Characterisation of Carbamate Degrading Aerobic Bacteria Isolated from Soils of Selected Horticultural Farms in Rift Valley and Central Kenya

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DECLARATION

This thesis is my original work and has not been presented for degree in any other University.

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DEDICATION

This work is dedicated to my beloved grandma Kristine Omedi (Ama) who laid in me a foundation of hardwork, humility, discipline and fear of God. You taught me the ways of patience, discipline, perseverance and respecting others. Thank you for moulding me to whom I am today.

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LIST OF ABBREVIATIONS AND ACRONYMS

µl	micro-liter
DNA	Deoxyribonucleic Acid
HPLC	High Pressure Liquid Chromatography
KOH	Potasium hydroxide
Min	Minutes
Ml	Mili liter
mM	milli molar
MR	Methyl Red
PCR	Polymerase Chain Reaction
v/v	volume by volume
VP	Voges Proskaeur
w/v	Weight by volume

ABSTRACT

The use of pesticides is very critical in protecting the farmers' investment in seeds, fertilizer and labour since they provide a sure cover from damage by pests. The use of pesticides is therefore inevitable and the environmental pollution due to pesticides and their residues will continue to be a challenge. In this study, bacterial strains capable of degrading methomyl (S-methyl-N-[(methylcarbamoyl) oxy]-thioacetimidate) and carbofuran (2, 3-dihydro-2, 2-dimethyl-7-benzofuranyl methylcarbamate) were isolated from soils sampled from horticultural farms with history of pesticide usage in Rift Valley and Central Kenya. High Pressure Liquid Chromatography (HPLC) was used to monitor biodegradation of both methomyl and carbofuran using reference standards and acetonitrile and water as mobile phases. Complete degradation of carbofuran was observed after 90 days while that of methomyl was observed after 40 days of incubation in mineral salt medium supplemented with either carbofuran or methomyl as the sole carbon source. Partial 16S rDNA sequence analysis indicated that the Carbofuran and Methomyl -degrading strains were closely related to members of the genera *Vagococcus, Paracoccus, Pseudomonas, Providencia, Alcaligenes, Bacillus* and *Flavobacterium.* The morphological and biochemical characteristics of the isolates also confirmed the phylogenetic signature. The biodegradation capability of the strains isolated in this study make them candidates for application in bioremediation trials in pesticide contaminated soils.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Organic and inorganic compounds are widely distributed in the environment as a result of their widespread use as pesticides, solvents, fire retardants, pharmaceuticals, and lubricants. Several of these chemicals cause considerable environmental pollution and human health problems due to their persistence and toxicity (Rossberg *et al*., 1986). Agricultural modernization due to increased market demand for agricultural products has greatly facilitated the industrial production and use of pesticides for pest management and vector control thus the diverse environmental contamination with pesticides (Ngowi *et al*., 2007).

Carbamates, synthetic organic chemicals, are highly poisonous pesticides that have found wide usage in agricultural farms as insecticides, fungicides, herbicides, nematicide and acaricides (WHO, 1986). The toxicity of carbamates extends from human beings to both aquatic and terrestrial organisms with high sensitivity in fish and earthworms (WHO, 1986). In humans, cholinergic symptoms, hyperpigmentation and increased sperm abnormalities have been reported. Therefore, carbamates can be potentially hazardous as a result of accidental spills and run off from areas of application. Also of concern is the general lack of disposal methods for excess carbamates and other pesticides.

In addition, several pesticides, including carbofuran and methomyl, may fail to control pests after they are used continuously for a number of years resulting in economic loss from failed crops. This reduction in efficacy could result from the fact that the target pest(s) may develop pesticide resistance analogous to the evolution of antibiotic resistant genes in microorganisms, and thus become insensitive to the pesticide.

