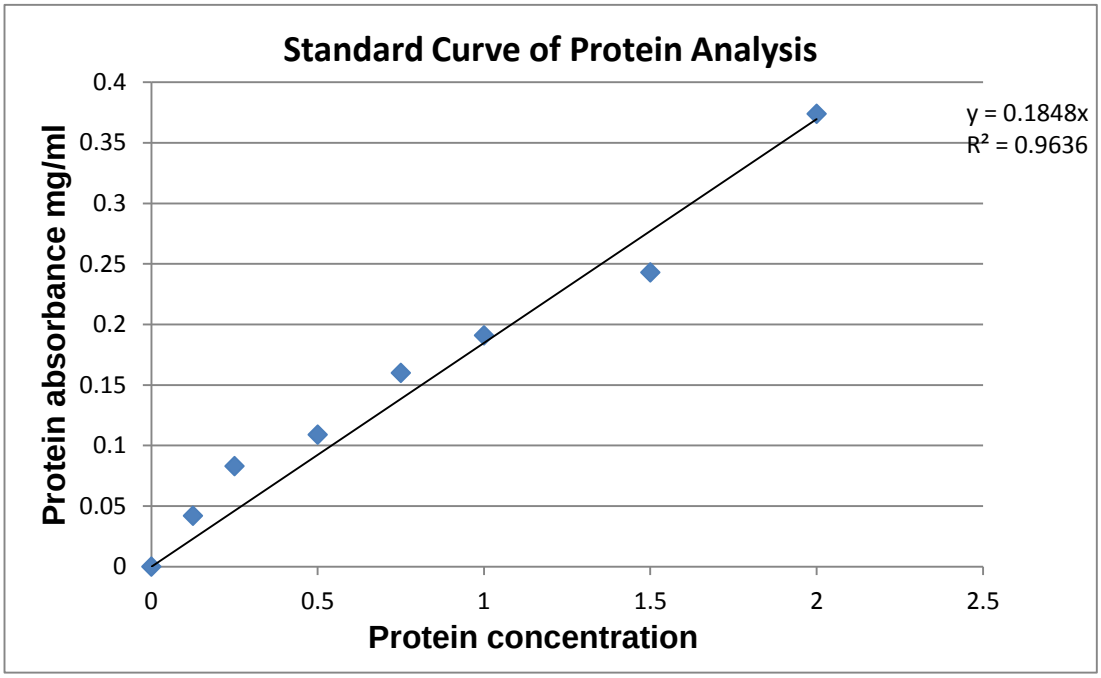


(k) Mortality rate of *Galleria mellonella* for formulation 53

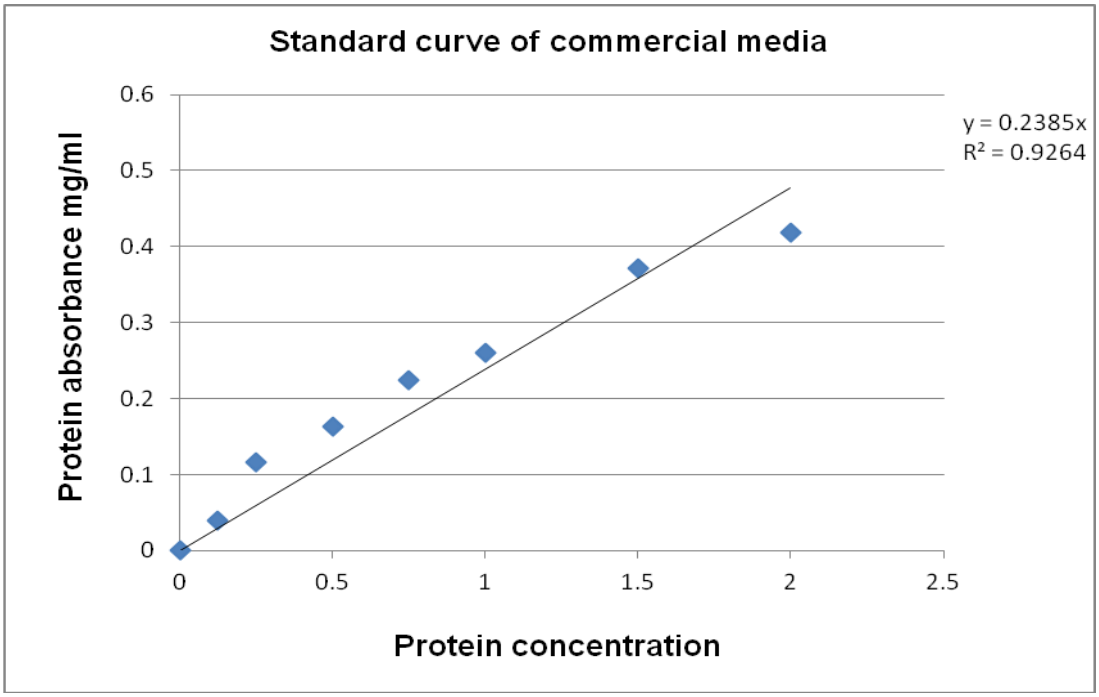


(l) Mortality rate of *Galleria mellonella* for formulation 45

APPENDIX B - Standard curve for protein analysis



(a) The correlation curve of the concentration of proteins (x-axis) and protein absorbance (y-axis). The correlation coefficient is 0.9636, with the slope of 0.1848. The protein concentration in this analysis ranges from 0.025 mg/mL to 0.200 mg/ml for raw material formulations.



(b) The correlation curve of the concentration of proteins (x-axis) and protein absorbance (y-axis). The correlation coefficient is 0.926, with the slope of 0.238. The protein concentration in this analysis ranges from 0.025 mg/mL to 0.200 mg/ml for commercial media.