Insect Control Using Chitinolytic Soil Actinomycetes as Biocontrol Agents

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Insect Control Using Chitinolytic Soil Actinomycetes As Biocontrol Agents

By

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A thesis

Submitted to

United Arab Emirates University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Environmental Sciences

2003-2004
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United Arab Emirates University
2003/2004
DEDICATION

By all love and loyalty I dedicate this thesis to my father

H.H. Sheikh Zayed bin Sultan Al-Nahyan, the president of the UAE, To my country, and to my dear parents.
Title: INSECT CONTROL USING CHITINOLYTIC SOIL ACTINOMYCETES AS BIOCONTROL AGENTS

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# TABLE OF CONTENTS

Abstract .......................................................................................................................... i

List of Figures .................................................................................................................. iii

List of Tables ................................................................................................................... v

Acknowledgement .......................................................................................................... vii

1.0 Introduction ................................................................................................................. 1

2.0 Literature review ........................................................................................................ 3

2.1 Methods of insects control ....................................................................................... 3

2.1.1 Chemical control .................................................................................................. 3

2.1.2 Biological control as alternative methods of insect control ......................... 5

2.1.2.1 Definition of biological control ................................................................... 5

2.1.2.2 Predators and parasites in insect control ..................................................... 6

2.1.2.3 Microbial control of insect pests ................................................................. 6

2.1.2.3.1 Bacterial pesticide .................................................................................. 7

2.1.2.3.1.1 Bacillus popilliae ............................................................................... 7

2.1.2.3.1.2 Bacillus thuringiensis (Bt) ................................................................... 8

2.1.2.3.1.3 Bacillus sphaericus ............................................................................. 9

2.1.2.3.1.4 Other bacterial pathogens .................................................................... 10

2.1.2.3.2 Actinomycetes insecticides ................................................................... 10

2.1.2.3.3 Viral insecticide ....................................................................................... 11

2.1.2.3.4 Nematode insecticides .......................................................................... 13

2.1.2.3.5 Fungal insecticides ............................................................................... 13

2.1.2.3.6 Protozoan Pesticides .............................................................................. 15

2.2 Biology and classification of actinomycetes ........................................................... 15

2.2.1 General properties of actinomycetes .................................................................. 15
2.2.2 Taxonomy of actinomycetes
2.2.3 Isolation of actinomycetes
2.2.4 Actinomycetes phage
2.3 Chitin
2.3.1 Chemical composition of chitin
2.4 Chitinase enzymes
2.5 Identification of actinomycetes by RAPD-PCR technique
2.6 Aims of thesis

3.0 Materials and methods
3.1 Materials
3.1.1 Isolation and characterization of chitinase producing actinomycetes
3.1.1.1 Composition of media
3.1.2 Application of the most promising chitinase producing streptomycete
and non-streptomycete actinomycetes as insect biological control agent
3.1.2.1 Insects
3.1.2.2 Drosophila rearing medium
3.1.2.3 Equipment
3.1.3 Identification of actinomycetes by RAPD-PCR technique
3.1.3.1 Buffers and solutions
3.1.3.2 Equipment

3.2 Methods
3.2.1 Isolation and characterization of chitinase producing actinomycetes
3.2.1.1 Isolation of streptomycete actinomycetes from tomato rhizosphere soil
3.2.1.2 Isolation of non-streptomycete actinomycetes by the Streptomyces phages technique
3.2.1.2.1 Soil samples for phage isolation
3.2.1.2.2 Propagation hosts

3.2.1.2.3 Phage isolation and purification

3.2.1.2.4 Phage assay

3.2.1.2.5 Plaque morphology

3.2.1.2.6 Host range

3.2.1.2.7 Preparation of the stock phage suspension and treatment of soil samples

3.2.1.3 Qualitative determination of chitinase production by actinomycetes

3.2.1.4 Quantitative determination of chitinase and β-1,3-glucanase

3.2.1.5 Identification of the most promising chitinase-producing actinomycete genera to species level

3.2.1.5.1 Morphological characteristics

3.2.1.5.2 Electron microscopy of spores

3.2.1.5.3 Cultural characteristics and pigmentation

3.2.1.5.4 Physiological characteristics

3.2.1.5.4.1 Utilization of carbon sources

3.2.1.5.4.2 Utilization of nitrogen sources

3.2.1.5.4.3 Tolerance to different concentrations of various inhibitors

3.2.1.5.4.4 Temperature sensitivity

3.2.1.5.4.5 Production of enzymes

3.2.1.5.4.5a Production of lipase enzymes

3.2.1.5.4.5b Production of keratinase enzymes

3.2.1.5.4.5c Production of cellulase enzymes

3.2.1.5.4.5d Production of chitinase enzymes

3.2.1.5.4.5e Production of pectinase enzymes

3.2.1.5.4.5f Production of nitrate reductase enzymes
3.2.1.5.4.6 Hydrogen sulphide production ................................................................. 44
3.2.1.5.4.7 Coagulation and peptonization of milk .................................................... 44
3.2.1.5.4.8 Degradation of complex insoluble compounds ........................................ 44
3.2.1.5.4.9 Degradation of other compounds ............................................................ 45
3.2.1.5.4.10 Antimicrobial activity ............................................................................. 46
3.2.1.5.4.11 Resistance to antibiotics ........................................................................... 47
3.2.1.5.5 Chemotaxonomical characteristics of actinomycete isolates ......................... 47
3.2.1.5.5.1 Detection of characteristic whole-cell sugars ........................................... 47
3.2.1.5.5.2 Determination of diaminopimelic acid (DAP) .......................................... 48
3.2.2 Application of the most promising chitinase producing streptomycete
and non-streptomycete actinomycetes as insect biological control agents ....................... 49
3.2.2.1 Collection of Drosophila melanogaster ............................................................ 49
3.2.2.2 Rearing of Drosophila melanogaster ................................................................ 49
3.2.2.3 Collection and counting of Drosophila eggs .................................................... 49
3.2.2.4 Production of actinomycete inoculum for in vitro experiments ......................... 49
3.2.2.5 Application of actinomycetes on insects .......................................................... 52
3.2.3 Identification of actinomycetes by RAPD-PCR technique ...................................... 53
3.2.3.1 Actinomycetes isolates ................................................................................... 53
3.2.3.2 DNA extraction .............................................................................................. 53
3.2.3.3 Measurement of DNA concentration ............................................................. 54
3.2.3.4 Agarose gel electrophoresis ............................................................................ 55
3.2.3.5 RAPD analysis ............................................................................................... 55
3.2.4 Statistical analysis .............................................................................................. 56

4.0 Results ................................................................................................................... 58

4.1 Isolation and characterization of chitinase producing actinomycetes ......................... 58
4.1.1 Isolation and enumeration of streptomycete and non-streptomycete actinomycetes from tomato rhizosphere soil .................................................. 58
4.1.2 Plaque morphology ............................................................................. 58
4.1.3 Host range ............................................................................................. 58
4.1.4 Qualitative determination of chitinase production by actinomycetes .... 64
4.1.5 Quantitative determination of chitinase and β-1,3-glucanase ............. 64
4.1.6 Identification of the most promising chitinase-producing actinomycete genera to species level ................................................................. 64
4.1.6.1 Characteristics and identification of isolate # 1 .............................. 64
4.1.6.2 Characteristics and identification of isolate # 7 .............................. 71
4.1.6.3 Characteristics and identification of isolate # 40 ............................ 71
4.2 Application of the most promising chitinase-producing streptomycete and non-streptomycete actinomycetes as insect biological control agent ................................................................. 90
4.3 Identification of actinomycetes by RAPD-PCR technique ..................... 94
5.0 Discussion .................................................................................................. 99
References ....................................................................................................... 106
Arabic summary ............................................................................................... 126
ABSTRACT
ABSTRACT

Several actinomycetes species were isolated from UAE soil using conventional microbiological techniques. Out of thirty-eight isolates, only three (one streptomycete actinomycete and two non-streptomycete actinomycetes which were isolated by the actinophages technique) were selected based on their high production of the chitinase enzyme. The three isolates were identified to the species level using cultural, physiological, biochemical and chemotaxonomical characteristics. Isolates 1, 7 and 40 were identified as *Actinoplanes philippinensis*, *Actinoplanes missouriensis* and *Streptomyces clavuligerus*, respectively.

Chitinolytic activity of the three isolates were tested against *Drosophila melanogaster* development as percentage of successful pupal formation. The three actinomycetes were applied individually against *Drosophila melanogaster*, then in pair wise combinations and then all together to study the synergistic and antagonistic effects between them on pupal formation.

The application of either *Actinoplanes philippinensis* or *Actinoplanes missouriensis* gave a good effect, shown as lowest pupal formation percentages, these were 39.43 ± 2.06% and 31.75 ± 3.79%, respectively. Whilst, *Streptomyces clavuligerus* was the least effective being, 55.71 ± 5.56% compared to control treatment.

*Streptomyces clavuligerus* which gave the least activity when applied individually, was synergized in the presence of *Actinoplanes philippinensis* (27.35 ± 3.95%) and *Actinoplanes missouriensis* (33.24 ± 2.97%). Meanwhile, an antagonistic effect was observed when *Actinoplanes philippinensis* and *Actinoplanes missouriensis* were combined. This antagonism resulted in a high percentage (51.06 ± 5.15%) of
successful pupal formation. When all three isolates were combined no antagonism 
\( (37.47 \pm 2.48\%) \) was observed compared to when the isolates were used alone.

The *Actinoplanes philippinensis* and *missouriensis* (isolate 1 and 7, respectively) were 
compared with their standards using the random amplified polymorphic DNA-
polymerase chain reaction (RAPD-PCR) to detect the genetic similarities and 
dissimilarities of DNA using six random primers. The study found that a total number 
of 43 scored bands for isolate1 and its standard, only 18 of them were polymorphic 
comprising 42% and 25 were monomorphic being 58%. As for isolate 7 and its 
standard, there were a total number of 11 scored bands. Eight (72.7%) of them were 
polymorphic while only three (27.3%) were monomorphic.

This work for the first time indicates the potential of screening actinomycetes as 
biocontrol agents against important insect pests of agricultural crops in the United 
Arab Emirates or elsewhere.
LIST OF FIGURES & TABLES
LIST OF FIGURES

Figure (1): Representative structure of a chitin chain as a special polysaccharide polymer composed of units of β-1,4-N-acetyl-D-glucosamine (after Cohen, 1991).

Figure (2): Rearing of the experimental fruit fly, *Drosophila melanogaster*, under laboratory conditions. Insects were kept under standard rearing conditions as described by Poulson (1950).

Figure (3): Plates of starch nitrate agar with colonies of actinomycetes isolated from tomato rhizosphere soil without treating the soil dilution with polyvalent *Streptomyces* phages illustrating the dominance of streptomycete actinomycetes colonies.

Figure (4): Plates of starch nitrate agar with colonies of actinomycetes isolated from tomato rhizosphere soil after treating the soil with polyvalent *Streptomyces* phages. Note the reduction of streptomycete actinomycete and the increase in non-streptomycete actinomycete. The arrows indicate the colonies of non-streptomycetes.

Figure (5): Plates of peptone yeast extract agar (PYCa) showing clear plaques after the addition of the actinophage S1 to plates containing a wide range of *Streptomyces* species.

Figure (6): Plates of colloidal chitin agar (CCA) inoculated with actinomycetes with large clear zones of chitinase enzyme production.

Figure (7): Plates of colloidal chitin agar (CCA) inoculated with isolate # 1 with clear zone of chitinase enzyme production.

Figure (8): Plates of colloidal chitin agar (CCA) inoculated with isolate # 7 with clear zone of chitinase enzyme production.

Figure (9): Plates of colloidal chitin agar (CCA) inoculated with isolate # 40 with clear zone of chitinase enzyme production.

Figure (10): Cultural characteristics of isolate # 1 on the upper (a) and lower (b) surfaces of oat meal yeast extract agar (OMYEA) after 10 days of incubation at 30°C.

Figure (11): Light microphotograph of *Actinoplanes philippinensis* (Isolate # 1) (400X). Note sporangia with attached sporangiophore (thick arrow) and germinating zoospores (thin arrow).

Figure (12): Cultural characteristic of isolate # 7 on the upper (a) and lower (b) surfaces of oat meal yeast extract agar (OMYEA) after 10 days of incubation at 30°C.
Figure (13): Light microphotograph of *Actinoplanes missouriensis* (Isolate # 7) (400X). Note sporangia with attached sporangiophore (thick arrow) and germinating zoospores (thin arrow).

Figure (14): Cultural characteristic of isolate # 40 on the upper (a) and lower (b) surfaces of oat meal yeast extract agar (OMYEA) after 10 days of incubation at 30°C.

Figure (15): Light microphotograph of *Streptomyces clavuligerus* (Isolate # 40) (400X) showing chains of spores characteristic for *Streptomyces* species.

Figure (16): Effect of chitinase producing actinomycete isolates (1, 7 and 40) on *Drosophila melanogaster*. Data are means ± SE.

Figure (17): Effects of combinations of the three actinomycete isolates on *Drosophila melanogaster*. Data are means ± SE.

Figure (18): Plates of oat meal yeast extract agar (OMYEA) inoculated with *Actinoplanes philippinensis* (Isolate # 1) and its standard, *Actinoplanes missouriensis* (Isolate # 7) and its standard. They were used for RAPD-PCR technique.

Figure (19): DNA fingerprints of two actinomycetes isolates (# 1 and 7) with their corresponding standards (*Actinoplanes philippinensis* (ATCC 12427) and *Actinoplanes missouriensis* (ATCC 145380)). PCR amplifications were performed using six random primers. Molecular weight are given as Kbp. Arrows point at stable and reproducible polymorphic bands.
LIST OF TABLES

Table 1: The major four types of insecticides being heavily used in the chemical control of insects (after Miller, 1993).

Table 2: Production of antibiotics by actinomycetes (Data taken from Cross, 1982; Goodfellow and Williams, 1986; Goodfellow, 1988; Nolan and Cross, 1988; Prescott et al., 1996; Kim et al., 2001; Saugar et al., 2002 and Garg et al., 2002).

Table 3: Examples of chitinase producing microorganisms with their target species compiled from several literature sources.

Table 4: Duration of Drosophila melanogaster stages throughout its life cycle under rearing conditions described by Poulson (1950).

Table 5: DNA sequences of the random RAPD primers used in the identification of the two actinomycetes isolates.

Table 6: Comparison of colony forming units (cfu) of streptomycete and non-streptomycete actinomycetes from starch nitrate agar plates with and without the treatment with polyvalent Streptomyces phages.

Table 7: Propagation hosts and host range of Streptomyces phages.

Table 8: Cell wall chemotype and whole cell sugar pattern for the three actinomycete isolates.

Table 9: Color of aerial mycelium of the three actinomycetes on five different agar media after 14 days of incubation at 30°C.

Table 10: Color of substrate mycelium of the three actinomycetes on five different agar media after 14 days of incubation at 30°C.

Table 11: Color of medium of the three actinomycetes on five different agar media after 14 days of incubation at 30°C.

Table 12: Melanin pigment production by the three actinomycetes and sensitivity of pigment to pH.

Table 13: Growth of the three actinomycete isolates at different temperatures.

Table 14: Growth of the three actinomycete isolates in the presence of different growth inhibitors.

Table 15: Production of enzyme by the three actinomycetes isolates.

Table 16: Growth of the three actinomycete isolates on different carbon sources.

Table 17: Growth of the three actinomycete isolates on different nitrogen sources.
Table 18: Degradation of complex compounds by the three actinomycete isolates.

Table 19: Sensitivity of the three actinomycetes isolates to different antibiotics measured in mm.

Table 20: Antimicrobial activities of the three actinomycetes isolates against a range of bacteria, yeast and moulds.

Table 21: A comparison between the applications of actinomycetes, *Actinoplanes philippinensis* (isolates 1), *Actinoplanes missouriensis* (isolate 7) and *Streptomyces clavuligerus* (isolate 40), individually, in combinations of two and all three together on the fruit fly *Drosophila melanogaster*. Controls were autoclaved spores suspended in the same amounts of distilled water. A water control was included for proper comparison. Statistical analysis shows Means ± Standard Errors. All treatments were significantly ($P < 0.05$) different from their controls.

Table 22: Characteristic polymorphic bands determined between standards (*Actinoplanes philippinensis* (ATCC 12427) and *Actinoplanes missouriensis* (ATCC 145380)) and samples of *Actinoplanes philippinensis* (isolates 1) and *Actinoplanes missouriensis* (isolate 7) of the highly active actinomycetes against *Drosophila melanogaster*. Bands were generated using six random primers.
Acknowledgements

I would like to introduce my special gratitude to my great father H.H. Sheikh Zayed bin Sultan Al-Nahyan, the president of UAE, for his support of higher education by providing all possible facilities in all branches of education and by encouraging his nation to escort the development of this era. I would also like to thank H.H. Sheikh Nahyan bin Mubarak Al-Nahyan, the Chancellor of UAE University, for his support of the scientific research. My thanks to Dr. Hadeef bin Ju'aan Al-Dhaheri, the Vice-Chancellor of UAE University, for his support to graduate programmes. I am truly thankful to the Graduate Studies Unit for its generous help to me and to all the programme's students to achieve our researches.

I am extremely grateful to my supervisors Dr. G.G. Gadelhak and Dr. K.A. El-Tarabily (Biology Department, Faculty of Science, UAE University) for providing me with their knowledge in different stages of this study, for encouraging me during my study and for helping me to get best results. For all that I am forever grateful to them.

My thanks to Prof. Mohammed H. Soliman who was before in (Biology Department, Faculty of Science, UAE University) for his help in writing the proposal of this study.

Many thanks to Ms. Huda Al-Hassani, Mrs. Latifa Al-Shamsi, Mrs. Naema Al-Shamsi, Ms. Wafaa Al-Dhaheri and Mrs. Rajaa (Biology Department, Faculty of Science, UAE University) for providing me with required tools and chemicals for my study to facilitate my work and for encouragement.

I would like to thank Mr. Shareef Al-Alfi (Biology Department, Faculty of Science, UAE University) for teaching me how to collect and rear the Drosophila, Mr. Mohammad Lotfi (Biology Department, Faculty of Science, UAE University) for teaching me the statistical analysis. Special thanks to Dr. Mohammad R. Anan (Biology Department, Faculty of Science, UAE University) for his help in doing the identification of actinomycetes by DNA.
Special thanks should go to my family, person by person, my parents for their support and encouragement, my brothers, sisters and friends for their encouragement and support.
CHAPTER ONE
INTRODUCTION
Chapter One

1. Introduction

As environmental contamination by toxic chemicals increases, alternative approaches for controlling pest populations have become research priorities. These have included biological and/or ecological control methods for limiting the destructive impacts of pest populations, especially in agriculture (Nakas and Hagedorn, 1990; Canaday, 1995; Hokkanen and Lynch, 1995).

Biological control methods take advantage of existing amensal, predatory and parasitic relationships that normally control pest populations and often involve the intentional release of pathogens or toxins aimed at specific pest populations. The use of biological insect control agents is likely to increase as farmers search for alternatives to chemical pesticides (Federici and Maddox, 1996).