It has also become increasingly possible to isolate microorganisms that are capable of degrading xenobiotic and recalcitrant compounds from environments polluted with toxic chemicals (Gibson and Harwood, 2002). Microbes have evolved catabolic pathways to degrade various foreign compounds based on the existence of catabolic plasmids that encode enzymes which aid in degradation of various natural compounds and xenobiotics (Kumar *et al*., 1996). The pesticide degrading genes (PDG) are highly transmissible to related and even unrelated microbial species and therefore cause changes in the vast and diverse gene pool among microbial community within the soil resulting into diverse microbial community capable of degrading natural and xenobiotic compounds (Don and Pemberton, 1981).

Members of genera *Flavobacterium, Pseudomonas* and *Achrobacterium* have been reported to degrade wide range of carbamates and other classes of pesticides with their degradative pathways, respective genetic determinants and regulation in these microorganisms have also been studied (Don and Pemberton, 1981).

Much of the recent research heve focused on microbial ecology, biochemistry and physiology of microbes capable of biodegrading different organic and inorganic pollutants (Ratledge, 1994) with little knowledge on genetic diversity of such biodegradative bacteria. This study focused on presence of methomyl and carbofuran degrading bacterial strains as well as their genetical and geographical diversity within horticultural soils in Rift Valley and Central Kenya.

1.2 Statement of the problem

Agriculture is the backbone of Kenyan economy. There is continuing increase in crop and horticultural farming in Kenya to meet the ever increasing market demand. This has led to modernization of agricultural practices which involves the use of both fertilizers and pesticides. Some of these chemicals pose threat to human health and have long term effect on the environment.

Research shows that some pollutants, such as Polyaromatic hydrocarbons (PAHs) have mutagenic and carcinogenic properties (Cerniglia, 1992) with carbamates being highly toxic to both humans and animals. The recalcitrance of these compounds is due to their chemical structure, environmental conditions and their concentration in the environment. A combination of two or all of these factors determines the bioavailability of these compounds for effective degradation. It has been shown that varied members of bacterial and fungal communities have inherent capability of using these compounds as source of nutrient for their survival. In effect, such microbes aid in biodegradation and biotransformation of these chemicals to a lesser toxic forms.

The great versatility of microorganisms offers a simpler, inexpensive and more environmentally friendly strategy to reduce environmental pollution than non-biological options. This study will unveil the pesticide-biodegrading potentials of aerobic bacteria and their underlying genetic diversity

1.3 Justification

Pesticides pose a major threat to the environment, being mutagenic, carcinogenic, and recalcitrant (Prashant *et al*., 2007). The negative impact associated with the use of pesticides requires the development of novel strategies for their management. Microorganisms are highly versatile and capable of utilizing various chemicals as a supplementary or sole source of nutrients. Though many conventional treatment methods such as adsorption, volatilization, incineration, and advanced oxidation are available for the abatement of pesticide contamination from aqueous and soil phases, these microorganisms can offer simpler, inexpensive and more environmentally friendly strategy for reducing environmental pollution from agricultural and industrial chemicals thus can be used for bioremediation.

Bioremediation enhances the rate of natural microbial degradation of contaminants in the environment by supplementing the environment with batch cultures of enriched microorganisms and sufficient nutrients. Establishing the presence, geographical and genetical diversity of carbamate-degrading bacterial strains within the selected regions in Kenya will enhance their application in bioremediating pesticide contaminated soils.

1.4 Hypothesis

There is no wide diversity of carbamate-degrading aerobic bacteria in horticultural soils.

1.5 Objectives:

1.5.1 General objective

To establish the presence, geographical and genetic diversity of carbamate-degrading aerobic bacteria and their possible application in bioremediation of carbamate-contaminated environments.

1.5.2 Specific objectives

1. To determine pesticide use in selected horticultural farms in Rift Valley and Central Kenya
2. To isolate carbofuran and methomyl degrading aerobic bacteria and characterize the isolates both morphologically and biochemically.
3. To monitor biodegradation of methomyl and carbofuran using High Performance Liquid Chromatography.
4. To identify and characterize molecular diversity of carbofuran and methomyl degrading bacteria using 16S rDNA based analysis.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Pesticide Usage

It is not known with exactitude the date of the introduction of synthetic pesticides into Africa (Wandiga, 2001). However, revolution in pest management occurred seven decades ago with the discovery and use of organochlorines, organophosphates, carbamates and more recently pyrethroids (Rhoda *et al.*, 2006). Pest and diseases are responsible for 30-40 % loss of agricultural produce in the tropics and the most common and conventional way of pest and disease control is through the use of pesticides (Rhoda *et al.*, 2006). Pesticides have become the main tool in pest management for public health, livestock and crop protection and their use has led to substantial suppression of pests and subsequent increase in agricultural production. Pesticide use in Kenya has developed correspondingly with cash crop and food production since the advent of colonialism and until today, Kenya is a net importer of pesticides except for pyrethrin-based products. It is assumed that this import combination ratio has remained the same (Wandiga, 2001). The bulk of the imported pesticides are consumed locally, with only 3% being exported to neighbouring countries. Development of pest resistance to specific pesticides has necessitated frequent change and introduction of new types of pesticides such as carbofurans, carbamates, or a new generation of organophosphorus pesticides, chlorfenviphos among many others (Wandiga, 2001).

Over 500 compounds are registered worldwide as pesticides, or metabolites of pesticides (Van der and Zoonen, 1999). Pesticides include all materials that are used to prevent, destroy, repel, attract or reduce pest organisms. Insecticides, herbicides, fungicides and rodenticides are some of the more well-known pesticides (Robert, 1995). Less well-known pesticides include growth regulators, plant defoliants, surface disinfectants and some swimming pool chemicals. Pesticides are grouped or classified according to the pests they control, their chemical structure, how/when they work, or their mode of action. Most pesticides are classified according to the pests they kill for instance insecticides, fungicides, herbicides among others (Hajslova *et al.*, 1999).

Pesticides can be grouped according to chemical structure. Pesticides with similar structures have similar characteristics and usually have a similar mode of action. Most pesticide active ingredients are either inorganic or organic pesticides (Robert, 1995). Inorganic pesticides do not contain carbon and are usually derived from mineral ores extracted from the earth. Organic pesticides contain carbon in their chemical structure. Most organic compounds are created from various compounds, but a few are extracted from plant material and are called botanicals (Ward *et al.*, 1993). Pesticides can also be classified according to how or when they work. Contact pesticides generally control a pest as a result of direct contact. Insects are killed when sprayed directly or when they crawl across surfaces treated with a residual contact insecticide. Weed foliage is killed when enough surface area is covered with a contact herbicide (Vander and Zoonen, 1999). Systemic pesticides are absorbed by pest and move to untreated tissues.

Systemic herbicides move within the plant to untreated areas of leaves, stems or roots and kill weeds with only partial spray coverage (Robert, 1995).

2.2 Diversity of biodegradative bacteria

Soil microbial communities are among the most complex, diverse, and important assemblages in the biosphere apparently because the soil matrix can promote and sustain diversification (Zhou *et al.,* 2003). Investigations using small-subunit (SSU) rRNA gene (rDNA)-based cloning and sequencing approaches have revealed astonishing diversity and considerable spatial variability of microbial distribution in soils and subsurface environments (Zhou *et al.,* 2003).

DNA re-association studies indicate that soils harbor highly diverse bacterial communities with up to millions of different 16S rRNA gene sequences (Gans *et al.,* 2005 and Sandaa *et al*., 1999). The high diversity of soil bacterial communities could be due to a multitude of ecological niches and adaptive mechanisms as well as high functional redundancy of the soil bacteria (Griffiths *et al*., 2001 and Dykhuizen, 1998). Of all soil and site variables examined, soil pH has been found to be a predictor of both soil bacterial diversity ($r^2 = 0.70$, $P < 0.0001$) and richness ($r^2 = 0.58$, $P < 0.0001$) with the lowest levels of diversity and richness observed in acid soils (Fierer and Jackson, 2005). Moreover, the soil with the higher pH (pH = 6.8) has an estimated bacterial richness of 60% higher than the more acidic soil (pH = 5.1). This is an evidence for a strong correlation between bacterial diversity and soil pH at the local scale (Fierer and Jackson, 2005).