Public and scientific concerns about the presence of toxic chemicals, including synthetic pesticides and herbicides in food and the environment, has increased in recent years (Falconer, 2002; Smith and Thomson, 2003). There is a growing concern, both in developed and developing countries, about the environmental impact and the environmental contamination resulting from the use of hazardous pesticides in agriculture for controlling plant diseases and insects pests (Whipps, 2001; Read, 2002; Smith and Thomson, 2003). Pesticides have already been proven to cause adverse environmental effects and result in health hazards to humans, as well as other organisms including beneficial natural enemies (Horrigan et al., 2002). The development of resistance in insect pests and plant pathogens to pesticides (Spotts and Cervantes, 1986) and the growing public concern over the health and environmental hazards associated with high
levels of pesticides (Falconer, 2002; Horrigan et al., 2002), have resulted in a significant interest in the development of alternative non-chemical plant protection strategies for pest control. This concern, concomitantly with fascinating progress in biotechnological developments, has lead researchers to develop safer and environmentally feasible control alternatives. Biological control, i.e., the use of biological processes to lower the pathogen inoculum density in order to reduce the disease producing activities (Whipps, 2001), thereby reducing crop loss, is a potential non-hazardous alternative. Biological control using microbial antagonists has emerged as one of the most promising alternatives, either alone or as a part of an integrated control strategy to reduce pesticides inputs (Whipps, 2001).

Several varieties of microorganisms including fungi, bacteria, nematodes and viruses that are antagonistic to insects have been reported as strategies to biologically control insects. However, screens for antagonism have focused primarily on bacteria, fungi, viruses and nematodes (Collier et al., 2001). There is a lack of published information with regard to the use of actinomycetes particularly, rare non-streptomycete actinomycetes as biocontrol agents of insect pests.

Therefore, the major objective of this study was to screen for chitinase-producing streptomycete and rare-slow-growing non-streptomycete actinomycetes from the UAE soils. Potentially active species were tried as biocontrol agents against a representative insect and were identified to the DNA level.
CHAPTER TWO
LITERATURE REVIEW
Chapter Two

2. Literature Review

2.1 Methods of insects control

2.1.1 Chemical control

Chemical insecticides have been classified by Miller (1993) into four major classes being chlorinated hydrocarbons, organophosphates, carbamates and pyrethroids (Table 1).

It has long been known that insects and other pathogens play an important role in food loss. More than 40% of all potential food production loss is caused by insects, weeds and plant pathogens before harvest and about 20% after harvest (Paoletti and Pimentel, 2000). Accordingly, chemical control was considered to be the solution for these problems, however, the extensive use of these chemicals causes many adverse effects to man and to the environment. They may cause human poisoning and cancer in addition to the environmental pollution along with high control costs for farmers (Paoletti and Pimentel, 2000). There are also many social and economical problems due to the continuous use of chemical control agents, such as, (i) their non-specific and broad-spectrum toxicity which can kill not only target pest species but also a number of natural predators and parasites, (ii) high persistence in the environment and (iii) the development of genetic resistance (Tare, 2001). The effects of chemical control on the diversity of insect pests and predatory natural enemies in cotton fields were studies by Su et al. (2002). Results showed that chemical control affected diversity and homogeneity indices, richness in the numbers of pests and predators in different cotton fields. Chemical control had a more significant effect on the diversity of predator than pest communities. The effects of chemical control varied in different types of cotton fields and in different stages of the growing cycle. Effects were more prominent in cotton monocultures or cotton-bean
Table 1. The major four types of insecticides being heavily used in the chemical control of insects (after Miller, 1993).

<table>
<thead>
<tr>
<th>Type</th>
<th>Examples</th>
<th>Persistence</th>
</tr>
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<tbody>
<tr>
<td>Chlorinated hydrocarbons</td>
<td>DDT, aldrin, dieldrin, endrin, heptachlor, toxaphene, lindane, chlordane, kepone, mirex.</td>
<td>High (2-15 years)</td>
</tr>
<tr>
<td>Organophosphates</td>
<td>Malathion, parathion, monocrotophos, methamedophos, methyl parathion.</td>
<td>Low to moderate (normally 1-12 weeks, but some can last several years).</td>
</tr>
<tr>
<td>Carbamates</td>
<td>Carbaryl, mane, profopoxor, mexicabate, aldicarb, aminocarb</td>
<td>Usually low (days to weeks)</td>
</tr>
<tr>
<td>Pyrethroids</td>
<td>Pemethrin, decamethrin</td>
<td>Usually low (days to weeks)</td>
</tr>
</tbody>
</table>
intercropped fields than in those with cotton-wheat intercropping, in late-sown than early sown fields, and in untilled than tilled fields. The effect was less on the edges of intercropped cotton fields than in the middle. The insect pest community was more sensitive to chemical insecticides at the early stage of the growing cycle, while chemical control significantly influenced the insect predator community throughout the growth period of cotton plants (Su et al., 2002).

2.1.2 Biological control as an alternative method of insect control

2.1.2.1 Definition of biological control

Since the first definition of biological control introduced by Smith (1919), there have been many other introduced definitions (Garrett, 1970; Mangenot and Diem, 1979; Freeman, 1981). In 1974, Baker and Cook introduced a sustained and more unified concept or definition of biological control stating that: "biological control is the reduction of the amount of inoculum or disease-producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host, or antagonist, or by mass introduction of one or more antagonists". Collier et al. (2001) identified biological control as the use of bacteria, fungi, viruses and nematodes to control pests and prevent insects from damaging field crops. Biological control can also refer to the application or manipulation of predators, parasitoids, or pathogens to suppress and control insect populations (Grace, 2003). Classical biological control involves deliberate release of exotic natural enemies into new environments in an attempt to limit the density of an invasive species (Louda et al., 2003). Various natural predators, parasites and pathogens (disease-causing bacteria and viruses) can be used to regulate the populations of specific pests. Worldwide, there more than 300 successful biological pest control projects. In the United States, natural enemies have been
used to control about 70 insect pests, and the use of biological control is increasing rapidly as more farmers seek alternatives for chemical pesticides (Miller, 1993).

2.1.2.2 Predators and parasites in insect control

Of all biological control attempts implemented against insects, control of the winter moth, *Operophthera brumata*, is one of the frequently cited and most successful biological agents’ (Caltagirone, 1981; Murdoch et al., 1985). Two of the successful examples of using entomophagous insects are (i) Coccinellidae, natural enemies of pest species especially mites (Chazeau, 1985), aphids (Frazer, 1988), white fly (Gerling, 1990), scale insects (Drea and Gordon, 1990) and mealy bugs (Herren and Neuenschwander, 1991) and (ii) Trichogrammatidae, which contains the egg parasitoid, *Trichogramma* sp. used for the management of Lepidoptera in various crops (Lenteren and Bueno, 2003).

Other major species used in commercial release are *Trissolcus basalis* against the heteropteran, *Nezara viridula*, *Telenomus remus* against the cotton leaf worm, *Spodoptera frugiperda* (Lenteren and Bueno, 2003) and the endoparasite *Micropletes rufiveutris* on the cotton leaf worm *Spodoptera littoralis* (Hegazi et al., 1998). Predatory arthropods have also been used to control one of the major insect pest which is the cabbage root fly (Collier et al., 2001). Other entomophagous insects have been used for the control of insect pests such as using the mite, *Metaseiulus occidentalis* against several pest populations (Loladze, 2003) and *Harmonia axyridis* against immature monarch butterfly, *Danaus plexippus* (Koch et al., 2003).

2.1.2.3 Microbial control of insect pests

Microbial control methods have been developed for the suppression of arthropod pests, especially insects (Longworth and Kalmakoff, 1978; Deacon, 1983; Aronson et al., 1986; Collier et al., 2001; Langewald et al., 2002; Zydenbos et al., 2003). Several commercial microbial insecticides have been developed and marketed. Microbial suppression of
insect populations is aimed at those that cause crop and other plant damage and at those that act as vectors of disease-causing microorganisms. Potentially, many bacterial, viral, nematodal, fungal, and protozoan populations can be used in the control of insect pests and vectors as described below.

2.1.2.3.1 Bacterial pesticides

There are several bacterial pathogens of insects that currently are used as insecticides or that have the potential for such use in the future (Aronson et al., 1986). They include *Bacillus lentiormbus*, *B. popiliae*, *B. sphaericus*, *B. thuringiensis*, *Clostridium malacosorne*, *Pseudomonas aeruginosa*, *Rickettsiella popiliae*, and *Xenorhabdus nematophilus*.

2.1.2.3.1.1 *Bacillus popilliae*

The use of the genus *Bacillus* in the production of bio-insecticides is well documented. An example is the use of *Bacillus popilliae* (Lazare et al., 1996), which are gram positive rods, aerobic or facultative anaerobic spore-forming bacteria (Wu et al., 1999). This bacterium causes an insect disease called the "milky disease" (Koppenhofer et al., 2000). *Bacillus popilliae* has been effectively used in the biological control of many insects i.e. Lepidopteran larvae (Steinkraus et al., 1998), white grubs (Koppenhofer et al., 2000) and Japanese beetle (Koppenhofer and Fuzy, 2002).

Theunis and Aloali’l (1999) reported the susceptibility of the taro beetle, *Papuana uninodis*, (Coleoptera, Scarabaeidae) to two new *B. popilliae* strains. They reported high infection and mortality rates among larvae and adults when they were subjected to the bacterial treatments.
2.1.2.3.1.2 *Bacillus thuringiensis* (Bt)

This bacterium is a facultative anaerobic, motile, gram positive, spore-forming rod. Bt is an important biological control agent since the late 1960s specifically for the control of lepidopteran pests (Aronson *et al*., 1986; Porter *et al*., 1993).

Feitelson (1993) and Zukowski (1995) have reported that the specific bioactivity of Bt is due to the insecticidal crystalline proteins (ICPs) that are encoded by the *cry* genes and are active against susceptible species in the insect orders Coleoptera, Diptera and Lepidoptera. Specific Bt activities against other insect orders i.e. Hymenoptera, Homoptera, Dictyoptera, Mallophaga and to nematodes like *Strongylida, Tylenchida*, mites, *Acari*, flatworms, *Digenea* and protozoa, and *Diplomonadida* have also been reported (Feitelson, 1993; Zukowski, 1995). The insecticidal action of Bt was first observed as the cause of the "Sotto disease" in the silkworm, *Bombyx mori* in 1901 and in the pupae of the flour moth *Anagasta kuehiella* in 1911 (Adams *et al*., 1996).

Schnepf *et al*. (1998) listed the mode of action of Bt in six steps: (1) ingestion of sporulated Bt and ICP by the insect larva, (2) solubilization of the crystalline ICP in the midgut, (3) activation of the ICP by proteases, (4) binding of the activated ICP to specific receptors in the midgut cell membrane, (5) insertion of the toxin in the cell membrane and formation of spores and channels in the gut cell membrane which followed by the destruction of the epithelial cells and (6) subsequent Bt spore germination and septicemia which may lead to mortality (Schnepf *et al*., 1998).

A wide range of insect biological control agents using Bt toxins have been developed. The effect of the toxin on the midgut of the gypsy moth, *Lymantria* was found to cause histological damage. Toxins bind to the midgut brush border membrane and change its electrical properties (Rausell *et al*., 2000). Bt was also used for the control of *Anomala cuprea* (Asano *et al*., 2003) and the economically important pinworm pest on tomatoes.
(Seal and Leibee, 2003). In the mean time, Mendez-Lopez et al. (2003) found that Bt var israelensis was highly toxic to the coffee berry borer Hypothenemus hampei.

2.1.2.3.1.3 Bacillus sphaericus

Bacillus sphaericus is a spore-forming aerobic bacterium. Several strains of this bacterium are pathogenic to mosquito larvae. During sporulation, the most active bacterial strains produce a crystal toxin with a high degree of larvicidal activity (Charles et al., 1996). The toxin is composed of two proteins which are encoded by highly conserved chromosomal genes. After B. sphericus is ingested, these proteins are released in the larva’s midgut where they bind to a specific receptor on midgut brush-border membranes, damaging the midgut epithelium and leading to high mortality (Charles et al., 1996). B. sphaericus was effectively used against other medically important mosquitoes like Culex pipiens, Anopheles pharoensis and Aedes caspius (Labib and Dawoud, 2003). Looking at the cellular structure, authors found that in A. pharoensis larvae midgut mucosa was enlarged, rough endoplasmic reticulum formed numerous concentrical structures as well as mitochondrial swelling. Large vacuoles appeared in the midgut of C. pipiens, and the rough endoplasmic reticulum broke into small vesicles. The midgut of A. caspius showed mitochondrial swelling and vacuolation of the smooth endoplasmic reticulum (Labib and Dawoud, 2003).

Fillinger et al. (2003) evaluated the efficacy of new water-dispersible granular (WDG) formulations of Bt var. israelensis and B. sphaericus for the control of Anopheles gambiae larvae. The larvae were more susceptible to B. sphaericus than to Bt, and the WDG formulations were slightly superior to the powder formulations. Bt WDG did not show a residual effect, irrespective of the concentration applied. The B. sphaericus WDG formulation showed effective significant larval reductions up to 11 days post-treatment (Fillinger et al., 2003).
2.1.2.3.1.4 Other bacterial pathogens

A group of bacterial pathogens that have received consideration, are the non-sporeulating bacterial pathogens of insects, *Pseudomonas aeruginosa*, but problems have been associated with its use as an insecticide because it is an opportunistic pathogen capable of producing disease in humans. It also tends to infect only stressed or injured insects, has a very limited shelf life, and has only a short survival on exposed dry surfaces such as leaves and stems of plants.

Another group of bacterial pathogens occur among the Rickettsiae. Rickettsial infections cause diseases in Coleoptera, Diptera, and Orthoptera, it also causes the blue disease of Japanese beetles, and infects the grubs of the European chafer. Problems similar to those discussed in connection with *P. aeruginosa* have prevented their use in biological control (Atlas and Bartha, 1998).

2.1.2.3.2 Actinomycetes insecticides

Actinomycetes represent a high proportion of the soil microbial biomass and have the capacity to produce insecticidal compounds (Doumbou *et al.*, 2001). Actinomycetes play an important role in biological control of insects through the production of insecticidally active compounds against the house fly *Musca domestica* (Hussain *et al.*, 2002). The mortality of larval and pupal stages were very high reaching up to 90% after actinomycetes treatments (Hussain *et al.*, 2002).

Actinomycetes were effectively used against *Culex quinquefasciatus* (Sundarapandian *et al.*, 2002). Bream *et al.* (2001) reported an insecticidal activity of selected actinomycete strains against the Egyptian cotton leaf worm *Spodoptera littoralis*. Actinomycete strains isolated from soil samples were used to investigate the biological activity of their secondary metabolites on the same insect. In their study, many actinomycete strains caused larval mortality ranging from 10-60%. In addition, considerable lethal effect of
some actinomycetes were observed on insect pupae. *Streptomyces* and *Streptoverticillium* were found to be the most potent actinomycetes affecting the biological and physiological criteria of the leaf worm (Bream et al., 2001).

### 2.1.2.3.3 Viral insecticide

Insect pathogenic viruses have the potential to become useful pesticidal agents (Tinsley, 1979; Deacon, 1983; Williams, 2002; Castillejos et al., 2002). More than 450 viruses have been described from approximately 500 arthropod species.

Viruses have been used in attempts to control outbreaks of a variety of pests, including gypsy moths, Douglas fir tussock moths, pine processionary caterpillars, red-banded leaf rollers, Great Basin tent caterpillars, alfalfa caterpillars, white butterflies, cabbage loopers, cotton bollworms, corn earworms, tobacco budworms, tomato worms, army worms, wattle bagworms, and others (Atlas and Bartha, 1998). A few viruses cause diseases in mites i.e. to European citrus fruit mite (Gustafsson, 1971; David, 1975). Virally induced infertility was proposed as a potential control measure against the economically important corn earworm (*Helicoverpa zea*) pest. Infection by a newly described gonad-specific virus (GSV) resulted in sterile, agonadal (AG) adult moths of the corn earworm that failed to reproduce. The virus did not invade other body cells and did not kill either the larval or moth stages of the corn earworm. The virus appears to infect immature eggs in the female. Mature eggs are not infected, and the infection of larvae or adults becomes manifest only in their offspring. The release of infected but otherwise normal adults could be used to spread infertility in corn earworm populations, thus controlling their numbers. This technique has yet to be field-tested (Raina and Adams, 1995).

Nuclear polyhedrosis viruses cause disease in sawflies. The accidental introduction of the European spruce sawfly (*Gilpinia hercyniae*) into North America in the twentieth century
threatened the spruce forests of North America. Introduction of nuclear polyhedrosis viruses into the European spruce sawfly population caused a spectacular epizootic that reduced the sawfly populations and saved many spruce forests. Similarly, the European pine sawfly *Neodiprion sertifer*, which causes serious damage to pines in Northern United States, has been controlled by introducing insects containing nuclear polyhedrosis viruses into the population, resulting in epizootics. Other sawfly populations have been subjected to similar controls (Atlas and Bartha, 1998). The use of nuclear polyhedrosis viruses for biological control can be optimized by using bioreporters (Yu-Chan *et al.*, 1996). The green fluorescent protein (GFP) introduced into the *Autographa californica* multiple nuclear polyhedrosis virus is useful for the biological control of lepidopterous pests. The baculovirus cause larval death, and the green fluorescence produced by GFP on excitation with light is used for the early detection of infected individuals among healthy larvae. GFP eliminates the need for molecular analysis to detect the presence of viruses in the larvae. Baculovirus containing similar GFPs can be used to predict the dispersal and presence of a virus that adversely affects the environment.

Viruses pathogenic to insects are found in families like Baculoviridae, Poxviridae, Reoviridae, Iridoviridae, Paroviridae, Picornaviridae and Rhabdoviridae. Some of these are nuclear polyhedrosis viruses (NPV), cytoplasmic polyhedrosis viruses (CPV), and granulosis viruses (GV). Nuclear polyhedrosis viruses usually develop in the host cell nuclei; the virions are occluded singly or in groups in polyhedral inclusion bodies (Atlas and Bartha, 1998). Cytoplasmic polyhedrosis viruses develop only in the cytoplasm of host midgut epithelial cells; the virions are occluded singly in polyhedral inclusion bodies (Atlas and Bartha, 1998). Granulosis viruses develop in either the nucleus of the cytoplasm of host fat, tracheal, or epidermal cells; the virions are occluded singly or rarely in pairs in small occlusion bodies called capsules (Atlas and Bartha, 1998).
Out of all virus families, only Baculoviridae have great potential pathogenicity against insects (Skrzecz, 2000). According to the new taxonomic classification, Baculoviridae are divided into two genera containing nuclear polyhedrosis viruses (NPV) and granulosis viruses (GV). The genus NPV is characterized by the presence of polyhedral-shaped viral occlusion bodies containing one or more viral particles. The genus GV has one viral particle in granule-shaped viral occlusion bodies. Viral occlusions are called polyhedra and granules (Skrzecz, 2000). The infection of insects with baculoviruses is initiated with the feeding of insects on leaves contaminated with the virus. In the host midgut, occlusion bodies are solubilized and virions enter the midgut cells, and then other tissues. Finally, infected larvae disintegrate, releasing occlusion bodies to the environment. Baculoviruses can cause diseases at a very low viral threshold against insect populations (Skrzecz, 2000).

2.1.2.3.4 Nematodal insecticides

Entomopathogenic nematodes are effective biological control agents against a number of important insect pests (Dasrat, 2001) such as cabbage root fly (Collier et al., 2001), several coleopteran larval species, the European chafer, Rhizotrogus majalis, the Japanese beetle (Cappaert and Koppenhofer, 2003), Lycoriella solani (Szyyk-Basalyga and Bednarek, 2003) and larvae and pupa of the Luperomorpha suturalis beetle (Yang et al., 2003). Entomopathogenic nematodes was effectively used to control over-wintering larvae of Thaumetopoea pityocampa, which have limited the larval population under the threshold of 50% (Triggiani and Tarasco, 2003).

2.1.2.3.5 Fungal insecticides

Fungi are potentially important in the control of pest populations (Deacon, 1983; Howell, 1990; Grimm, 2001; Maccheroni, 2002; Fargues, 2003). Most studies on entomogenous
fungi (fungi that live on insects) have been concerned with members of the fungal genera *Beauveria, Metarrhizium, Entomophthora*, and *Coelomomyces*.

*Beauveria bassiana* is an entomopathogenic fungus that has great potential as a biological control agent against insect pests (Zydenbos et al., 2003) such as stored-grain beetles, Colorado potato beetle, the codling moth and the corn earworm which are major pests in the United States and the old Soviet Union (Lord, 2001) and the red imported fire ant *Solenopsis invicta* (Bextine and Thorvilson, 2002). Broadcast applications and individual ant mound treatments of *B. bassiana* in alginate pellets coated with peanut oil reduced activity ratings of *S. invicta* populations (Bextine and Thorvilson, 2002).

There were several attempts to use the fungus *Entomophaga maimaiga* for the control of the gypsy moth, with mixed results (Carrington, 1993). Early (1910-1911) attempts in Massachusetts, USA seemed completely infective, yet many years later spectacular epizootics were caused by this fungus in the original release area. More recent (1989-1991) introductions in Michigan, USA were partially successful. The degree of control seems to depend strongly on the weather. The successful sporulation and spread of the pathogen requires periods of 100% humidity (Carrington, 1993).

Fungi of the genus *Aschersonia* were used to control pests of citrus trees in the Soviet Union near the Black Sea. In Florida, *Hirsutella* has been used against the citrus rust mite.

The fungus *Metarrhizium* was used in Brazil to control populations of leaf hoppers and frog hoppers. Members of the genus *Entomophthora* showed promise as pathogens of aphids. Some members of the genus *Coelomomyces* are pathogenic to the larvae of the mosquito populations of *Anopheles*, *Opifex*, and *Aedes*. Because these mosquitoes are important vectors for microbial pathogens of humans, control of these pests by fungal pathogens could become an important contribution to disease control. Similarly, several
fungal species have been shown to produce diseases in mites, which also are important vectors for microbial diseases of humans (Atlas and Bartha, 1998).

2.1.2.3.6 Protozoan Pesticides

Many protozoa are pathogens of arthropods, but for the most part, protozoa are not suited for use as short-term, quick-acting microbial insecticides; few act with sufficient speed to prevent severe crop damage. Protozoa must be applied before outbreaks of disease to affect proper control. The relatively slow development of protozoan infections of pest animal populations- and difficulties with storage and environmental stability-limit the prospects of their use in biological control (Pramer and Al-Rabai, 1973).

Few attempts have been made to use protozoa as a practical measure of pest suppression. There does appear to be some potential for use of protozoa on grasshoppers, mosquitoes, and boll weevils to augment other methods of controlling these pest populations. Some attempts have also been made to control lepidopteran pests of fruit trees using sporozoan protozoa (Pramer and Al-Rabai, 1973).

2.2 Biology and classification of actinomycetes

2.2.1 General properties of actinomycetes

Actinomycetes are a varied group of Gram positive eubacteria that show wide morphological differences ranging from relatively simple rods and cocci to complex mycelial organization similar to that of some eukaryotic microorganisms (Locci and Sharples, 1984; Wenzel et al., 2002). Actinomycetes are placed in the order Actinomycetales. They reproduce by fragmentation of the hyphae or by spores formed in specialized areas of the substrate or aerial mycelium. Most species are aerobic, chemooorganotrophic, mesophilic and grow optimally at a pH near neutrality (Williams and Wellington, 1982; Solans and Vobis, 2003; Pulverer et al., 2003).
Actinomycete are ubiquitous, they are found in soils, fresh and marine environments, cold and warm-blooded animals, and composts (Williams and Wellington, 1982; Doumbou et al., 2001). However, they seem to prefer the soil, litter, dung, and rock surfaces as substrates (Lechevalier, 1981).

Common genera of actinomycetes include: Streptomyces, Actinomadura, Actinoplanes, Micromonospora, Pilimelia, Streptosporangium, nocardioforms, Thermobifida and Thermomonospora (Solans and Vobis, 2003; Trujillo and Goodfellow, 2003). Most Streptomyces spp., which is the largest group in the order, and other members of the Actinomycetales are biologically active components of the soil microflora (Solans and Vobis, 2003). They give a strong odour of freshly cultivated soils which is a characteristic property of compounds produced by streptomyces (Gerber, 1979), and indicates that they are in a physiologically active state. They contribute to the breakdown and recycling of complex organic materials such as proteins, nucleic acids, polysaccharides and lignocellulose found in soils (McCarthy et al., 1986; Doumbou et al., 2001), making the constituents of these compounds available for growth of all the microbiota. Viable counts of several millions per gram are common, and over 20 actinomycete genera have been obtained from soil. Many studies on actinomycetes in soil have in fact been on streptomycele actinomycetes. However, there is little doubt that the genus Streptomyces is the most widespread and important in soil (Williams, 1978).

Actinomycetes are an important group of microorganisms, not only as degraders of organic matter in the natural environment, but also as producers of antibiotics (Table 2), and other useful compounds of commercial interest (Saugar et al., 2002; Bentley et al., 2002; Basilio et al., 2003). In addition, actinomycetes are important for the production of enzymes, such as chitinase (eg. Streptomyces viridificans), cellulases (eg. Thermomonospora spp.), peptidases, proteases (Nocardia spp.), Xylanases (Microbispora spp.), ligninases

16
Table 2. Production of antibiotics by actinomycetes (Data taken from Cross, 1982; Goodfellow and Williams, 1986; Goodfellow, 1988; Nolan and Cross, 1988; Prescott et al., 1996; Kim et al., 2001; Saugar et al., 2002 and Garg et al., 2002).

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Genera selected</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin + polymyxin</td>
<td>Actinomycetes</td>
<td>Williams and Davies (1965)</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>Thermodactylomycetes</td>
<td>Cross (1968)</td>
</tr>
<tr>
<td>Rote Bengal</td>
<td>Streptomyces</td>
<td>Ottow (1968)</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>Actinomadura</td>
<td>Lavrova (1971)</td>
</tr>
<tr>
<td>Tellurile</td>
<td>Actinoplanes</td>
<td>Willoughby (1971)</td>
</tr>
<tr>
<td>Rubomycin</td>
<td>Actinomadura</td>
<td>Lavrova et al. (1972)</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>Nocardia asteroidi</td>
<td>Orchard and Goodfellow (1974)</td>
</tr>
<tr>
<td>Bruneomycin</td>
<td>Actinomadura</td>
<td>Preobrazhenskaya et al. (1975)</td>
</tr>
<tr>
<td>Benzamate</td>
<td>Micromonomospora</td>
<td>Sandrah (1977)</td>
</tr>
<tr>
<td>Penicillin + NaCl</td>
<td>Streptomyces</td>
<td>Mackay (1977)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Micromonomospora</td>
<td>Ivanistkaya et al. (1978)</td>
</tr>
<tr>
<td>Dihydroxymethyl furatrazine</td>
<td>Microtetraspora</td>
<td>Tomita et al. (1980)</td>
</tr>
<tr>
<td>Nalidixic acid + penicillin + tellurite</td>
<td>Actinomadura</td>
<td>Barton and Hughes (1981)</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>Micromonomospora</td>
<td>Athalye et al. (1985)</td>
</tr>
<tr>
<td>Tunicamycin</td>
<td>Micromonomospora</td>
<td>Wakisaka et al. (1982)</td>
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<td>Oxytetracycline</td>
<td>Streptoverticillium</td>
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</tr>
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<td>Vancomycin</td>
<td>Amycolatopsis</td>
<td>Lechevalier et al. (1986)</td>
</tr>
<tr>
<td>Novobiocin and streptomycin</td>
<td>Glycomyces</td>
<td>Labeda (1987)</td>
</tr>
<tr>
<td>Benzylpenicillin + nalidixic acid</td>
<td>Saccharolohrix</td>
<td>Labeda (1987)</td>
</tr>
<tr>
<td>Carbomycin</td>
<td>Streptomyces halstedii</td>
<td>Prescott et al. (1996)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Streptomyces venezuelae</td>
<td>Prescott et al. (1996)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>Streptomyces erythroaues</td>
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</tr>
<tr>
<td>Kanamycin</td>
<td>Streptomyces kanemyceticus</td>
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<tr>
<td>Nystatin</td>
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<td>Streptomycin</td>
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<td>Puromycin</td>
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<tr>
<td>Valaninemycin</td>
<td>Streptomyces viridifaciens</td>
<td>Garg et al. (2002)</td>
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(Nocardia autotrophica), amylases (Thermomonospora curvata), sugar isomerases (Actinoplanes missouriensis), pectinase, hemicellulase and keratinase (Solans and Vobis, 2003). Actinomycetes have also been reported to degrade various biopolymers (Solans and Vobis, 2003) and to have protein synthesis inhibitory activity (Reis et al., 2003). Actinomycetes are also important for transforming aromatic, sterol and steroid compounds and for the degradation of recalcitrant molecules (Lechevalier and Lechevalier, 1985). Actinomycetes are also infectious to humans and animals, causing tuberculosis, leprosy and farmer’s lung disease (Pulverer et al., 2003). Pulmonary actinomycosis caused by Actinomyces meyeri in which hematogenous dissemination caused multiple brain abscesses resembling metatstatic lung cancer (Colmegna et al., 2003) have been reported. In plants, they also cause diseases such as potato scab (Streptomyces scabies). However, they are far more beneficial as crop symbionts and enzyme, antibiotic producers (Goodfellow and Williams, 1983).

2.2.2 Taxonomy of actinomycetes

Taxonomy of actinomycetes has changed in the last decade according to the re-evaluation of the value and significance of criteria used for the characterization of genera and species of actinomycetes (Goodfellow and Cross, 1984). The criteria of classification for the identification of actinomycetes into genera and species were thoroughly reviewed and discussed by Goodfellow and Cross (1984). These included morphological, cultural, physiological, ecological criteria, reliable biochemical, chemical and genetical characters (Goodfellow et al., 1988).

Bergey’s Manual of Systematic Bacteriology (Anon. 1989) divided the actinomycetes into eight groups based on such properties as conidial arrangement, the presence of a sporangium, cell wall type and cell extract sugars. There groups are: actinobacteria, nocardioform actinomycetes, actinomycetes with multilocular sporangia, actinoplanetes,
streptomyces and related genera, maduromycetes, thermomonospora and related genera and thermoactinomycetes (Anon, 1989).

2.2.3 Isolation of actinomycetes

Stages for the isolation of actinomycetes include: (i) selecting the microorganism containing material (ii) growth on laboratory selective media; (iii) substrate pre-treatment; (iv) incubation; and (v) colony selection and purification (Williams and Wellington, 1982; Cross, 1982).

Li et al. (2002) showed that a new method employing extremely high irradiation frequencies was proposed for the selective isolation of actinomycetes from soil. The pretreatment of soil suspensions with extremely high frequencies wavelengths of 5.6 and 7.1 nm led to a non selective isolation of actinomycetes. However, the irradiation of soil suspensions within wavelength bands of 3.8-5.8 and 8-11.5 nm, considerably reduced the total number of isolated actinomycetes and increased the fraction of isolated rare genera up to seven times (Li et al., 2002).

2.2.4 Actinomycetes phage

Actinophages are viruses that infect members of the order Actinomycetales (Ackermann et al., 1985). Since their first isolation by Wieringa and Wiebols (1936), many actinophages have been reported, particularly for members of the genus Streptomyces. Actinophages have been used to investigate relationships between different genera of the Actinomycetales (Bradley and Anderson, 1958). Early studies on activity spectra of Streptomyces phage reported that members of the genera Actinoplanes, Mycobacterium and Streptosporangium to be resistant to Streptomyces phage (Bradley et al., 1961). Prasuer and Falta (1968) examined 19 phages against members of 20 actinomycete genera and demonstrated that phage were active only on hosts with similar cell wall chemotype as defined by Lechevalier and Lechevalier (1970). The generic host ranges of polyvalent
phages isolated against a variety of *Streptomyces* species were studied by Wellington and Williams (1981). They examined the host ranges of polyvalent phages isolated to genera sharing the cell wall chemotype (I) with *Streptomyces* and to genera with different chemotypes. They also reported that polyvalent phages isolated against *Streptomyces* species have lysed only genera of the cell wall chemotype (I). Strains of *Streptomyces*, *Chainsia*, *Actinopycnidium*, *Actinosporangium*, *Microellobosporia*, *Elytrarosporangium*, *Microechinospora*, *Kitasatoa* and *Streptoverticillium* were susceptible to sets of phages which were referred to as family-specific for the streptomycetaceae (Wellington and Williams, 1981). It has been proven difficult to obtain phage which lyses all genera within a given chemotype or all species of a genus. Very few phage have been found to possess a narrow host range and thus to be considered as species specific. However, most of the phages isolated to the genus *Streptomyces* are highly polyvalent and attack a wide range of *Streptomyces* species and also *Streptoverticillium* species (Combes et al., 2002).

In general, phages are usually characterized according to their physiochemical properties, plaques, morphology and their host range (El-Sayed et al., 2001).

The isolation of *Streptomyces* phages are of practical importance for a variety of reasons such as the problems they cause to fermentation industries (Chater, 1986), their value for typing streptomycetes in taxonomic studies (Korn-Wendish and Schneider, 1992), their use for the detection and understanding of host controlled restriction-modification systems (Diaz et al., 1989), their utilization as tools for genetic exchange and analysis in *Streptomyces* spp. (Herron and Wellington, 1990), the study of their general and molecular biology (Lomovskaya et al., 1980) and ecology (Williams et al., 1987) and most recently, for their ability to reduce the numbers of streptomycete actinomycetes on isolation plates in order to select non-streptomycete actinomycetes (Kurthbokie et al., 1992; Long and Amphlett, 1996).
2.3 Chitin

2.3.1 Chemical composition of chitin

Chitin is the major structural polysaccharide of the cuticle of arthropods. It is also an important constituent of the insect cuticular structures, gut lining and midgut peritrophic matrix. Synthesis of cuticular chitin is strictly coordinated with the ecdycone-regulated molting cycle of insect development (Tellam et al., 2000; Gagou et al., 2002).

Chitin formation and deposition are part of well aligned cellular processes associated with growth, molting and metamorphosis that are controlled by insect ecdysone and juvenile hormones (Tripathi et al., 2002). It is composed of unbranched polymer of β-1,4-N-acetyl-D-glucosamine residues (Lezica and Quesaka-allué, 1990; Gooday, 1990).

The β-1,4-N-acetyl-D-glucosamine residues are joined by glycosidic bondings (Figure 1). The structural strength of this material, is due to the fact that the molecules aggregate through hydrogen-bonding (Solomon et al., 1996). Chitin molecules are grouped into bundles and assembled into flexible micro-fibrils which are embedded in and intimately linked to a protein matrix, giving great tensile strength, the commonest arrangement of chitin microfibrils is in a sheet, in which the microfibrils are parallel (Gullan and Cranston, 1994). Chitin formation can be inhibited by diverse groups of compounds and can generally be degraded rapidly by chitinase group of enzymes (Zhang et al., 2000; Tripathi et al., 2002).

2.4 Chitinase enzymes

Chitinase is originally an enzyme used by insects to degrade the structural polysaccharide chitin during the molting process (Zhang et al., 2002). The largest chitinase activity among bacteria has been observed in species of Streptomyces, Serratia, Vibrio and Bacillus (Table 3) (Yang Jiahui et al., 1997; Reguera and Leshrine, 2001).
Figure (1): Representative structure of a chitin chain as a special polysaccharide polymer composed of units of β-1,4-N-acetyl-D-glucosamine (after Cohen, 1991).
**Table 3:** Examples of chitinase producing microorganisms with their target species compiled from several literature sources

<table>
<thead>
<tr>
<th>Genera</th>
<th>Pathogen</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Enterobacter aerogenes</em></td>
<td>Insect and fungi</td>
<td>Yang Jiahui <em>et al.</em> (1997)</td>
</tr>
<tr>
<td><em>Streptomyces</em></td>
<td>Fungi</td>
<td>Gomes <em>et al.</em> (2000)</td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td></td>
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<tr>
<td><em>Streptomyces viridodiasticus</em></td>
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<tr>
<td><em>Micromonospora carbonacea</em></td>
<td>Fungi</td>
<td>El-Tarabily <em>et al.</em> (2000)</td>
</tr>
<tr>
<td><em>Bacillus thuringiensis</em></td>
<td></td>
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<tr>
<td><em>serovar</em></td>
<td>Insects</td>
<td>Sirichotpakorn <em>et al.</em> (2001)</td>
</tr>
<tr>
<td><em>Bacillus thuringiensis</em></td>
<td>Insects</td>
<td>Liu <em>et al.</em> (2002)</td>
</tr>
<tr>
<td><em>Nocardiosis prasina</em></td>
<td>Fungi</td>
<td>Tsujibo <em>et al.</em> (2003)</td>
</tr>
</tbody>
</table>
Chitinase enzyme is very important in the biological control of insects (Reguera and Leschine, 2001) and plant pathogenic fungi (El-Tarabily et al., 2000; El-Tarabily, 2003). Chitinase enzyme(s) have great biotechnological interest for several reasons: (1) they can be used to convert chitin-containing biomass into useful depolymerised components, (2) and for the control of fungal and insect pathogens of plants (Melchers and Stuiver, 2000). Actinomycetes and other bacteria have chitinolytic activity against insects (Gomes et al., 2000), for example. Shternshis (2002) showed the effects of some of these products in the biological control of the raspberry midge blight where metabolites of Bacillus thuringiensis subsp. israelensis and Streptomyces avermitilis were used. The author reported that chitinase 1% spray caused a 4-fold decrease in the severity of midge blight. In addition, the use of chitinase caused a significant suppression of the independent spur blight (Shternshis, 2002).

Species of Streptomyces showed high multiplicity of chitinase genes (Williamson et al., 2000; Saito et al., 2003), as in the case of Streptomyces coelicolor and Streptomyces griseus (Itoh et al., 2003). Meanwhile chitinase over expression through genetic engineering could play an important role in plant defense responses (Mondal et al., 2003).