Many microorganisms have evolved catabolic pathways to degrade various aromatic compounds. The versatility and adaptability of bacteria is based on the existence of catabolic plasmids most of which are self-transmissible and have a broad host range, related as well as unrelated, and thereby propagate the unique metabolic capacity into new species (Ramon, 1984). Catabolic plasmids have been found to encode enzymes that aid in degradation of various natural and xenobiotics. Foght *et al*., (1990) postulated that bacteria have multidegradative capabilities that are not necessarily mutually exclusive (Sotsky *et al.,* 1994). This means that given degradative bacteria can utilise two or more foreign compounds as an energy source.

With the extensive pollution of the environment with agricultural and industrial chemicals and the transmissability of pesticide degrading genes (PDG) among microbial community, soil microorganisms that repeatedly or continuously encounter synthetic toxic chemicals develop new capabilities to degrade such chemicals and this may cause changes in the vast and diverse gene pool that is present in soil.Pesticide degrading genes are those genes that code for enzymes involved in biodegradation.Such enzymes include the hydrolases, oxygeneses among others. This influences gene expression, regulation, and genetic recombination in and among the soil microorganisms, thus, may play an important role in the evolution and distribution of new degradative traits (Chaudhry and Ali, 1988).

2.3 Physiology of biodegradative bacteria

A number of bacteria with biodegradative potentials have been described (Gibson and Harwood, 2002). Many of these bacteria capable of degrading carbamate pesticides have also been isolated from soil around the world (Desaint *et al.*, 2000).

The biodegradation of various compounds depends largely on their physicochemical properties which determine their hydrophobicity and solubility thus the bioavailability (Robert *et al.*, 1990). Many hydrocarbon-degrading microorganisms have highly hydrophobic cell surfaces that enable them associate with hydrophobic compounds for effective degradation. Extensive change in membrane lipid composition occurs during the growth of microbes on foreign compounds thus enabling their adaptability to contaminated environments (Singer and Finnerty, 1984). Bacterial cells have both intracellular and extracellular enzymes that readily decompose various compounds. These enzymes are either encoded for by the bacterial plasmids or chromosomal DNA (Janssen *et al*, 2005; Smith, 1990). The extracellular enzymes are mainly oxygenases that are involved in the oxidative decomposition of aromatic hydrocarbons (Smith, 1990) while Intracellular enzymes are mainly hydrolases that mediate the hydrolysis of labile bonds of degradative intermediates. The inherent biodegradative capability of microorganisms has molecular basis and this has been evident by the presence of pesticide degrading genes (PDG) for specific pesticides located on the plasmid, transposons and/ or chromosomes (Kumar *et al*., 1996).

2.4 Biodegradation of pesticides

2.4.1 Biodegradation of Benzenes

All of the important aromatic hydrocarbons that occur in pesticides are derivatives of benzene thus a reaction that is common to all pathways that lead to mineralization of aromatic substrates is cleavage of the benzene ring (Alexander, 1985). Molecular oxygen serves as reactant in two steps in the pathways for benzene catabolism. In each of these reactions, both atoms from molecular oxygen become incorporated into the substrate. Enzymes that catalyze such reactions are called di-oxygenases (Sheldon and Kochi, 1981). Molecular oxygen results in destabilization and cleavage of benzene ring to benzene dihydrodiol (*cis* -1, 2-dihydroxycyclohexa-3, 5-diene) (Ribbons and Eaton, 1982; Gottschalk, 1986). The aromaticity of benzene is restored by a dehydrogenase-catalyzed conversion of benzene dihydrodiol to catechol (1, 2-dihydroxybenzene), which is the ring cleavage substrate (Figure 1).