2.5 Identification of actinomycetes by RAPD-PCR technique

Polymerase chain reaction (PCR) has been demonstrated to be a reliable and highly sensitive tool for the detection of gene sequences (Vinette et al., 2004). There are many techniques of PCR like repetitive-element PCR (rep-PCR) (Rodriguez-Barradas et al., 1995), enterobacterial repetitive intergenic consensus (ERIC-PCR) (Rodriguez-Barradas et al., 1995), quantitative PCR (Rawer et al., 2003) and real-time PCR (RTm-PCR) (Mackay, 2004). Of all the PCR techniques, RAPD (random amplified polymorphic DNA) is the most quick and reliable (Welsh and McClelland, 1990; Williams et al., 1990). It was developed in 1990, in which DNA fragments are amplified by a polymerase
chain reaction (PCR) using a single arbitrary primer (Welsh and McClelland, 1990; Williams et al., 1990). It does not require any sequence data because arbitrary DNA sequences are used as single primers which target unspecified genomic sequences to generate a genetic profile (Caetano-Anolles et al., 1991).

The RAPD assay was used in many fields such as other detection of genetic instability in tumors (Luceri et al., 2000) as well as detecting DNA alterations induced by benzo[a]pyrene (Atienzar et al., 2002b), copper (Atienzar et al., 2001), mitomycin C (Becerril et al., 1999), 4-n-nonylphenol and 17-β estradiol (Atienzar et al., 2002c), chrysotile asbestos (Yoshida et al., 2001), UV or X-rays (Atienzar et al., 2000; Jones and Kortenkamp, 2000), and radionuclide exposure (Theodorakis, 2001). It has been used also to identify many organisms like arthropods (Benecke, 1998), microorganisms such as Brucella species (Tcherneva et al., 2000), mollusca such as Balea biplicata (Hille et al., 2003), protozoa such as Leishmania species (Martinez et al., 2003) and plants (Barcaccia et al., 2003).
2.6 Aims of thesis

The aims of the present investigation were to:

1. Isolate streptomycete actinomycetes using the normal soil dilution plate method.
2. Isolate non-streptomycete actinomycetes using the actinophage method.
3. Screen the isolates for their ability to produce chitinase and B-1,3 glucanase.
4. Apply the most promising chitinase producing actinomycetes as bio-control agents on *Drosophila melanogaster* under laboratory conditions.
5. Identify the most promising chitinase producing actinomycetes using conventional identification methodologies and RAPD DNA-fingerprinting.
CHAPTER THREE
MATERIALS AND METHODS
3. Materials and Methods

3.1 Materials

3.1.1 Isolation and characterization of chitinase producing actinomycetes

3.1.1.1 Composition of media

3.1.1.1.1 Inorganic salt-starch agar (Starch nitrate agar) (SNA) (Küster, 1959)

Soluble starch 10 g, potassium nitrate 2 g, dipotassium hydrogen phosphate 1 g, magnesium sulphate 0.5 g, sodium chloride 0.5 g, calcium carbonate 3 g, ferrous sulphate 0.01 g, trace salt solution 1 ml, agar 20 g in 1 liter of distilled water (pH = 7.2).

*Trace salt solution as adopted by Pridham et al. (1957) was composed of: 0.1 mg per liter of each of the following salts: ferrous sulphate, magnesium chloride, copper sulphate and zinc sulphate.

3.1.1.1.2 Inorganic salt-starch broth (Starch nitrate broth) (SNB) (Küster, 1959)

The medium composition was similar to the medium of starch nitrate agar (SNA) described above, but without the addition of agar.

3.1.1.1.3 Colloidal chitin agar (CCA) (Makkar and Cross, 1982)

Crude chitin from crab shells (Sigma chemical Co., St. Louis, USA) was washed alternately in 1 N NaOH and 1 N HCl for 24 h periods each, on five occasions. It was then washed four times with 95% (v/v) ethanol. Fifteen grams of the purified white chitin was then dissolved with 100 ml of concentrated HCl and stirred in an ice bath for 20 min. The mixture was then filtered through glass wool, and the solution was poured into cold distilled water in order to precipitate the chitin. The insoluble chitin on the glass wool was treated again with HCl, and the process was repeated until no more precipitate was obtained when the filtrate was added to cold water. The colloidal chitin was allowed to
settle overnight and the supernatant was decanted. The remaining suspension was neutralized to pH 7.0 with NaOH. The precipitated chitin was centrifuged, washed with sterile distilled water, and stored as a paste at 4°C. The medium contained: Colloidal chitin (Dry weight) 2 g, calcium carbonate 0.02 g, ferrous sulphate 0.01 g, magnesium sulphate 0.05 g, potassium chloride 1.71 g, disodium hydrogen phosphate 1.63 g, agar 20 g in 1 liter of distilled water (pH = 7.2).

3.1.1.4 Peptone-yeast extract calcium nitrate (PYCa) (Bradley et al., 1961)
Peptone 5 g, yeast extract 3 g, calcium nitrate 0.5 g, agar 15 g in 1 liter of distilled water.

3.1.1.5 Glucose nitrate (Küster, 1959)
Glucose 10 g, potassium nitrate 2 g, dipotassium hydrogen phosphate 1 g, magnesium sulphate 0.5 g, sodium chloride 0.5 g, calcium carbonate 3 g, ferrous sulphate 0.01 g, agar 20 g in 1 liter of distilled water.

3.1.1.6 Basal medium (Küster, 1959)
Potassium nitrate 2 g, dipotassium hydrogen phosphate 1 g, magnesium sulphate 0.5 g, sodium chloride 0.5 g, calcium carbonate 3 g, ferrous sulphate 0.01 g, agar 20 g in 1 liter of distilled water.

3.1.1.7 Glycerol asparagine agar (Pridham and Lyons, 1961)
Glycerol 10 ml, L-asparagine 1 g, dipotassium hydrogen phosphate 1 g, magnesium sulphate 0.5 g, sodium chloride 0.5 g, calcium carbonate 3 g, ferrous sulphate 0.01 g, agar 20 g in 1 liter of distilled water.

3.1.1.8 Oat-meal yeast extract agar (OMYEA) (Küster, 1959)
Twenty grams of oatmeal were steamed in 1 liter of distilled water for 20 min. After filtration, the oatmeal was filtered through cheese cloth, and distilled water was added to restore the filtrate again to 1 liter. One gram of yeast extract, 20 g of agar were added and the pH was adjusted to 7.2.
3.1.1.9 Tyrosine agar (Shirling and Gottlieb, 1966)
Glycerol 15 ml, L-Tyrosine 0.5 g, L-Asparagine 1 g, dipotassium hydrogen phosphate 0.5 g, magnesium sulphate 0.5 g, sodium chloride 0.5 g, ferrous sulphate 0.01 g, agar 20 g in 1 liter of distilled water.

3.1.1.10 Peptone-yeast extract iron agar (Tresner and Danga, 1958)
Peptone 15 g, protease peptone 5 g, ferric ammonium citrate 0.5 g, dipotassium hydrogen phosphate 1 g, sodium thiosulphate 0.08 g, yeast extract 1 g, agar 20g in 1 liter of distilled water.

3.1.1.11 Nutrient agar
Beef extract 1 g, peptone 5 g, yeast extract 2 g, sodium chloride 5 g, agar 20 g in 1 liter of distilled water.

3.1.1.12 Yeast extract malt extract agar (Pridham et al., 1957)
Glucose 4 g, yeast extract 4 g, malt extract 10 g, agar 20 g in 1 liter of distilled water.

3.1.1.13 Hutchinson medium (Hutchinson and Clayton, 1919)
Calcium nitrate 2.5 g, dipotassium hydrogen phosphate 1 g, calcium chloride 0.1 g, magnesium sulphate 0.3 g, sodium chloride 0.1 g, ferrous sulphate 0.1 g and filter paper strips 2 x 10 cm in 1 liter of distilled water.

3.1.1.14 H2S production medium (Küster and Williams, 1964)
Beef extract 1 g, peptone 5 g, yeast extract 2 g, sodium chloride 5 g, potassium nitrate 2 g in 1 liter of distilled water.

3.1.1.15 Pectic enzymes medium (Hankin et al., 1971)
Potassium dihydrogen phosphate 4 g, disodium hydrogen phosphate 6 g, pectin from citrus rind 5 g, yeast extract 1 g, magnesium sulphate 0.2 g, ferrous sulphate 0.001 g, calcium chloride 0.001 g, disodium phosphate 2 g, agar 13 g in 1 liter of distilled water.
3.1.1.16 Nitrate reduction medium (Bacto-nitrate broth) (Gordon and Mihm, 1957)
Beef extract 3 g, peptone 5 g, potassium nitrate 1 g in 1 liter of distilled water.

3.1.1.17 Reagents associated with bacto-nitrate broth

i- Sulfanilic acid-acetic acid
Eight grams of sulfanilic acid were dissolved in 1 liter of 5 N acetic acid (1 part chemically pure glacial acid to 2.5 parts distilled water) and stored in a brown glass bottle.

ii- Dimethyl naphthylamine
Six ml of dimethyl naphthylamine were dissolved in 1 liter of 5 N acetic acid (1 part chemically pure glacial acid to 2.5 parts distilled water) and stored in a brown glass bottle.

iii- Zinc dust (powdered zinc)

3.1.1.18 Tryptone agar (Goodfellow et al., 1979)
Tryptone 20 g, sodium chloride 5 g, agar 20 g in 1 liter of distilled water.

3.1.1.19 Urea broth (Rustigan and Stuart, 1941)
Potassium dihydrogen phosphate 9.1 g, disodium hydrogen phosphate 9.5 g, yeast extract 0.1 g, phenol red 0.01 g in 1 liter of distilled water.

3.1.1.20 Carbon utilization agar basal medium (Shirling and Gottlieb, 1966)
Dipotassium hydrogen phosphate 5.56 g, magnesium sulphate 1 g, potassium dihydrogen phosphate 2.38 g, disodium sulphate 2.64 g, ferrous sulphate 1.1 mg, copper sulphate 6.4 mg, manganese chloride 7.9 mg, zinc sulphate 1.5 mg, agar 15 g in 1 liter of distilled water.

3.1.1.21 Nitrogen utilisation agar basal medium (Williams et al., 1983)
D-glucose 10 g, magnesium sulphate 0.5 g, sodium chloride 0.5 g, ferrous sulphate 0.01 g, dipotassium hydrogen phosphate 1 g, agar 15 g in 1 liter of distilled water.

3.1.1.22 Yeast extract-dextrose broth (Waksman, 1950)
Yeast extract 10 g and dextrose 10 g in 1 liter of distilled water.
3.1.1.23 Basal medium (Luedemann and Brodsky, 1964)

Yeast extract 5 g, calcium carbonate 1 g, agar 15 g in 1 liter of distilled water.

3.1.1.24 Fishmeal extract agar (FMEA) (El-Tarabily et al., 1996).

Fishmeal extract 20 g, glucose 20 g, peptone 5 g, sodium chloride 0.5 g, calcium carbonate 3 g, agar 20 g in 1 liter of distilled water.

3.1.2 Application of the most promising chitinase producing streptomycete and non-streptomycete actinomycetes as insect biological control agent

3.1.2.1 Insects

Wild type of the fruit fly Drosophila melanogaster were collected and kept in plastic vials.

3.1.2.2 Drosophila rearing medium:

The insect colony was kept in the laboratory on an instant medium (Ward’s Inc., New York, USA). The medium constituents were, flaked potatoes and assorted cereals (>90%), yeast (<2%), water-absorbing polymer (<5%) and mold inhibitors plus blue pigment (<5%).

3.1.2.3 Equipment

Insect colonies and experiments were kept in an incubator (Sheldon Manufacturing Inc., USA) at 25 ± 2 °C. Eggs larvae and pupae were monitored using a binocular microscope (Leica Inc., USA).

Regular laboratory dissection tools were used to count, collect and transfer Drosophila eggs during rearing and experimentation.

3.1.3 Identification of actinomycetes by RAPD-PCR technique

3.1.3.1 Buffers and solutions

Tris-base Boric acid EDTA (10 X TBE) and Tris-base EDTA (TE) buffers:
For the 10 X TBE, 108 g of Tris-Base, 55 g of Boric acid and 40 ml of 0.5 M EDTA at pH 8.0 were prepared. While the TE was prepared by mixing 0.01 M Tris-Base at pH 8.0 with 0.001 M EDTA.

3.1.3.2 Equipment

Chemicals used were agarose, Tris-base, Boric acid (Promega, USA), isopropanol (Scharlau, Barcelona, Spain), EDTA, ethidium bromide (Bio Basic, Inc, Swiss), chloroform and isoamylalcohol.

Genomic prep cells and tissue DNA isolation kit and Ready-To-Go RAPD analysis beads (Amersham Pharmacia Biotech, UK).

Water bath (Grant Instruments Ltd, UK), vortex (Autovortex SA6, Stuart Scientific, UK), eppendorf centrifuge (IEC, International Equipment Company, USA), UV-spectrophotometer (WPA, WPA Cambridge, U.K.) were used.

Electrophoresis apparatus, UV-ray transilluminator (Lifetechnology, USA), microwave (National, Japan), thermal cycler (Techne, UK), lambda DNA/ Hind III plus marker and Kb-DNA ladder (Bio Basic Inc, Swiss) and gel camera (Polaroid, UK).
3.2 Methods

3.2.1 Isolation and characterization of chitinase producing actinomycetes.

3.2.1.1 Isolation of streptomyces actinomycetes from tomato rhizosphere soil

Rhizosphere soil samples were collected from tomato fields at ten random sites from Al-Ain city, 140 Kilometers east of Abu Dhabi, United Arab Emirates and were placed into plastic bags. The soil used was a light brownish yellow sandy soil (Satchell, 1978). In the laboratory, soil samples were mixed to ensure uniformity, passed through a 3-mm sieve to remove stones and root debris. The soil was air dried for 4 days at 25°C to reduce gram negative bacteria and stored in sealable plastic bags at 15°C. Soil pH was found to be 7.5 (0.01 M CaCl₂).

For the isolation of streptomyces actinomycetes, three 10 g replicates of each rhizosphere soil sample were dispensed into 100 ml of sterile 1 g l⁻¹ agar (Sigma) solution in deionized water containing 20 g glass beads (3 mm in diameter). The soil suspension was placed in an ultra-sonicator (Virsonic 60, the Virtis Company, Inc., Gardiner, NY, USA) at a frequency of 55,000 cycles sec⁻¹ for 20 sec, and then shaken on a gyratory shaker (Model D-30938, Gesellschaft Für Labortechnik mbH, Burgwedel, Germany) at 250 rpm for 30 min at 28°C. Ten-fold serial dilutions (10⁻² - 10⁻⁵) were made in sterile deionized water and 0.2 ml aliquots were spread with a sterile glass rod over the surface of inorganic salt starch agar (SNA) in sterile plastic, 9 cm diameter Petri-plates. Cooled (45°C) agar was amended with cycloheximide (50 mg l⁻¹) and nystatin (Sigma) (50 mg l⁻¹) immediately prior to pouring plates. Ten plates were used per dilution and dried in a laminar flow-cabinet for 20 min before incubation at 30°C in the dark for 10 days.
Actinomycete colonies were transferred onto oatmeal agar plates supplemented with 1 g l⁻¹ yeast extract (OMYEA). Hyphae and spores of actinomycetes from five plates were removed from the culture surface, suspended and stored in 20% glycerol (cryoprotectant) at -20°C (Wellington and Williams, 1978).

3.2.1.2 Isolation of non-streptomyces actinomycetes by the actinophages technique

3.2.1.2.1 Soil samples for phage isolation

Fresh rhizosphere soil samples were collected from tomato field as described above. The soil was air dried for 4 days at 25°C to reduce Gram negative bacteria, mixed to ensure uniformity, and passed through a 3-mm sieve to remove stones and root fragments. Soil samples were stored as described above at 15°C.

3.2.1.2.2 Propagation hosts

Type strains of *Streptomyces diastaticus* (ATCC 3315), *S. griseus* (NTCT 7807), and *S. hygroscopicus* (ATCC 31955) were used as propagation hosts. All hosts were grown on OMYEA in the dark for 10 days at 30°C which gave optimal growth and sporulation. Spores were harvested by scraping the surface of ten plates into 10 ml of sterile 20% (v/v) glycerol and stored at -20°C.

3.2.1.2.3 Phage isolation and purification

Flasks (250 ml) containing 20 ml of sterile peptone-yeast extract calcium nitrate (PYCa) broth were inoculated with 1 ml of the spore suspension of the prospective *Streptomyces* host and 2 g of the bulk soil sample (Bradley *et al*., 1961). The flasks were then incubated in a gyratory shaker at 200 rpm at 28°C for 48 h. After incubation, the suspensions from each flask were centrifuged for 1 h at 2000 g and the supernatant was filtered through sterile 0.22 μm Millipore membrane filters (Millipore Corporation, MA, USA) and collected in sterile tubes. A 0.3 ml glycerol suspension of each of the three prospective *Streptomyces* hosts were inoculated separately onto PYCa plates and dried for 30 min in a
laminar flow (Vickers and Williams, 1987). After drying the plates, 0.2 ml of each soil filtrate was spotted onto the plates (Bradley et al., 1961). The plates were then incubated at 30°C for 48 h and examined for the presence of plaques (Williams et al., 1980). After incubation, lysis zones on lawns of the Streptomyces hosts were cut out and soaked at 4°C for 2 h in PYCa broth. The broth was then filtered through Whatman paper No. 1 to remove agar particles and the filtrate was again membrane filtered through sterile 0.22 μm Millipore membrane filters. The concentration obtained was \( \times 10^8 \) plaque forming units (pfu ml\(^{-1}\)) of phage suspension. To obtain higher concentrations, the same procedure was repeated by increasing the soaking period in PYCa broth for 24 h. To minimize the bacterial growth which would impede filtration, phage lysate were kept at 4°C. The following day, new lysates were added to the same broth after removing the previously soaked agar particles. This procedure was repeated until titres of \( \times 10^{12} \) pfu ml\(^{-1}\) were achieved; these were used immediately to avoid a decrease in the concentration of the phage suspension.

### 3.2.1.2.4 Phage assay

Phage assays were carried out using the serial ten-fold dilutions (up to \( 10^{15} \)) prepared in PYCa broth. A 0.3 ml of each Streptomyces host spore suspension was inoculated onto PYCa agar and dried in a laminar flow cabinet for 30 min. A 0.2 ml aliquot of phage dilutions were then placed onto PYCa plate and spread carefully with a glass rod. Three replicate plates were used for each dilution. All plates were incubated in the dark at 30°C for 48 h. After incubation, pfu were counted and single plaques were removed from the agar plates and resuspended in 1 ml of PYCa broth at 4°C for 36 h and these purified phage suspensions were stored at 4°C (Williams et al., 1980). A sample of this broth was then filtered and spotted on the PYCa plates previously inoculated with the prospective hosts.
3.2.1.2.5 Plaque morphology

Purified phage suspensions (0.2 ml) containing $10^7$ pfu ml$^{-1}$ were spotted onto PYCa agar seeded with the *Streptomyces* host species (X $10^6$ cfu ml$^{-1}$). The plates were then incubated for 48 h at 30°C and the plaque morphology was noted.

3.2.1.2.6 Host range

All *Streptomyces* hosts including *Streptomyces acrimycini, S. aeruleus, S. albidoflavus, S. champavattii, S. chromofuscus, S. cinerochromogenes, S. diastaticus, S. fimbriatus, S. flaveolus, S. fulvissimus, S. griseoruber, S. griseoviridis, S. griseus, S. hygroscopicus, S. inusitatus, S. roseoflavus, S. torulosus* *Streptoverticillium netropsis* and *Streptoverticillium* sp. were grown on (OMYE A) and the spores were harvested by scraping the surface of ten plates into 10 ml of sterile 20% (v/v) glycerol and stored at -20°C.

The host range of the three phages were studied by spotting 0.2 ml of each phage suspensions containing $10^7$ pfu ml$^{-1}$ onto a PYCa agar plates each previously seeded with a glycerol suspension (X $10^7$ cfu ml$^{-1}$) of one of the host strains used. Each phage suspension was added to the dried *Streptomyces* host seeded plates, 30 min after seeding the plates. The plates were then incubated at 30°C for 48 h and examined for lysis.

3.2.1.2.7 Preparation of the stock phage suspension

A stock phage suspension of about $10^{12}$ pfu ml$^{-1}$ was prepared by mixing each of the individual high-titre phage suspensions. The resulting titre of the stock phage suspension was $10^{12}$ pfu ml$^{-1}$. This titre was chosen because it has been shown that higher reduction rates are obtained for the targeted actinomycetes through the use of high titres (Kurtböke and Williams, 1991). The stock phage suspension was prepared by mixing high-titre phage suspensions of the three polyvalent phages (x$10^{12}$ plaque forming units ml$^{-1}$) and then used to treat tomato rhizosphere soil suspensions in dilution tubes as described by Kurtböke *et al.* (1992). Briefly, 5 g of the air dried rhizosphere soil was placed into 99 ml
of (PYCa) broth (Bradley et al., 1961) in (250 ml) Erlenmeyer flasks and shaken at 200 rpm at 28°C for 30 min in a gyratory shaker. The PYCa broth was used to minimize experimental error, since phage suspensions had to be prepared in this medium. From the soil suspension, two sets of ten-fold dilutions were prepared. The first used the phage suspension as a diluent, and the second used the PYCa broth alone, which acted as a control treatment. One ml of each soil suspension (phage suspension and control suspension) was diluted in a ten fold series, to produce dilutions of \(10^{-3}\), \(10^{-4}\) and \(10^{-5}\) g dry weight of soil ml\(^{-1}\) of the phage suspension or g/dry weight of soil ml\(^{-1}\) of the control broth. The soil dilution tubes were left to stand for 60 min to expose the soil samples to the phages suspension (Kurtbøke et al., 1992).

Starch nitrate agar was used as the isolation medium and contained cycloheximide and nystatin as described above. The plates were then inoculated with 0.2 ml of the selected dilutions (\(10^{-2}\), \(10^{-3}\) and \(10^{-4}\)) of the soil samples treated with and without phages, and dried in a laminar flow cabinet for 30 min (Vickers and Williams, 1987). Plates without phages were used as control treatments. Five plates were used per dilution and incubated at 30°C in the dark for 3 weeks. Actinomycete colonies were counted and were expressed as cfu g dry\(^{-1}\) soil. All colonies were transferred onto OMYEA and all the isolates were tentatively identified.

### 3.2.1.3 Qualitative determination of chitinase production by actinomycetes

The aim of this experiment was to screen the streptomycete and non streptomycete actinomycete isolates for their capacity to produce chitinase enzyme on colloidal chitin agar (CCA). Five-day-old isolates grown on Starch nitrate agar (SNA) were inoculated onto CCA and incubated for 5 days until zones of chitin clearing were seen around and beneath the colonies. Clear zone diameters were measured in (mm) and used as an indicator of chitinase activity. Large diameters represented high activity and small diameters...
represented low activity. According to the results obtained from the qualitative test, three highly active chitinase-producing isolates (HC) were selected for further quantitative studies.

3.2.1.4 Quantitative determination of chitinase and β-1,3-glucanase.

Individual 250-ml Erlenmeyer flasks containing 50 ml of minimal synthetic medium (MSM) (Tweddell et al., 1994) amended with 2 mg ml\(^{-1}\) of colloidal chitin were prepared. Flasks containing colloidal chitin were inoculated with 2 ml of a 20% glycerol suspension of each actinomycete isolate and incubated on a rotary shaker at 250 rpm for 7 days. After incubation, the suspensions from each flask were centrifuged for 30 min at 12 000 \(g\). The supernatant was filtered through sterile Millipore membranes, collected in sterile tubes and was used as a source of the crude enzymes.

Chitinase specific activity was determined according to the method described by Tweddell et al. (1994) and modified by Singh et al. (1999). Chitinase activity was calculated by measuring the release of N-acetyl-D-glucosamine (NAGA). The reaction mixture contained 1 ml of culture supernatant from a colloidal chitin amended MSM and 1 ml of colloidal chitin (10 mg ml\(^{-1}\)) in 50 mM sodium acetate buffer at pH 6.8 and was incubated in a water bath for 1 h at 50°C. After boiling for 15 min the mixture was centrifuged at 2500 \(g\) for 20 min. The concentration of NAGA in the supernatant was determined by the procedure of Reissig et al. (1955). Specific activity \((U = 1\text{ unit of chitinase})\) was defined as the amount of the enzyme that released 1 \(\mu\text{mol of NAGA mg}^{-1}\text{ protein h}^{-1}\).

The specific activity of β-1,3-glucanase was determined by measuring the amount of reducing sugars liberated using dinitrosalicylic acid solution (DNS) (Miller, 1959). The reaction mixture contained 1 ml of laminarin solution (10 mg ml\(^{-1}\)) in 0.2M acetate buffer (pH 5.4). The mixture was incubated in a water bath at 40°C for 1 h and the reaction

38
terminated by adding 3 ml of DNS solution. The color of the end product was developed by boiling for 10 min. Reducing sugar concentration was determined by optical density at 530 nm using a scanning spectrophotometer (UV-2101/3101 PC; Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan). Glucose was used as the calibration standard. Specific activity (U=1 unit of β-1,3-glucanase) was defined as the amount of the enzyme that released 1 μmol of glucose mg⁻¹ protein h⁻¹. The protein content of the enzyme solution was determined with the Folin phenol reagent (Lowry et al., 1951).

3.2.1.5 Identification of the chitinase-producing actinomycete genera to species level
Identification of the three actinomycetes to species level was based on morphological, cultural, physiological and chemotaxonomical characteristics as presented in Bergy’s Manual of Systematic Bacteriology (Anon., 1989). In all the experiments described in this section, the cultures were incubated at 30°C, unless otherwise stated. For the cultural, morphological, physiological, biochemical and nutritional tests described in this section, actinomycete isolates were inoculated onto OMYEA and incubated in the dark for 10 days. Spores and/or mycelial fragments from these plates were used to inoculate solid and liquid media used for these tests.

3.2.1.5.1 Morphological characteristics
Actinomycete isolates were morphologically examined as described by Williams and Davies (1967). This involved cultivating the isolates on (SNA). A number of sterile cover slips were inserted at 45° angle immediately after inoculation of the plates with the isolate. After incubation for 14 days, cover slips colonized by each isolate were carefully removed from the plates, air dried for 10 min, mounted on a glass slide (upper surface down) and examined with high-power objective lenses. The formation of aerial hyphae and fragmentation of the aerial or substrate mycelia were recorded.
For *Actinoplanes* isolates, the cultures were flooded with 20 ml of sterile distilled water for 30 min, and the agar surface then scraped gently. The suspension was transferred to sterile McCartney bottles and allowed to settle for 30 min at room temperature. One drop of the suspension was then examined microscopically for sporangia and zoospores (Vobis, 1989).

### 3.2.1.5.2 Electron-microscopy of spores

A Phillips electron microscope (5600 MD, Eindhoven, The Netherlands) at 80 KV was used for the examination of spores by the method of Tresner *et al.* (1961). This method involved gently pressing electron microscope specimen grids coated with formvar to the sporulating surface of cultures grown on OMYEA. Spore chains which adhered to the coated surfaces of the grids were observed and photographed without fixing or shadowing.

Electron microscope studies were conducted only on *Streptomyces* species.

### 3.2.1.5.3 Cultural characteristics and pigmentation

The type and intensity of growth, color of substrate and aerial mycelia and the reverse color of the media were determined for each actinomycete isolate using the 7 color wheels of Tresner and Backus (1963). These were determined on 7 and 21 day-old cultures grown on SNA, glycerol asparagine agar (GAA) (Pridham and Lyons, 1961), OMYEA, and yeast-extract malt-extract agar (YEMA) (Pridham *et al*., 1957). The plates were incubated in the dark at 30°C. The pH sensitivity of the substrate mycelium and diffusible pigments was assessed by noting any color changes induced by the addition of acid or alkali (Shirling and Gottlieb, 1966).

Melanin pigments production was determined after 3 days incubation at 30°C in the dark on peptone-yeast-extract iron agar (Tresner and Danga, 1958) and tyrosine agar (Shirling and Gottlieb, 1966).
3.2.1.5.4 Physiological characteristics

3.2.1.5.4.1 Utilization of carbon sources

The ability of the actinomycete isolates to assimilate a range of carbon sources was examined by inoculating them onto a carbon utilization agar basal medium amended with one of the following carbon sources: L-arabinose, raffinose, sucrose, D-xylose, meso-inositol, mannitol, maltose, D-fructose, L-rhamnose, D-mannose, D-ribose, D-lactose, inulin, salicin, trehalose, dextran, D-galactose, cellobiose, adonitol, xylitol, sodium acetate, sodium propionate, sodium citrate, sodium malonate and sodium pyruvate (Shirling and Gottlieb, 1966).

Each carbon source was added at 1.0% (w/v) with the exception of sodium citrate, sodium propionate, sodium acetate, sodium malonate and sodium pyruvate, which were added at 0.1% (w/v). The carbon compounds were sterilized by filtration through sterile 0.22 μm Millipore membrane filters and added to the basal medium after autoclaving and cooling to 45°C. Results were recorded after 14 days incubation in the dark at 30°C by comparing growth intensity with that on un-amended basal agar medium (negative control) and on a positive control amended with 1% D-glucose. A positive result was recorded when growth was greater than that on the negative control.

3.2.1.5.4.2 Utilization of nitrogen sources

The ability of actinomycete isolates to utilize a range of nitrogen sources (0.1%, w/v) was examined by inoculating a spore suspension onto a nitrogen utilization agar basal medium (Williams et al., 1983) amended with one of the following nitrogen sources: DL-amino-n-butyric acid, potassium nitrate, L-cysteine, L-valine, L-threonine, L-serine, L-phenylalanine, L-methionine, L-histidine, L-arginine and L-hydroxy proline. Growth was measured after 14 days in the dark at 30°C by comparing test plates on each source with that on the non-supplemented basal medium (negative control) and on a basal medium.
containing L-asparagine (positive control). A positive result was recorded when growth was greater than that in the negative control.

3.2.1.5.4.3 Tolerance to different concentrations of various inhibitors

Different concentrations of sodium chloride (0, 0.5, 5, 10 or 15% w/v), sodium azide (0.01 or 0.02% w/v), phenol (0.1% w/v), potassium tellurite (0.01 or 0.001% w/v), thallous acetate (0.01 or 0.001% w/v), and crystal violet (0.0001% w/v) were used and incorporated into SNA (Williams et al., 1983). Actinomycete growth was recorded after 14 days incubation in the dark at 30°C. Growth in the presence of chemical inhibitors at one or more concentrations was recorded as positive when it was greater than that in the un-supplemented negative control.

3.2.1.5.4.4 Temperature sensitivity

Inoculated SNA plates were incubated at 5, 10, 30, 37, 45 or 52°C in the dark. Growth of each isolate was recorded after 14 days and indicated a positive result.

3.2.1.5.4.5 Production of enzymes

3.2.1.5.4.5a Production of lipase enzymes

One ml of corn oil was added to 3 ml of glucose nitrate broth (GNB) (Küster, 1959) in which the oil replaced the carbon source (glucose). The tubes were inoculated with each isolate and incubated for 2 weeks in the dark at 30°C. Growth of each isolate as well as the disappearance of oil droplets indicated the production of the enzyme which was recorded as a positive result.

3.2.1.5.4.5b Production of keratinase enzymes

Small pieces of washed fine chicken feathers of the white Leghorn breed, were added to the starch nitrate broth in which the feathers replaced starch and potassium nitrate (carbon and nitrogen source, respectively). The feather pieces were inoculated with each isolate
and incubated in the dark at 30°C for 2 weeks. Growth of each isolate and pigment production of the actinomycete isolates on the feathers indicate a positive result.

3.2.1.5.4.5c Production of cellulase enzymes

Strips (20 X 100 mm) of Whatman No. 1 filter paper in 10 ml of Hutchinson liquid medium (Hutchinson and Clayton, 1919) in 25 ml McCartney vials were inoculated with each isolate. Inoculation was carried out by transferring a loop of spore suspension to the surface of the filter paper, as close as possible to the level of the liquid medium. After 21 days of incubation at 30°C in the dark, the filter strips were examined. The maceration of paper revealed cellulolytic activities. Pigmentation on the cellulose filter paper was also recorded (Hutchinson and Clayton, 1919).

3.2.1.5.4.5d Production of chitinase enzymes

These were recorded from 14 day-old cultures of each organism grown on colloidal chitin agar (CCA) at 30°C in the dark by the appearance of clearing zones. Growth intensity was recorded visually by comparing plates of each isolate. Positive colonies have a clear zone around them (Williams et al., 1983).

3.2.1.5.4.5e Production of pectinase enzymes

A solid medium for the detection of pectinolytic activity was based on that of Hankin et al. (1971). Actinomycete isolates were streak inoculated onto the agar medium and the pectinolytic activity was determined after 4-6 days incubation at 30°C in the dark by flooding the culture surface for at least 4 h with a 1% (w/v) aqueous solution of hexadecyltrimethylammonium bromide (Sigma). This reagent precipitates undegraded pectin, leaving a clear zone around colonies with pectinolytic activity.

3.2.1.5.4.5f Production of nitrate reductase enzymes

Six tubes of Bacto nitrate broth (Gordon and Mihm, 1957) were inoculated with 0.2 ml of spore suspension for each actinomycete isolate. The tubes were shaken vigorously and
incubated at 30°C in the dark. After 14 days duplicate tubes were removed and tested, along with uninoculated control tubes, for the presence of nitrite. One ml of sulfanilic acid (Sigma) was added to the contents of each tube and mixed, then 1 ml of dimethyl 1-naphthylamine (Sigma) was added to each tube and mixed. The formation of a distinct pink or red color indicated the reduction of nitrate. Broths cultures which showed a negative nitrite test were then tested for the presence or absence of residual nitrate. This was done by adding a small amount of zinc dust to the tube to which the reagents have been added then allowed to stand for few minutes. The formation of a characteristic pink or red color would indicate that nitrate was not reduced (Gordon and Mihm, 1957).

3.2.1.5.4.6 Hydrogen sulphide production

Filter paper strips (20 X 100 mm) moistened with lead acetate were inserted into the necks of 25 ml McCartney vials containing 15 ml of nutrient broth supplemented with 0.2% potassium nitrate. The tubes were inoculated with spore suspension of each isolate and examined for the appearance of a black color after 14 days at 30°C in the dark (Küster and Williams, 1964). The black color indicated that hydrogen sulphide was produced.

3.2.1.5.4.7 Coagulation and peptonization of milk

Test tubes containing sterile skimmed milk (5 ml per tube) were inoculated with the test isolates and examined after 7 and 14 days at 30°C for coagulation and subsequent liquefaction of milk (Williams et al., 1983).

3.2.1.5.4.8 Degradation of complex insoluble compounds

The degradation of adenine, tyrosine (0.5% w/v), hypoxanthine, xanthine (0.4% w/v), elastin (0.3%), casein and glycogen (1%), testosterone (0.1% w/v) and guanine (0.05% w/v) (Sigma), was detected on SNA in which the complex insoluble compounds replaced starch and potassium nitrate. Plates were inoculated with the selected isolates and
examined after 7 and 14 days at 30°C for the clearing of the insoluble compounds around and beneath each colony growth which was scored as positive result.

3.2.1.5.4.9 Degradation of other compounds

Gelatin (0.4% w/v) and starch (Sigma) (1.0% w/v) degradation were detected on the same agar medium used for degradation of insoluble complex compounds. After 7 days the cultures were flooded with acidified HgCl₂ solution (Sigma) (Frazier, 1926) and iodine solution (0.13 g of iodine and 0.3 g of KI in 100 ml of distilled water) (Cowan, 1974) respectively, and scoring for cleared zones as positive results.

The degradation of DNA (0.2% w/v), and RNA (Sigma) (0.3% w/v) were observed using the Bacto DNase test agar (Difco) and tryptone agar, respectively. In each case, 7 day-old colonies were flooded with 1M HCl and the presence of a clear zone was recorded as positive (Goodfellow et al., 1979).

The degradation of aesculin (0.1%) and arbutin (Sigma) (0.1%) were determined by the method of Kutzner (1976) using a medium containing yeast extract 3 g, ferric ammonium citrate 0.5 g and agar 7.5 g per 1 liter of distilled water. Tubes were examined after 7 and 21 days after inoculation and incubation at 30°C in the dark. A positive result was recorded when the test medium turned black.

The degradation of Tween 80 (Sigma) (1% w/v), was tested on a nutrient agar medium supplemented with Tween 80. After inoculation and incubation at 30°C in the dark for 14 days, growth and opacity of the tubes were examined.

The degradation of urea (0.4% w/v) was recorded after 14 days using the method of (Gordon, 1967). Briefly, 10 ml of a 15% (w/v) solution of urea sterilized by filtration, was added to 75 ml of sterile urease broth (Rustigan and Stuart, 1941). The mixture was pipetted aseptically into sterile tubes and inoculated with actively growing cultures. An alkaline reaction after incubation at 30°C demonstrated the decomposition of urea.
The degradation of allantoin was determined in 3 ml of Rustigan and Stuart's broth (Rustigan and Stuart, 1941) and 0.01 of allantoin (Sigma), autoclaved and inoculated with spores from actively growing cultures. Decomposition of allantoin was indicated by an alkaline reaction after 14 days at 30°C (Gordon, 1967).

3.2.1.5.4.10 Anti microbial activity

The antimicrobial activities of the actinomycete isolates were tested against a range of test-organisms which included:

(i) Gram negative bacteria: *Escherichia coli* and *Pseudomonas fluorescens*.

(ii) Gram positive bacteria: *Staphylococcus aureus* and *Bacillus subtilis*.

(iii) Yeast fungi: *Candida albicans* and *Saccharomyces cerevisiae*.

(iv) Mould fungi: *Fusarium solani*, *Aspergillus niger*, *Rhizoctonia solani* and *Pythium ultimum*.

For bacteria and yeast antagonism, the actinomycete isolates were grown for 6 days on fish meal extract agar at 30°C in the dark after which agar blocks of the cultures (7 mm in diameter) were transferred to the surface of agar plates, freshly seeded with the test organism (Hasegawa et al., 1990). The width of inhibition zones around the test colonies were measured after 24 h for the bacteria and yeasts. Bacteria were cultivated on nutrient agar, and yeast fungi on malt-extract agar.

For fungal antagonism, the actinomycete isolates were streak-inoculated to one side of fishmeal extract agar (FMEA). The plates were then incubated for 8 days to allow the production and diffusion of metabolites into the agar. An agar disk (6 mm diameter) containing mycelium of the test fungus was then placed onto the opposite side of the actinomycete inoculated plates. Mycelial plugs were also placed on uninoculated FMEA separately as uninhibited controls. Cultures were incubated in the dark at 30°C for 7 days and the plates were examined for inhibition of growth after 2 and 6 days. Inhibition was
indicated when mycelial growth of the test fungi in the direction of the actinomycete colony was retarded.