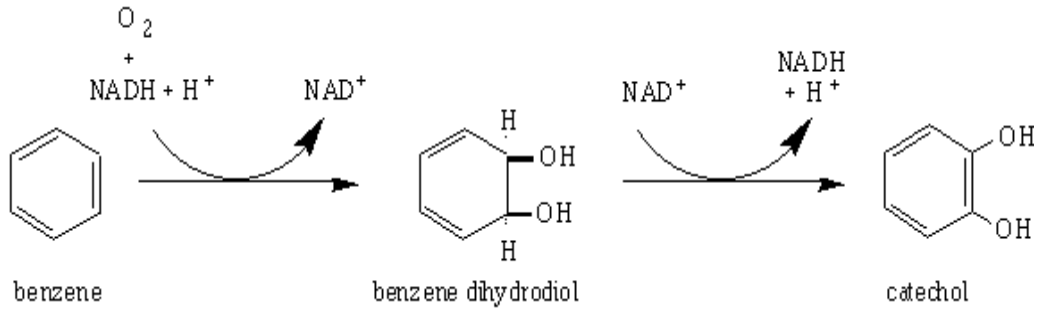


Figure 1. Oxidation of benzene to catechol by *Pseudomonas Sp.* (Sheldon and Kochi, 1981).

Catechol is then catabolized by ring cleavage, in which the aromatic ring is broken. This occurs in two major pathways: the ortho-cleavage pathway, in which the aromatic ring is split between the two carbon atoms bearing hydroxyl groups, or the meta-cleavage pathway, in which the ring is broken between a hydroxylated carbon atom and an adjacent unsubstituted carbon atom (Gottschalk, 1986). Each of these ring-cleavage reactions is catalyzed by a dioxygenase. The final products of catechol catabolism are TCA cycle intermediates (Figure 2).

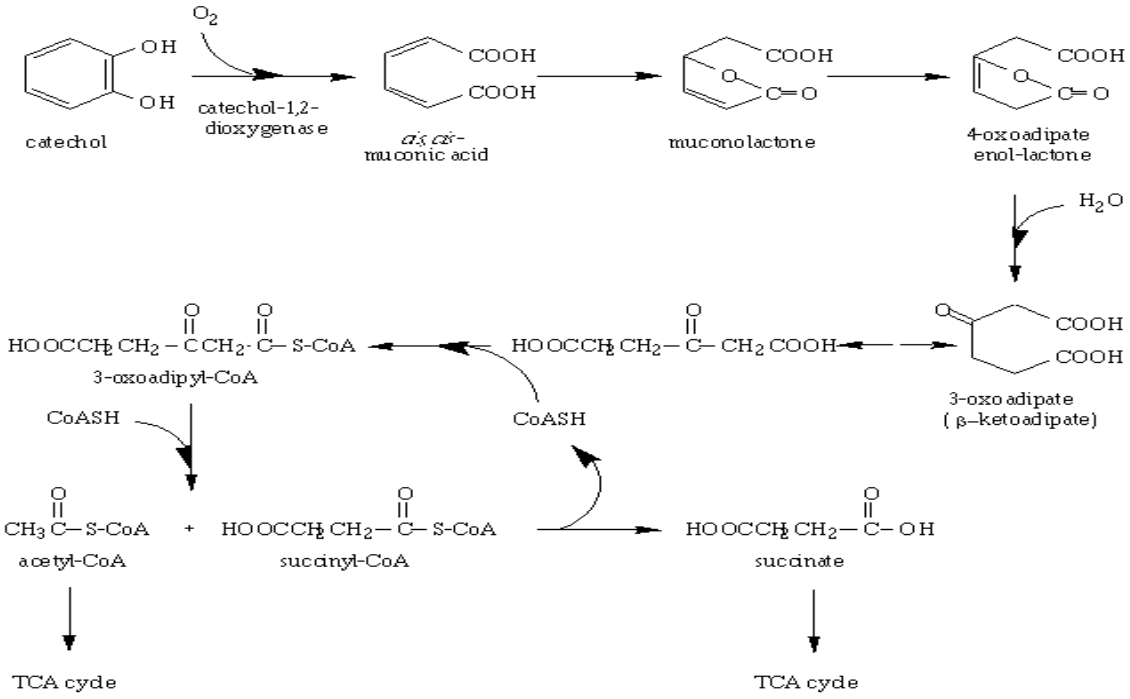


Figure 2. Flow diagram showing the degradation of catechol to TCA cycle intermediates ((Sheldon and Kochi, 1981).