3.2.1.5.4.11 Resistance to antibiotics

The resistance of the actinomycete isolates to ten antibiotics was tested using the filter paper disc method of Goodfellow and Orchard (1974). Briefly, sterile Whatman No. 1 filter paper discs (3 mm in diameter) were soaked in solutions of the following antibiotics: gentamycin sulphate (100 µg ml⁻¹), neomycin sulphate (50 µg ml⁻¹), streptomycin sulphate (100 µg ml⁻¹), rifampicin (Merrell Dow, N.S.W., Australia) (50 µg ml⁻¹), cephaloridine (100 µg ml⁻¹), tetracycline hydrochloride (500 µg ml⁻¹), oleandomycin (Sigma) (100 µg ml⁻¹), penicillin G (Sodium salt, Instituto Biochimico Italiano, Milano, Italy) (10 i.u ml⁻¹), and vancomycin hydrochloride (David Bull Laboratories Pty Ltd, Melbourne, Australia) (50 µg ml⁻¹). The discs were placed over the surface of inorganic salt-starch agar plates, freshly inoculated with the isolates. Plates were examined for inhibition zones after 4 days incubation at 30°C in the dark.

3.2.1.5.5 Chemotaxonomical characteristics of actinomycete isolates

3.2.1.5.5.1 Detection of characteristic whole-cell sugars

The method of Lechevalier (1968) was used to determine the whole-cell sugar pattern for the actinomycete isolates. Actinomycete cells were cultivated in yeast extract-dextrose broth (Waksman, 1950) for 10 days in the dark at 30°C. Samples were washed twice with sterile distilled water, the cells separated by centrifugation, dehydrated by the addition of 95% ethanol for 24 h, and dried for 2 h at 60°C. Fifty milligrams of dry cells were added to an open Pyrex test tube with 1 ml of 1 N H₂SO₄ and the tubes were placed in a boiling water bath for 1 h. The acid hydrolysate was adjusted with saturated barium hydroxide solution to pH = 5. The white precipitate was centrifuged off, and the supernatant fluid plus 5 ml chloroform (as anti-contaminant) was dried at 37°C for 5 h. The dry residue
was taken up in 0.4 ml distilled water and 20 µL were spotted on a sheet of Whatman No. 1 filter paper, drying each fraction of the sample with heated air in such a way that it formed a ring. A standard mixture of sugars, including galactose, glucose, mannose, arabinose, xylose, madurose (3-O-methyl-D-galactose) and ribose (Sigma) were spotted as references. The components of the samples were separated by descending chromatography in the top phase of the system "n-butanol: water: pyridine: toluene" (5 : 3 : 3 : 4 v/v). After 48 h, the visualisation of the sugars was accomplished by spraying the dried, developed chromatograms with acid aniline phthalate (3.25 g phthalic acid dissolved in 100 ml water-saturated butanol plus 2 ml aniline) and heating at 120°C for 5 min. Hexoses appeared as brown spots and pentoses as pink spots.

3.2.1.5.5.2 Determination of diaminopimelic acid (DAP)

The method of Becker et al. (1964) was followed. Actinomycete cells were cultivated, prepared and dried as described for the determination of whole-cell sugars. For each actinomycete isolate, dried cells (10 mg) were hydrolysed for 18 h with 1 ml of 6N HCl in a sealed pyrex tube held at 100°C in an oven. After hydrolysis and cooling, the content of the tube was filtered through Whatman No. 1 filter paper, the sediment was washed with three drops of distilled water and the filtrate was dried three consecutive times on a steam bath to eliminate the HCl. The residue was taken up in 0.3 ml distilled water, and 20 µL of the liquid was spotted and dried on Whatman No. 1 filter paper as described above. Ten µL of a 0.01 M mixture of meso and LL-diaminopimelic acid was spotted to serve as a reference standard. Descending chromatography was carried out overnight using the solvent system: "methanol: water :10 N HCl: pyridine" in ratios of (v/v) (80 : 17.5 : 2.5 : 10). Amino acids were detected by spraying the chromatogram with ninhydrin (Sigma) dissolved in acetone, followed by heating for 2 min at 100°C. Spots of DAP showed olive green colour fading to yellow, whilst other amino acids in the hydrolysate appeared as
purple spots and migrated faster than did the DAP. LL-DAP migrated faster than meso-
DAP.

3.2.2 Application of the most promising chitinase producing streptomycete and non-
streptomycete actinomycetes as insect biological control agents

3.2.2.1 Collection of Drosophila melanogaster

Wild type Drosophila melanogaster was collected in spring 2002, using fruits such as
apples or banana in uncapped bottles to provide Drosophila adults with nourishment and
oviposition sites. Trapped insects were then used to start a laboratory colony.

3.2.2.2 Rearing of Drosophila melanogaster

All Drosophila stages were kept under 25 ± 2 °C (Strickberger, 1962) (Figure 2). The life
cycle of Drosophila, in days is shown in Table 4 according to Poulson (1950).

3.2.2.3 Collection and counting of Drosophila eggs

Drosophila eggs were collected by covering a piece of solidified media with muslin
fabric. Adult male and female Drosophila were allowed into the bottle for oviposition on
the muslin fabric. Insects were left for 24 h and then transferred to a fresh culture bottle.
The fabric with the medium were carefully removed and the muslin with the eggs was
removed. A female Drosophila may lay about 50-75 eggs/day and about 400-500 in 10
days (Strickberger, 1962). Eggs were counted using binocular microscope and were
removed with a spatula.

3.2.2.4 Production of actinomycete inoculum for in vitro experiments

To produce the inoculum for further in vitro experiments, 4 mL aliquots of 20% glycerol
 suspension of each actinomycete isolate were inoculated into 500 mL Erlenmeyer flasks
containing 250 mL of sterilized OMYE broth. Cultures were incubated on a rotary shaker
at 250 rpm for 4 days and then centrifuged at 1300 g for 10 min. Cell pellets were
resuspended, washed twice in sterile distilled water and centrifuged again.
Figure (2): Rearing of the experimental fruit fly, *Drosophila melanogaster*, under laboratory conditions. Insects were kept under standard rearing conditions as described by Poulson (1950).
Table 4: Duration of *Drosophila melanogaster* stages throughout its life cycle under rearing conditions described by Poulson (1950).

<table>
<thead>
<tr>
<th>Days</th>
<th>Approximate Days</th>
<th>Stages</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>Eggs laid.</td>
</tr>
<tr>
<td>2</td>
<td>0-1</td>
<td>Embryogenesis.</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>Hatching from egg (first instar).</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>First molt (second instar).</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>Second molt (third instar).</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>Formation of puparium</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>Prepupal molt (fourth instar)</td>
</tr>
<tr>
<td>8</td>
<td>5 ½</td>
<td>Pupa, Formation of head, wings and legs</td>
</tr>
<tr>
<td>9</td>
<td>7</td>
<td>Pigmentation of pupal eyes</td>
</tr>
<tr>
<td>10</td>
<td>9</td>
<td>Adults emerge from puparium with folded wings</td>
</tr>
<tr>
<td>11</td>
<td>9</td>
<td>Wings unfold to adult size</td>
</tr>
</tbody>
</table>
Concentrations of cells in the washed suspension were determined using a dilution series-plate count method. A concentration of approximate $10^6$ cfu ml$^{-1}$ of each isolate was used as the inoculum.

### 3.2.2.5 Application of actinomycetes on insects

In this experiment, the three most promising actinomycete isolates (1, 7, and 40), which produced the highest level of chitinase activity, were tested to evaluate their potential and ability to inhibit the growth and development of *Drosophila* under controlled laboratory conditions. The three actinomycete isolates were included singly or in various combinations with each other to determine whether biological control could be enhanced by combining the actinomycete isolates.

In total there were fifteen treatment combinations, as following:

1. Water control (*insect only*),
2. Control (*Drosophila* + autoclaved actinomycete number 1),
3. *Drosophila* + actinomycete number 1,
4. *Drosophila* + autoclaved actinomycete number 40,
5. *Drosophila* + actinomycete number 40,
6. *Drosophila* + autoclaved actinomycete number 7
7. *Drosophila* + actinomycete number 7,
8. *Drosophila* + autoclaved actinomycete 7 + autoclaved actinomycete 40,
9. *Drosophila* + actinomycete 7 + actinomycete 40,
11. *Drosophila* + actinomycete 1 + actinomycete 7,
(14) Drosophila + autoclaved actinomycete number 1 + autoclaved actinomycete number 40 + autoclaved actinomycete number 7.

(15) Drosophila + actinomycete number 1 + actinomycete number 40 + actinomycete number 7.

For application, actinomycetes were cultured on OMYEA and incubated at 30°C until sporulation. Spores were then suspended in 10 ml of sterilized distilled water by scraping each plate separately. Controls for each actinomycete isolate were prepared by taking the same amount of autoclaved spore suspension.

After preparing the spore suspensions, seven replicates (each contain 7 ml) for each active and heat-killed actinomycetes were added to 1.5 g of Drosophila media and mixed thoroughly. Drosophila eggs were counted as described above and placed gently on the prepared spores + media vials. Experiments were kept at 25°C for approximately 7 days, until pupation. Successful pupations were counted at the end of the experiment.

3.2.3 Identification of actinomycetes by RAPD-PCR technique

3.2.3.1 Actinomycetes isolates

Pellets of actinomycete isolates 1 and 7 and their standards (Actinoplanes philippinensis (ATCC 12427) and Actinoplanes missouriensis (ATCC 145380)) were prepared by adding sterile distilled water with the spores of actinomycetes and were centrifuged (IEC clinical centrifuge, USA) for 15 min at 2500 g. The supernatant were poured and the pellets were washed twice and were kept at -80°C. Isolate 40 was not tested due to unavailability of a wide range of Streptomyces standards.

3.2.3.2 DNA extraction

This was carried out using Genomic Prep Cells and Tissue DNA Isolation Kit according to the manufacturer's procedure.
Tube f 1. 5 ml containing 600 µl of cell lysis-solution was chilled on ice. The actinomycetes were added to the solution and heated in water bath (Grant Instruments Ltd, England) at 65°C for one hour. The RNase was treated by adding 3 µl of RNase solution to the cell lysate and heating for 1 h at 37°C. The samples were cooled to room temperature and were added to the RNase treated cell lysate. Protein precipitation solution was mixed uniformly with the cell lysate by vortex (Auto vortex SA6, Stuart Scientific, U.K) at high speed for about 20 seconds. The solution was centrifuged at 12,000 g for five min (IEC, International Equipment Company, USA), the proteins formed a tight pellet. The supernatant which contain the DNA was poured into a clean 1.5 ml tube containing 600 µl of 100% isopropanol (Scharlau, Barcelona, Spain) for DNA precipitation and was mixed by inverting gently 50 times until the white threads of DNA form a visible clump. It was then centrifuged at 12,000 g for ten min, the DNA was visible as a small white pellet. The supernatant was poured and the DNA pellet was washed by adding 600 µl of 70% ethanol and inverting the tube several times. Again the tubes were centrifuged at 12,000 g for 15 min, the ethanol was carefully poured off without dislodging the DNA pellet. Tubes were drained on clean absorbent paper and samples were allowed to air dry 15 min. Finally 100 µl of sterile distilled water were added to the DNA pellets.

### 3.2.3.3 Measurement of DNA concentration

Ten µl of DNA was added to each tube containing 990 µl sterile distilled water for dilution. The spectrophotometer (WPA, WPA Cambridge, U.K.) was adjusted to λ = 260 nm to measure the absorbance of the samples. The concentration of DNA (µg/ ml) was measured by the following equation:

\[ OD \text{ (Optical Density)} \times 50 \times \text{Dilution Factor} \]
3.2.3.4 Agarose gel electrophoresis

To prepare 1% agarose gel, one gram of agarose (Promega, USA) was weighed and added to 100 ml of autoclaved 1X TBE (Tris base, Boric acid, EDTA) buffer. The solution was heated in a microwave for 2 min to dissolve the agarose and was then left to cool at 60°C. The ethidium bromide (5 μl) of 10 mg/ml was added. The solution was poured into the electrophoresis apparatus (Lifetechnology, U.S.A). After the gel had solidified, 1 X TBE was poured into the gel box to cover the gel then the comb was removed. The samples were loaded into the gel by taking 10 μl of sample with 3 μl of the loading dye and the volt was adjusted at 120v. The bands of DNA were checked by UV-ray transilluminator (Lifetechnology, U.S.A).

3.2.3.5 RAPD Analysis

The Ready-To-Go RAPD Analysis Kit provided six RAPD primers, each primer is a 10-basepairs of arbitrary sequence. DNA sequences of RAPD primers are shown in Table 5. Beads were checked if it is visible in the bottom of the tube of RAPD analysis tubes (Amersham Biosciences, USA), then the following items were added to the tube contents: 5 μl (5 pm/μl) of a single RAPD primer, 5 μl (50 ng) of template DNA and 15 μl of distilled water. The contents of the tube was mixed by repeatedly pipetting the mixture up and down. Tubes were then centrifuged briefly to precipitate the contents. Samples were placed in a thermal cycler (Techne, UK) using the following profile: 1 cycle: 95°C for 5 min then 35 cycles: 95°C for 1 min, 36°C for 1 min and 72°C for 2 min.

Agarose gel 2% which was prepared using 1x TBE buffer stained with ethidium bromide (0.5 μg/ml) was added and the samples were loaded on to the gel in addition to the Lambda DNA/ Hind III plus marker and KbDNA ladder (Bio Basic Inc, Swiss) and the volt was adjusted at 120v. Bands of DNA were checked by UV-ray transilluminator.
(Lifetechnology, USA) and an image was also captured using the gel camera (Polaroid, UK).

3.2.4 Statistical analysis

A randomized complete block design was used and analysis of variance was carried out using Superanova® (Abacus Concepts, Inc., Berkeley, California, U.S.A). Percentage data were arcsine transformed before analysis of variance was carried out. Significant differences between means were determined by Fisher’s Protected LSD Test at $P = 0.05$. 
Table 5: DNA sequences of the random RAPD primers used in the identification of the two actinomycetes isolates.

<table>
<thead>
<tr>
<th>RAPD Primers</th>
<th>5'-3' DNA Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer 1</td>
<td>5'-d[GGTGCGGGA]-3'</td>
</tr>
<tr>
<td>Primer 2</td>
<td>5'-d[GGGCGCTCC]-3'</td>
</tr>
<tr>
<td>Primer 3</td>
<td>5'-d[GTCAGCCGTA]-3'</td>
</tr>
<tr>
<td>Primer 4</td>
<td>5'-d[AAGAGCCCGT]-3'</td>
</tr>
<tr>
<td>Primer 5</td>
<td>5'-d[AACGCACGCA]-3'</td>
</tr>
<tr>
<td>Primer 6</td>
<td>5'-d[CCGTCAGCA]-3'</td>
</tr>
</tbody>
</table>
CHAPTER FOUR
RESULTS
Chapter Four

4. Results

4.1 Isolation and characterization of chitinase producing actinomycetes

4.1.1 Isolation and enumeration of streptomycete and non-streptomycete actinomycetes from tomato rhizosphere soil

Twenty-three Streptomyces isolates were isolated from SNA plates without the application of Streptomyces phages (Figure 3), whilst fifteen non-streptomycete actinomycetes were isolated from SNA plates with the application of Streptomyces phages (Figure 4). The use of Streptomyces phages with high polyvalency significantly \( P<0.05 \) reduced the numbers of streptomycetes on the isolation plates with a concurrent increase in the numbers of non-streptomycete actinomycetes isolated (Table 6). With this reduction, non-streptomycete actinomycetes were able to form discrete colonies and were easy to isolate and enumerate. Actinoplanes, Micromonospora, Nocardia, and Rhodococcus were readily isolated from soil samples treated with the polyvalent Streptomyces phages.

4.1.2 Plaque morphology

The phages S1 selected to inhibit Streptomyces griseus (NTCT 7807) and S2 selected to inhibit S. diasitaticus (ATCC 3315) formed small circular clear plaques (0.7 and 0.5 mm, respectively), whilst the phage S3 selected to inhibit S. hygroscopicus (ATCC 31955) formed round plaques with sharp edges 0.8 mm in diameter (Figure 5).

4.1.3 Host range

Phages S1, S2 and S3 were not species specific and lysed a wide range of Streptomyces species (Table 7). However, they did not lyse Streptoverticillium species.
Figure (3): Plates of starch nitrate agar with colonies of actinomycetes isolated from tomato rhizosphere soil without treating the soil dilution with polyvalent *Streptomyces* phages illustrating the dominance of streptomycete actinomycetes colonies.
Figure (4): Plates of starch nitrate agar with colonies of actinomycetes isolated from tomato rhizosphere soil after treating the soil with polyvalent *Streptomyces* phages. Note the reduction of streptomyce actinomycete and the increase in non-streptomycete actinomycete. The arrows indicate the colonies of non-streptomycetes.
Table 6: Comparison of colony forming units (cfu) of streptomycete and non-streptomycete actinomycetes from starch nitrate agar plates with and without the treatment with polyvalent *Streptomyces* phages.

<table>
<thead>
<tr>
<th>Number of colony forming units of streptomycetes</th>
<th>Without phage</th>
<th>With phage</th>
</tr>
</thead>
<tbody>
<tr>
<td>cfu / plate</td>
<td>42.35 ± (2.25)*</td>
<td>10.52 x 10^3 ± (1.22)</td>
</tr>
<tr>
<td>cfu / g. dry weight. soil</td>
<td>10.03 x 10^3 ± (0.13)</td>
<td>13.4 ± (1.25)</td>
</tr>
</tbody>
</table>

*Values in the parentheses are the standard error of the mean. The reduction of streptomycetes and the increase of non-streptomycete actinomycetes in soil after treatment with the polyvalent *Streptomyces* phages were significant (P<0.05) using Fisher’s protected PLSD Test.
Figure (5): Plates of peptone yeast extract agar (PYCa) showing clear plaques after the addition of the actinophage S1 to plates containing a wide range of *Streptomyces* species.
Table 7: Propagation hosts and host range of *Streptomyces* phages

<table>
<thead>
<tr>
<th>Species</th>
<th>S 1</th>
<th>S 2</th>
<th>S 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptomyces acrimycinii</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>S. aeruleus</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>S. albidoflavus</em></td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>S. champavatii</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>S. chromofuscus</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>S. cinerochromogenes</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>S. diastaticus</em></td>
<td>+</td>
<td>PH</td>
<td>-</td>
</tr>
<tr>
<td><em>S. fimbriatus</em></td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>S. flaveolus</em></td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>S. fulvissimus</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>S. griseoruber</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>S. griseoviridis</em></td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>S. griseus</em></td>
<td>PH</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>S. hygroscopicus</em></td>
<td>+</td>
<td>-</td>
<td>PH</td>
</tr>
<tr>
<td><em>S. inusitatus</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>S. roseoflavus</em></td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>S. torulosus</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Streptoverticillium netropsis</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Streptoverticillium sp.</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Phage; S1-S3 *Streptomyces* phage. (+): host species susceptible to phage lysis; (-): host species not susceptible to phage lysis; PH: propagation host;
4.1.4 Qualitative determination of chitinase production by actinomycetes

Of the thirty-eight isolates, only three isolates (one streptomycete and two non-streptomycete actinomycetes) were ranked as HC isolates (Figure 6) and chosen for further study. These isolates produced large clear zones on CCA (Figures 7, 8, 9). These three isolates (Isolates 1, 7 and 40) were chosen for further studies. The remaining isolates either produced no chitinase or produced small amounts of chitinase and were not included in subsequent studies.

4.1.5 Quantitative determination of chitinase and β-1,3-glucanase

The three most promising isolates, number 1, 7 and 40 grew well on the MSM containing colloidal chitin, and laminarin. Their chitinase specific activities were 5.23U, 4.77U and 4.65U, whilst the β-1-3 glucanase specific activities were 0.52U, 0.44U and 0.39U, respectively.

4.1.6 Identification of the most promising chitinase-producing actinomycete genera to species level

The actinomycetes isolates were identified to species level using Bergey's Manual of Systematic Bacteriology (Anon., 1989).

4.1.6.1 Characteristics and identification of isolate # 1

Microscopic examination revealed that isolate 1 produced stable branched substrate mycelium. Aerial mycelium was absent. Spores were produced within sporangia. Sporangia were subglobose, borne on short sporangiophores formed above the surface of the culture (Figure 10). Spores were spherical and arranged in irregular coils. Upon immersion in water, motile spores were released from the sporangia (Figure 11). Cell wall contained meso-diaminopimelic acid and glycine. D-xylose and L-arabinose were present in the whole-cell-hydrolysates (cell wall chemotype II). Additional information on the
Figure (6): Plates of colloidal chitin agar (CCA) inoculated with actinomycete isolates with large clear zones of chitinase enzyme production.
Figure (7): Plates of colloidal chitin agar (CCA) inoculated with isolate #1 with clear zone of chitinase enzyme production.
Figure (8): Plates of colloidal chitin agar (CCA) inoculated with isolate #7 with clear zone of chitinase enzyme production.
Figure (9): Plates of colloidal chitin agar (CCA) inoculated with isolate # 40 with clear zone of chitinase enzyme production.
Figure (10): Cultural characteristics of isolate #1 on the upper (a) and lower (b) surfaces of oat meal yeast extract agar (OMYEA) after 10 days of incubation at 30°C.
Figure (11): Light microphotograph of *Actinoplanes philippinensis* (Isolate # 1) (400X). Note sporangia with attached sporangiophore (thick arrow) and germinating zoospores (thin arrow).
cultural, physiological and morphological characteristics is given in Tables 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20.

By consulting the references of the taxonomy of actinomycetes and on the basis of its biological characteristics, the isolate # 1 was identified as *Actinoplanes philippinensis* Couch.

4.1.6.2 Characteristics and identification of isolate # 7

Microscopic examination revealed that isolate 7 produced stable branched substrate mycelium. Aerial mycelium was absent (Figure 12). Spores were produced within sporangia. Sporangia were subglobose, borne on short sporangiophores formed above the surface of the culture. Spores were spherical and arranged in irregular coils. Upon immersion in water, motile spores were released from the sporangia (Figure 13). Cell wall contains meso-diaminopimelic acid and glycine. D-xylose and L-arabinose were present in the whole-cell-hydrolysates (cell wall chemotype II). Additional information on the cultural, physiological and morphological characteristics is given in Tables 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20.

By consulting the references of the taxonomy of actinomycetes and on the basis of its biological characteristics, the isolate # 7 was identified as *Actinoplanes missouriensis* Couch.

4.1.6.3 Characteristics and identification of isolate # 40

Microscopic examination revealed that the isolate 40 produced stable branched substrate mycelium and well developed aerial mycelium (Figure 14). It produced short straight to flexuous chains of spores (rectiflexibles) (Figure 15). Electron microscopy of the spores revealed that the isolate produced cylindrical spores with more or less rounded ends. The spore surface was smooth. The spore mass is dark grayish green with abundant sporulation. The reverse is generally pale yellow to grayish yellow. Melanin pigment is
Figure (12): Cultural characteristic of isolate # 7 on the upper (a) and lower (b) surfaces of oat meal yeast extract agar (OMYEA) after 10 days of incubation at 30°C.
Figure (13): Light microphotograph of *Actinoplanes missouriensis* (Isolate # 7) (400X). Note sporangia with attached sporangiophore (thick arrow) and germinating zoospores (thin arrow).
Figure (14): Cultural characteristic of isolate # 40 on the upper (a) and lower (b) surfaces of oat meal yeast extract agar (OMYEA) after 10 days of incubation at 30°C.
Figure (15): Light microphotograph of *Streptomyces clavuligerus* (Isolate # 40) (400X) showing chains of spores characteristic for *Streptomyces* species.
not produced. The cell wall peptidoglycan contains major amounts of L-diaminopimelic acid.

No characteristics sugars were present in the whole-cell-hydrolysates (cell wall chemotype I). Additional information on the cultural, physiological and morphological characteristics is given in Tables 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20.

By consulting the references of the taxonomy of actinomycetes and on the basis of its biological characteristics, the isolate # 40 was identified as *Streptomyces clavuligerus* Higgens and Kastner.
Table 8: Cell wall chemotype and whole cell sugar pattern for the three actinomycete isolates.

<table>
<thead>
<tr>
<th>Actinomycete</th>
<th>Cell wall chemotype *</th>
<th>Whole cell sugar pattern **</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Actinoplanes philippinensis</em></td>
<td>II</td>
<td>D</td>
</tr>
<tr>
<td><em>Actinoplanes missouriensis</em></td>
<td>II</td>
<td>D</td>
</tr>
<tr>
<td><em>Streptomyces clavuligerus</em></td>
<td>I</td>
<td>No</td>
</tr>
</tbody>
</table>

* Major constituents in wall chemotypes, I, L-DAP and glycine; and II, meso-DAP and glycine; (Lechevalier and Lechevalier, 1970). ** Whole cell sugar patterns of actinomycetes containing xylose and arabinose; D and no, not applicable Lechevalier et al., 1971).
Table 9: Color of aerial mycelium of the three actinomycetes on five different agar media after 14 days of incubation at 30°C.

<table>
<thead>
<tr>
<th>Actinomycetes</th>
<th>Color of aerial mycelium on</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Starch nitrate agar</td>
</tr>
<tr>
<td><strong>Actinoplanes philippinensis</strong></td>
<td>Absent</td>
</tr>
<tr>
<td><strong>Actinoplanes missouriensis</strong></td>
<td>Absent</td>
</tr>
<tr>
<td><strong>Streptomyces clavuligerus</strong></td>
<td>Dark grayish green</td>
</tr>
</tbody>
</table>
Table 10: Color of substrate mycelium of the three actinomycetes on five different agar media after 14 days of incubation at 30°C.

<table>
<thead>
<tr>
<th>Actinomycetes</th>
<th>Color of substrate mycelium on</th>
<th>Starch nitrate agar</th>
<th>Oatmeal agar</th>
<th>Glycerol asparagines agar</th>
<th>Yeast malt agar</th>
<th>Fishmeal extract agar</th>
</tr>
</thead>
</table>
| *Actinoplanes*
| philippinensis      | Orange brown           | Yellow to orange   | Orange brown | Orange brown              | Orange brown   | Orange brown         |
|                     | Orange               | Orange brown        | Orange        | Yellow to orange brown     | Orange          | Orange               |
| *Actinoplanes*
| missouriensis       | Yellowish brown       | Yellowish brown    | Yellow       | Yellowish brown           | Brown           |                      |

79
Table 11: Color of medium of the three actinomycetes on five different agar media after 14 days of incubation at 30°C.

<table>
<thead>
<tr>
<th>Actinomycetes</th>
<th>Color of medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Starch nitrate agar</td>
</tr>
<tr>
<td><em>Actinoplanes</em></td>
<td>Light brown</td>
</tr>
<tr>
<td><em>philippinensis</em></td>
<td>Non pigmented</td>
</tr>
<tr>
<td><em>Actinoplanes</em></td>
<td>Non pigmented</td>
</tr>
<tr>
<td><em>missouriensis</em></td>
<td>Non pigmented</td>
</tr>
<tr>
<td><em>Streptomyces</em></td>
<td>Non pigmented</td>
</tr>
<tr>
<td><em>clavuligerus</em></td>
<td>Non pigmented</td>
</tr>
</tbody>
</table>
Table 12: Melanin pigment production by the three actinomycetes and sensitivity of pigment to pH

<table>
<thead>
<tr>
<th>Actinomycetes</th>
<th>Melanin production on</th>
<th>Sensitivity of</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peptone-yeast-iron agar</td>
<td>Tyrosine agar</td>
<td>Diffusible substrate pigment to pH</td>
<td>Diffusible pigment to pH</td>
</tr>
<tr>
<td>Actinoplanes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>philippinensis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Actinoplanes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>missouriensis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Streptomyces</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>clavuligerus</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 13: Growth of the three actinomycete isolates at different temperatures

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Actinoplanes philippinensis</th>
<th>Actinoplanes missouriensis</th>
<th>Streptomyces clavuligerus</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 °C</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10 °C</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>28 °C</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>37 °C</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>45 °C</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>52 °C</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(+)= growth, (-) = no growth
Table 14: Growth of the three actinomycete isolates in the presence of different growth inhibitors.

<table>
<thead>
<tr>
<th>Growth inhibitor</th>
<th>Actinoplanes philippinensis</th>
<th>Actinoplanes missouriensis</th>
<th>Streptomyces clavuligerus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride (0%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sodium chloride (0.5%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sodium chloride (5%)</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Sodium chloride (10%)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Sodium chloride (15%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sodium azide (0.01%)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Sodium azide (0.02%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phenol (0.1%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Potassium tellurite</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.001%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potassium tellurite (0.01%)</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Thallous acetate (0.001%)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Thallous acetate (0.01%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Crystal violet (0.0001%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(+) = growth, (-) = no growth
Table 15: Production of enzyme by the three actinomycetes isolates.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th><em>Actinoplanes philippinensis</em></th>
<th><em>Actinoplanes missouriensis</em></th>
<th><em>Streptomyces clavuligerus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipolytic</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Keratinolytic</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Cellulolytic</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pectinolytic</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Chitinolytic</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Production of H2S</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Coagulation of milk</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Peptonization of milk</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

(+)= enzyme produced, (-)= enzyme not produced
Table 16: Growth of the three actinomycete isolates on different carbon sources.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Actinoplanes philippinensis</th>
<th>Actinoplanes missouriensis</th>
<th>Streptomyces clavuligerus</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-arabinose</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Raffinose</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-xylose</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Meso-inositol</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mannitol</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-fructose</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-rhamnose</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>D-mannose</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ribose</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-Lactose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Inulin</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Salicin</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trehalose</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Dextran</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-galactose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cellulose</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Celllobiose</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Adonitol</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Xylitol</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Sodium propionate</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Sodium malonate</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(+)= growth, (-) = no growth
Table 17: Growth of the three actinomycete isolates on different nitrogen sources.

<table>
<thead>
<tr>
<th>Nitrogen sources</th>
<th><em>Actinoplanes philippinensis</em></th>
<th><em>Actinoplanes missouriensis</em></th>
<th><em>Streptomyces clavuligerus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-α-amino-n-butyric acid</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Potassium nitrate</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-valine</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>L-threonine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-serine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-phenylalanine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-asparagine</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-methionine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-histidine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-arginine</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>L-hydroxyproline</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(+) = growth, (-) = no growth
Table 18: Degradation of complex compounds by the three actinomycete isolates.

<table>
<thead>
<tr>
<th>Complex compounds</th>
<th>Actinoplanes philippinensis</th>
<th>Actinoplanes missouriensis</th>
<th>Streptomyces clavuligerus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Xanthine</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Elastin</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Casein</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glycogen</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Testosterone</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Guanine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Starch</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DNA</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>RNA</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Aesculin</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Arbutin</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tween 80</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Allantoin</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Urea</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

(+)= degradation of the compound, (-)= no degradation of the compound
Table 19: Sensitivity of the three actinomycetes isolates to different antibiotics measured in mm.

<table>
<thead>
<tr>
<th>Antibiotic (µg mL⁻¹)</th>
<th>Actinoplanes philippinensis</th>
<th>Actinoplanes missouriensis</th>
<th>Streptomyces clavuligerus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gentamicin sulphate (100)</td>
<td>15</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>Neomycin sulphate (50)</td>
<td>30</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Streptomycin sulphate (100)</td>
<td>35</td>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td>Rifampicin (50)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cephaloridine (100)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dimethyl chlorotetracycline (500)</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Oleandomycin (100)</td>
<td>25</td>
<td>21</td>
<td>35</td>
</tr>
<tr>
<td>Penicillin G (10 i.u)</td>
<td>30</td>
<td>25</td>
<td>40</td>
</tr>
<tr>
<td>Vancomycin (50)</td>
<td>45</td>
<td>30</td>
<td>45</td>
</tr>
</tbody>
</table>
Table 20: Antimicrobial activities of the three actinomycetes isolates against a range of bacteria, yeast and moulds.

<table>
<thead>
<tr>
<th></th>
<th>Actinoplanes philippinensis</th>
<th>Actinoplanes missouriensis</th>
<th>Streptomyces clavuligerus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Fusarium solani</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Rhizoctoma solani</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Pythium ultimum</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

(+) = inhibition of growth, (-) = no inhibition of growth
4.2 Application of the most promising chitinase producing streptomycete and non-streptomycete actinomycetes as insect biological control agents

The three isolates of actinomycetes (1, 7 and 40) were selected based on the chitinase activity studies carried out in the previous section. These isolates were propagated under standard laboratory conditions and were thoroughly mixed with the Drosophila medium. A known number of the insect eggs were carefully transferred to each treatment. Treatments included the application of the three isolates individually and in various combinations with each other. Isolate controls were autoclaved spores suspended in distilled water and treated the same way as their active spores.

The most effective biocontrol treatment of D. melanogaster was the synergistic interaction of isolates 1 and 40 which reduced pupal formation by 27.35 ± 3.95%. Isolate 1, 7 and 40 when used alone reduced pupal formation by 39.43 ± 2.06%, 31.75 ± 3.79% and 55.71 ± 5.56%, respectively. When isolates 1 and 7 were combined together an antagonistic interaction was observed with 51.06 ± 5.15% of pupal formation compared to when isolate 1 alone (39.43 ± 2.06%) and isolate 7 alone (31.75 ± 3.79%) (Figure 17 and Table 21). When all three isolates were used in combination a pupal formation was 37.47 ± 2.48% (Figure 17 and Table 21).

There was no significant differences between pupal formation in the water control (91.13 ± 3.15%) and the autoclaved spores of actinomycete isolate 40 (83.09 ± 5.61%) and isolate 7 (89.57 ± 2.68%). However, the autoclaved control of isolate 1 did reduce the amount of pupa formed (73.81 ± 2.72%) compared to the water control (91.13 ± 3.15%).

The application of individual isolates is presented in Figure (16) and Table (21). The water control gave 91.13 ± 3.15%. Autoclaved spores controls data are shown as the percentage of successful pupal formation being 73.81 ± 2.72% for isolate 1, 83.09 ± 5.61% for isolate 40 and 89.57 ± 2.68 for isolate 7. The active spores of isolates 1 and 7
Figure (16): Effect of chitinase producing actinomycete isolates (1, 7 and 40) on *Drosophila melanogaster*. Data are means ± SE. Column number 1 is the water control for all other treatments. A: (2) *Drosophila* + autoclaved actinomycete 1, (3) *Drosophila* + actinomycete 1. B: (4) *Drosophila* + autoclaved actinomycete 40, (5) *Drosophila* + actinomycete 40. C: (6) *Drosophila* + autoclaved actinomycete 7, (7) *Drosophila* + actinomycete 7.
Figure (17): Effects of combinations of the three actinomycete isolates. Data are means ± SE.
Table 21: A comparison between the applications of actinomycetes. *Actinoplanes philippinensis* (isolates 1), *Actinoplanes missouriensis* (isolate 7) and *Streptomyces clavuligerus* (isolate 40), individually, in combinations of two and all three together on the fruit fly *Drosophila melanogaster*. Controls were autoclaved spores suspended in the same amounts of distilled water. A water control was included for proper comparison. Statistical analysis shows Means ± Standard Errors. All treatments were significantly (*P* < 0.05) different from their controls.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Pupal Formation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) water control (insect only)</td>
<td>91.13 ± 3.15&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>(2) control (<em>Drosophila</em> + autoclaved actinomycete 1)</td>
<td>73.81 ± 2.72&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
<td>(3) <em>Drosophila</em> + actinomycete 1</td>
<td>39.43 ± 2.06&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td>(4) <em>Drosophila</em> + autoclaved actinomycete 40</td>
<td>83.09 ± 5.61&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
<tr>
<td>(5) <em>Drosophila</em> + actinomycete 40</td>
<td>55.71 ± 5.56&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>(6) <em>Drosophila</em> + autoclaved actinomycete 7</td>
<td>89.57 ± 2.68&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>(7) <em>Drosophila</em> + actinomycete 7</td>
<td>31.75 ± 3.79&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>(8) <em>Drosophila</em> + autoclaved actinomycetes 7 + 40</td>
<td>81.78 ± 5.75&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
<tr>
<td>(9) <em>Drosophila</em> + actinomycetes 7 + 40</td>
<td>33.24 ± 2.97&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>(10) <em>Drosophila</em> + autoclaved actinomycetes 1 + 7</td>
<td>87.29 ± 2.62&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>(11) <em>Drosophila</em> + actinomycetes 1 + 7</td>
<td>51.06 ± 5.15&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>(12) <em>Drosophila</em> + autoclaved actinomycetes 1 + 40</td>
<td>69.04 ± 5.01&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>(13) <em>Drosophila</em> + actinomycete 1 + 40</td>
<td>27.35 ± 3.95&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(14) <em>Drosophila</em> + autoclaved actinomycetes 1 + 40 + 7</td>
<td>85.93 ± 5.09&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>(15) <em>Drosophila</em> + actinomycetes 1 + 40 + 7</td>
<td>37.47 ± 2.48&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values with the same letter are not significantly (*P* > 0.05) different according to Fishers protected LSD test.
gave the lowest pupal formation percentages, 39.43 ± 2.06% and 31.75 ± 3.79%, respectively. Isolate 40 was least effective of the three and gave a 55.71 ± 5.56% reduction in pupal formation compared to control treatments.

To study the synergistic or antagonistic effects of the three actinomycetes, isolates were introduced to the insect medium in a combination of treatments of two and all three together. The data of this experiment are shown in Figure (17) and Table (21). Isolate 40 which gave the least activity when applied individually, was synergized by the presence of isolate 1 (27.35 ± 3.95) Figure (17c) and 7 (33.24 ± 2.97) Figure (17a). Meanwhile, the combination of isolates 1 and 7, gave an indication of antagonism (51.06 ± 5.15) shown as high percentage of successful pupal formation. Whilst the application of all isolates together showed no antagonism (37.47 ± 2.48) Figure (17d) compared to control treatments.

A highly significant at (P < 0.05) difference between the combination treatment of isolates 1 + 40 (27.35 ± 3.95) and all the other treatments. This makes this combination the best treatment of all. The results for all are shown in Table 21.