2.4.2 Degradation of Resorcinol and its derivatives

Resorcinol is natural as well as man-made phenolic compound (Hans, 1994). Phenolic compounds enter the environment during the biodegradation of natural polymers containing aromatic rings, (Paula *et al.*, 1998) such as lignin and tannins and from aromatic amino acid precursors. Industries such as pulp mills, refineries and wood preservation plants as well as chemical industries have been known to release phenolic compounds into the environment (Vasil'eva *et al*., 1999). The degradation of resorcinol has been demonstrated in a number of screening studies (Larway and Evans, 1965; Chapman and Ribbons, 1976; Gorney *et al*., 1992; Heider and Fuchs, 1997; Philip and Schink, 1998).

Resorcinol is present in most soils and sediments and only a few aerobic resorcinol degrading microorganisms have been isolated and characterized (Maeda and Massey, 1993). Microorganisms capable of anaerobic degradation were described as early as 1976 (Chapman and Ribbons, 1976), pertaining to a bacterium of the genus *Pseudomonas*. Tschech and Schink (1985) documented the degradation pathway of a resorcinol degrading fermenting bacteria of the genus *Clostridium sp.* Cell –free extracts of this bacteria convert resorcinol to dihydroresornicol (Klunge *et al.*,1990) to form cyclohexanedione which further hydroxylated to 5-Oxohexanoate by nucleophilic attack on one of the carbonyl-carbonyl carbon atoms (Schink *et al*., 2000). The resorcinol reductase of this bacterium consists of sub-units of 49.5 kDa and contains flavin adenine dinucleotide (FAD), but iron sulfur centers have not been detected (Schuler, 1997).

The resorcinol ring is destabilized by the introduction of a further hydroxyl group to form hydroxyhydroquinone (HHQ). HHQ is oxidized to hydroxybenzoquinone, which is further oxidized to succinate and acetate, and a number of other yet unidentified products (Philip and Schink, in press). Reichenbecher and Schink (1997) also found a further reducing activity with HHQ in the sulphate-reducing bacterium *Desulfovibirio inopinatus.* Szewzyk and Pfenning (1987) have similarly reported a sulphate-reducing resorcinol degrading bacterium, *Desulfobacterium catecholicum*, which also degrades catechol and hydroquinone as well.

2.4.3 Degradation of methomyl

Methomyl (S-methyl-N-[(methylcarbamoyl) oxy]-thioacetimidate) is a broad-spectrum insecticide which is extensively used as an acaricide to control ticks and spiders and it is employed for foliar treatment of vegetables, fruits and field crops, cotton, and commercial ornamental plants (Farré, 2002). Methomyl is considered very toxic to mammals and it is classified as a restricted-use pesticide (RUP) or Class I. Reported oral 50% lethal dose (LD_{50}) values are 17 to 24 mg kg^{-1} in rats. Methomyl is moderately to highly toxic to fish, with reported LD_{50} values of 3.4 mg L^{-1}.

The high water-solubility (10 mg L^{-1}) of methomyl and its low soil organic carbon partition coefficient (Koc=0.16) make methomyl a candidate for groundwater contamination (Farré, 2002; Tomlin, 2003; Strathmann and Stone, 2001). Biodegradation of methomyl is documented with members of the genera *Pseudomanas* and *Agrobacterium* being the main methomyl- degrading bacteria (Chewapat,*.et al.*, 2004).