### 4.3 Identification of actinomycetes by RAPD-PCR technique:

Two species (isolates 1 and 7) of the effective chitinolytic actinomycetes were compared with their standards using random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR).

RAPD analysis was performed to detect the genetic similarities and dissimilarities at the DNA level between each effective actinomycete (isolates 1 and 7) and their available standard (Figure 18). Six random primers were used to amplify the actinomycetes genome. All primers gave sufficient and reproducible RAPD patterns.

RAPD primers produced visible fragments that varied in size between 0.3 up to 2.5 Kbp. The total number of fragments scored in the RAPD profile was 45 bands (Figure 19 and
Out of these bands, only three fragments (6.7%) were considered monomorphic while the rest (42 bands, 93.3%) were polymorphic.

Out of a total number of 43 scored bands for isolate 1 and its standard (S2), only 18 of them were polymorphic comprising 42% and 25 were monomorphic being 58%. For isolate 7 and its standard (S1), there were a total number of 11 scored bands. Eight (72.7%) of them were polymorphic while only three (27.3%) were monomorphic.

Primers 1 and 2 were not amplified and hence, did not show any banding with isolate 7 and its standard (S1). While primer 3 showed only two bands at 0.7 and 0.8 Kbp with isolate 7 and not with its standard (S1). As for Primer 5, there was a monomorphic band (0.7 Kbp) for isolate 7 and its corresponding standard. Whilst primers 4 and 6 showed clear banding with the same isolate (isolate 7) that was not represented with its standard.

In the case of isolate 1 and its standard (S2), there was a high frequency of monomorphic banding with primers 4 and 5 at the lower Kbp (0.3, up to 0.85), while primer 1 gave the same monomorphic banding but at higher Kbp (0.75, up to 2.5).

In the mean time, isolate 1 and its standard (S2) had a high frequency of polymorphic banding with primers 2 and 3 at the lower Kbp (0.3, up to 0.85), while primer 5 gave the same polymorphic banding but at higher Kbp (0.65, up to 2.5).

When isolates 1 and 7 were compared, the highest number of polymorphic bands were observed with RAPD primers 1 and 2 and the lowest number was recorded for RAPD primers 3 and 6. On the other hand, comparing the two standards (S1 and S2) showed that the highest number of scored polymorphic bands was observed with RAPD primers 1 and 3 and the lowest with RAPD primers 2 and 6.
Figure (18): Plates of oat meal yeast extract agar (OMYEA) inoculated with *Actinoplanes philippinensis* (Isolate # 1) and its standard, *Actinoplanes missouriensis* (Isolate # 7) and its standard. They were used for RAPD-PCR technique.
Figure (19): DNA fingerprints of *Actinoplanes philippinensis* and *missouriensis* with their corresponding standards (*Actinoplanes philippinensis* (ATCC 12427) and *Actinoplanes missouriensis* (ATCC 145380)). PCR amplifications were performed using six random primers. Molecular weight are given as Kbp. Arrows point at stable and reproducible polymorphic bands. A: using primer 1 and 2; B: using primer 3 and 6; C: using primer 4; D: using primer 5.

M1: is the high Kbp reference.

M2: is the low bp reference.
Table 22: Characteristic polymorphic bands determined between standards (*Actinoplanes philippinensis* (ATCC 12427) and *Actinoplanes missouriensis* (ATCC 145380)) and samples of *Actinoplanes philippinensis* (isolates 1) and *Actinoplanes missouriensis* (isolate 7) of the highly active actinomycetes against *Drosophila melanogaster*. Bands were generated using six random primers.

<table>
<thead>
<tr>
<th>Bands MW (Kbp)</th>
<th>Random Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>0.3</td>
<td>S₁</td>
</tr>
<tr>
<td>0.35</td>
<td>-</td>
</tr>
<tr>
<td>0.4</td>
<td>-</td>
</tr>
<tr>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>0.55</td>
<td>-</td>
</tr>
<tr>
<td>0.6</td>
<td>-</td>
</tr>
<tr>
<td>0.65</td>
<td>-</td>
</tr>
<tr>
<td>0.7</td>
<td>-</td>
</tr>
<tr>
<td>0.75</td>
<td>-</td>
</tr>
<tr>
<td>0.8</td>
<td>-</td>
</tr>
<tr>
<td>0.85</td>
<td>-</td>
</tr>
<tr>
<td>0.9</td>
<td>-</td>
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<td>1.0</td>
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</tr>
<tr>
<td>1.5</td>
<td>-</td>
</tr>
<tr>
<td>2.3</td>
<td>-</td>
</tr>
<tr>
<td>2.5</td>
<td>-</td>
</tr>
</tbody>
</table>

S₁: standard type of isolate #7  S₂: standard type of isolate #1
CHAPTER FIVE
DISCUSSION
Chapter Five

5. Discussion

The present study appears to be a first record of an insect being controlled by a rare slow-growing chitinolytic non-streptomycete actinomycete belonging to genus *Actinoplanes*. The two isolates of *Actinoplanes philippinensis* and *Actinoplanes missouriensis* used in the present study produced high levels of chitinase and were capable of killing the insect under laboratory conditions. Meanwhile, the inability of the autoclaved preparation of each chitinase-producing isolate to kill the insect indicates that the reduction in the insect population by the chitinase-producing isolates may be associated with their chitinase production.

The process of cuticular chitin deposition is coordinated with the ecdysteroid regulated molting during insect development. Major protein subunits of the chitin-synthase were proven to be integral membrane proteins on the epidermal cell layer underlying the procuticle region of the integument in insects (Tellam *et al.*, 2000). Chitin production has been one of the plausible targets for insect control.

Chitin synthase genes in an insect like *Drosophila* were elucidated by Gagou *et al.* (2002) where they identified them (DmeChSA and DmeChSB) to be on the other side of centromere of the third chromosome. Chitin formation inhibition is usually achieved either by affecting the catalytic site of the synthase or by interfering at the sulfhydryl-sensitive sites of the synthase during polymerization of the β-1,4-N-acetyl-D-glucosamine residues. Whilst, chitin formation can be inhibited by diverse groups of compounds (cyromazine) and can generally be degraded rapidly by chitinase group of enzymes (Zhang *et al.*, 2000; Tripathi *et al.*, 2002).

In the present study, the actinomycetes *A. philippinensis* (isolate 1), *A. missouriensis* (isolate 7) and *Streptomyces clavuligerus* (isolate 40) were effective producers of
chitinase and caused extensive mortality in *Drosophila melanogaster* under controlled laboratory conditions. In addition, all three isolates were effective against the insect when applied individually or in combinations, and significantly reduced the percentage of pupal formation under the same conditions.

Since the cuticle of insect species consists largely of chitin, it was postulated that chitinase produced by these isolates could be involved in insect control. Therefore, the production of chitinases was used as the criteria for the selection of potential biocontrol agents of insects. Microbial chitinolytic enzymes have been considered important in the biological control of many insects because of their ability to interfere with chitin deposition (Tripathi *et al*., 2002).

Many fungi, bacteria and soil actinomycetes have been screened for their chitinolytic activities (Fabre *et al*., 1988; Vijayan and Balaraman, 1991). Many bacterial genera such as *Serratia, Rickettsiella, Bacillus, Clostridium Pseudomonas,* and *Xenorhabdus* (Aronson *et al*., 1986) have also been reported to be effective biological control agents of many insects from different orders using more than one mode of action. The chitinase enzyme is produced by several fungi and bacteria (Inbar and Chet, 1991; Chernin *et al*., 1997; Frändberg and Schntürer, 1998; Singh *et al*., 1999; El-Tarabily *et al*., 2000). Many actinomycetes produce chitinase in culture (Gupta *et al*., 1995; Singh *et al*., 1999; El-Tarabily *et al*., 2000; Gomes *et al*., 2000) or in sterilized soil (Lockwood and Lingappa, 1963). Chitinolytic actinomycetes previously used for laboratory studies on insects have included several *Streptomyces* sp. (Hussain *et al*., 2002, Sundarapandian *et al*., 2002; Bream *et al*., 2001). Several chitinase producing fungi such as *Beauveria bassiana* were used for the control of fire ants, colorado potato beetle and the codling moth (Zydenbos *et al*., 2003; Bextine and Thorvilson, 2002; Lord, 2001).
The effective mechanism of these chitinolytic isolates may be explained by their ability to produce chitinas enzymes which interfere with the formation of the insect cuticle. It might be possible that additional mechanism(s), that were not the subject of the present study, be involved in the reduction of insect pupal formation such as production of cuticular proteins and the involvement of hormones.

The isolate 1 control, or autoclaved spores, caused a pupal formation percentage of 73.81%. This could be due to heat-resistant materials that may be produced by the organisms and may cause some effects on the insect development. This point may need further investigation.

The application of chitinase producing actinomycetes to the rearing medium of the fruit fly *Drosophila melanogaster*, had a significant effect on their mortality. The actinomycete isolates were all considerably effective compared to their controls. Both *Actinoplanes philippinensis* and *A. missouriensis* significantly reduced insect pupal when applied to the medium individually. Similar data were generated by Regev *et al.* (1996), and Sampson and Gooday (1998) on their work on the effect of the endogenous chitinase activity of Bt against caterpillars of *Spodoptera littoralis* and the larval midges of *Culicoides nubeculosus*. In the present study, synergistic or antagonistic effects of the three actinomycetes isolates alone or in combinations showed that *S. clavuligerus*, which gave the least individual activity was synergized in the presence of *A. philippinensis* or *A. missouriensis*. In contrast, the combination of *A. philippinensis* and *A. missouriensis* was antagonistic as shown by the high percentage of successful pupal formation. There was no antagonism observed when the three actinomycetes were applied together.

Methods commonly used for the enumeration and isolation of soil actinomycetes deal almost exclusively with those suitable for *Streptomyces* species. *Streptomyces* species comprise from 50% (Goodfellow and Cross, 1984) up to 95.3% (Williams and
Wellington, 1982) of the total population of actinomycetes in the soil. They grow relatively rapid, and compete successfully on soil-dilution isolation plates and present an unbalanced picture of the spectrum of actinomycetes which inhabit the soil and the rhizosphere (Goodfellow and Cross, 1984). Streptomyces spp. normally account for 70 to 90% and rarely as low as 5% of the actinomycetes colonies recovered on most agar media inoculated with soil suspensions. This in part could be due to the nature of the isolation media or the methods employed. The nature of the isolation media may also explain why Nocardia spp. are considered to account for 10 to 30% and Micromonospora for 1 to 15% of the actinomycetes colonies growing in solid media. Actinomyces are also considered to be uncommon with the exception of a few aerobic species (Alexander, 1977). Lechevalier and Lechevalier (1967) examined 5000 isolates of actinomycetes from the soil, and found that genera which can be considered as rare, such as Thermomonospora, Actinoplanes, Microbispora, Thermoactinomyces, Streptosporangium, Micropolyspora, Pseudonocardia, and Microellobosporia constitute less than 0.2% of the total isolations. In Japan, Nonomura and Ohara (1971) found that Microbispora and Thermomonospora constituted less than $10^3$ propagules per gram soil. All these reports further support the view that isolation techniques can bias the proportion of non-streptomycete actinomycetes isolated in such studies.

Most studies that have examined the activities of soil actinomycetes have mainly examined the role of streptomycete actinomycetes. In contrast, the present study highlights the importance of using selective isolation techniques, such as the use of polyvalent Streptomyces phages, for the selective isolation of non-streptomycete actinomycetes and to investigate their effect on insect development. Apart from actinomycetes obtained by the application of the conventional soil dilution method, other rare slow-growing non-streptomycete actinomycetes were easily obtained using the
phages technique. It is important to note that the two techniques mentioned above yielded different genera of actinomycetes. The use of the phages technique in the present study, clearly indicate that the application of phage increased the recovery of *Actinoplanes, Micromonospora, Rhodococcus,* and *Nocardia* species, whilst the majority of the colonies obtained by the soil dilution method were found to belong to the genus *Streptomyces.*

The three actinomycete isolates used in the present study were identified on the basis of morphological, cultural, physiological and chemotaxonomical characteristics. The isolates were tentatively identified and grouped to the genus level on the basis of their standard morphological criteria and according to the absence or presence of aerial mycelium, distribution (aerial/substrate) and form of any spores present and stability or fragmentation of substrate mycelium (Cross, 1989). The identification of these taxa was also based on the practical guide to generic identification of actinomycetes by Lechevalier (Anon., 1989). Lechevalier's key based on simple morphological characteristics such as mycelium, conidia, sporangia and other morphological structures as well as cell chemistry characteristics were used to identify the taxa to genus level. The three actinomycete isolates were identified to species level as *A. philippinensis, A. missouriensis* and *S. clavuligerus* based on characteristics specific for each species as presented in Bergy's manual (Anon., 1989) for individual species using morphological, cultural, physiological, biochemical and chemotaxonomical characteristics. No attempt was made to characterize species based on peptidoglycan type, fatty acid pattern, major menaquinone, phospholipid type and molecular % of G + C of DNA (Goodfellow, 1989).

The DNA fingerprinting using the random amplified polymorphic DNA (RAPD) technique to confirm the identification of *A. philippinensis* and *A. missouriensis* by comparing them to their corresponding standards generated enough numerical data to
indicate the similarities and dissimilarities between the four actinomycete species. The PCR amplification using primer 2 gave seven characteristic polymorphic bands while primers 3 and 5 gave four polymorphic bands between the four organisms. This supports the conclusion that these primers could be used to differentiate between actinomycetes species.

Based on that, it seems like primers 2, 3 and 5, which gave many polymorphic bandings, could be the choice primers to separate between actinomycete species as in the case of *A. philippinensis* and its standard (S2). When comparing *A. philippinensis* and *A. missouriensis* specifically, RAPD primers 1 and 2 could also be the characteristic random primers to differentiate between them. This is based on the fact that these two primers gave eight and ten polymorphic bands, respectively. Heuer *et al.* (1997) developed a group-specific primer for the detection of actinomycetes in the environment through using temperature or denaturing gradient gel electrophoresis.

It could be concluded that the comparison of *A. philippinensis* with its standard gave a good degree of certainty to support the notion that both could belong to the same species. In contrast, comparing *A. missouriensis* with its corresponding standard (S1) did not show enough similarities to support the notion that they belonged to the same species. Using conventional identification criteria during the course of this study, have previously confirmed the identification of *A. missouriensis* to species level. However, using DNA-RAPD fingerprinting might have needed further confirmation in this regard.

Although this study has concentrated on actinomycetes at the expense of other groups of potential microbial biocontrol agents such as bacteria, fungi and viruses, it has yielded some interesting information on the taxonomy and biological activity of streptomycete and non-streptomycete actinomycetes. This study highlights the potential of using
chitinase-producing actinomycetes for the biological control of insects having chitin as a major component of their cuticle in the United Arab Emirates or elsewhere.
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114


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ARABIC ABSTRACT
نسبة تكون المذارى (pupae) عالية، 15.1 ± 5.15%. أما توحيد العزلة الثلاثة مم ببعضها لم يعثر على تنازع حيث بلغت 37.47 ± 2.48%.

بعد ذلك تم مقارنة العزلة Actinoplanes missouriensis وأ. Actinoplanes philippinensis تنوعت عزلة في (ATCC 12427) Standards العزلة Actinoplanes والقابلية باستخدام PCR RAPD-ATCC 145380 missouriensis

من خلال الدراسة وجدت أن المجموع الكلي للحزم من العزلة (Bands) الناتجة عن العزلة Actinoplanes والقابلية الخاص بما بلغ 43 من ضمن 25 مختلفه 18 و25 متشابهه 58%. أما philippinensis بالنسبة للعزلة Actinoplanes missouriensis فبلغ المجموع الكلي للحزم (bands) الناتجة 11 منها 8 مختلفه 72.7% و3 متشابهه 27.3%.

وعتبر هذه الدراسة من الدراسات التي تناولت للمرة الأولى استخدام اكتيفوبسيتات التربة كأحد عناصر المحاكاة البيولوجية ضد الحشرات. حيث أثبتت إمكانية تطوير هذه الكائنات الدقيقة لاستخدامها ضد آفات حشرية اقتصادية في المستقبل.
الخلاصة بالعربية:

ت(result) من نوعة (Actinomycetes) 

الإمارات باستخدام تقنيات الكائنات الدقيقة، من 38 عزلة. تم اختيار ثلاث عزلات تبعاً لنتائج (Chitinase enzyme) 

غلاف الفيبر-six) باستخدام 

العديد من الخواص البيولوجية ولون الأصباغ والخواص الفيزيولوجية، والكيمياء الحيوية والخواص 

المورفولوجية. وتم حُددت العزلات الثلاث إلى رقم (1) Actinoplanes philippinensis 

والعزلة رقم (40). Actinoplanes missouriensis 

تلت ذلك تحليل العزلات الثلاث باستخدام 

للعزلة ثلاث لدبيبة الفاكهة (Chitinolytic activity) حيث تم اختبار العزلات 

لمعرفة نسبة نجم تكون العذراء (Drosophila melanogaster) 

الثلاث بشكل فردي ضد الخربة، تم دم العزلات بشكل ثلاثي وثلاثي لدراسة تأثير التحفيز أو 

التحترم.

وقد أُحدثت النتائج أنه في التطبيق الفردي للعزلة أعطى كل من 

تأثير جيد من خلال نسبة المتفقنة لتكوين Actinoplanes missouriensis، philippinensis 

العذراء (Pupae) حيث بلغت 2.06 ± 1.43 في حالة استخدام (Pupae) Actinoplanes philippinensis 

Streptomyces (Actinoplanes missouriensis) فحصت الأقل تأثيراً حيث كانت نسبة تكون العذراء (Pupae) عالية وصلت إلى 55.71 ± 5.56% 

في تجربة دم عزلتين أعطت العزلة Streptomyces clavuligerus 

وكانت نسبة تكون العذراء (pupae) متفقة معنويًا حيث بلغت Actinoplanes philippinensis 127
جامعة الإمارات العربية المتحدة
كلية العلوم
قسم علوم الحياة

عنوان الرسالة: استخدام الاكتئوميسيتات التي تفرز الكيتين في المكافحة البيولوجية للحشرات

اسم الطالبة: فاطمة خميس عبد الله الكعبي

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<th>الوظيفة</th>
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الإهـادة

بفضل الحب والبناء، أهدي هذه الرسالة إلى والدي سمو الشيخ
زايد بن سلطان آل نهيان، رئيس دولة الإمارات العربية المتحدة، وإلي
البلدي، وإلي والدتي العزيزين.

صاحب السمو
الشيخ زايد
بن سلطان آل نهيان
رئيس دولة الإمارات العربية المتحدة

PRESIDENT OF UAE
HIS HIGHNESS
SHEIKH ZAYED
BIN SULTAN AL NAYAN
استخدام الأكتينوميسيتات التي تفرز الكيتيين في المكافحة البيولوجية للحشرات

رسالة مقدمة منطالبة

فاطمة أحمد عبده أحمد الصغيري
بكالوريوس علوم الحيوان كلية العلوم- جامعة الإمارات العربية المتحدة (1999 - 2000)

إلى

جامعة الإمارات العربية المتحدة
استكمالاً لمتطلبات الحصول على درجة الماجستير في العلوم
علوم البيئة

2004 - 2005