In the soil, methomyl degrades primarily by microbial degradation with carbon dioxide as the principal end product (WHO, 1996). The initial degradation product of methomyl is *S*-methyl- *N*-hydroxythioacetimidate (MHTA) followed by its breakdown to carbon dioxide.

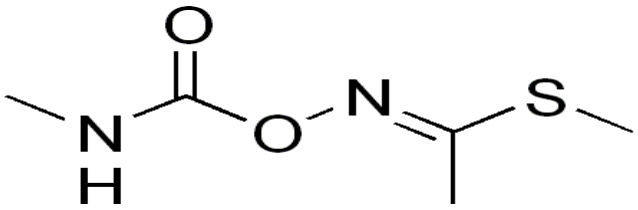


Figure 3: Chemical structure of methomyl (Extoxnet, 1996)

The rate of biotransformation of methomyl varies with the type of soil with high rate of transformation in silt loam soil. Fast transformation of methomyl has been recorded in moist soil suggesting a combination of chemical hydrolysis and biotransformation. Aerobic degradation in soil is about twice as fast as anaerobic degradation (WHO, 1996).

Reported half-life of methomyl in soil varies from a few days to more than 50 days depending on the concentration and frequency of methomyl usage in given soil.

Photocatalytic and hydrolytic degradation of methomyl has also been documented (Tamimi *et al.*, 2005). Methomyl is photodegraded to methomyl oxime which undergoes Beckmann rearrangement to acetonitrile. Acetonitrile is then transformed to acetamide, acetic acid, oxalic acid, formic acid and carbon dioxide respectively (Tamimi *et al.*, 2005).

The photolysis of methomyl has been studied at pH 5, 7 and 9, at concentrations of 10 and 100 mg/litre, and at 25°C. Methomyl photolyses rapidly at both concentrations with a half-life of 2-3 days at 100 mg/litre. The principal photo-product is acetonitrile.

Duplicate preparations of methomyl, kept in the dark, did not decompose (WHO, 1996). Methomyl degrades rapidly in slightly alkaline solution (pH 8.85). The degradation rate increase with increased temperature (WHO, 1996).

2.4.4 Degradation of Carbofuran

Carbofuran (2, 3-dihydro-2, 2-dimethyl-7-benzofuranyl methylcarbamate) is abroad-spectrum N-methyl carbamate that has been widely used as insecticide, miticide and acaricide. It is a potent inhibitor of cholinesterase and as a result highly toxic to mammals having an oral 50% lethal dose of $2mgKg^{-1}$ in mice (Fahmy *et al.*, 1970). High avian death from granular formulation has also been reported in United States (Mineau, 1993).

Cabofuran and its metabolites have also been reported in aquatic environments where carbofuran disrupts enzyme and lipid metabolism in fish (Eisler, 1985; DPR, 2002). For terrestrial species, honeybees are extremely sensitive to carbofuran (LD_{50}=0.16µg/bee) (Eisler, 1985). Earthworms (*Lumbricus herculeus)* are also particularly susceptible, with an LC_{50} value in soil of 0.5 ppm at 5 hours. Earthworm mortality could result in an increased likelihood of secondary poisoning in many species (Eisler, 1985).

Biodegradation and photodecomposition of carbamates to amines and alcohols have been reported, although, photolysis is a lesser degradative pathway (Raha and Das, 1990). There is high specificity in microbial degradation which is due to diverse chemical configurations within carbamates.

Different bacterial and fungal communities with inherent capability to degrade carbofuran have been recently isolated from agricultural soils (Seo *et al*., 2007; Chapalmandugu and Chaudhry, 1992; Chaudhry and Ali, 1988). Members of genera *Flavobacterium*, *Pseudomonas* and *Achrobacterium* have been isolated and shown to degrade carbofuran to carbofuranphenol, 3-ketocarbofuran and 3-hydroxycarbofuran as the major carbofuran metabolites (Seo *et al*., 2007; Chaudhry and Ali, 1988).

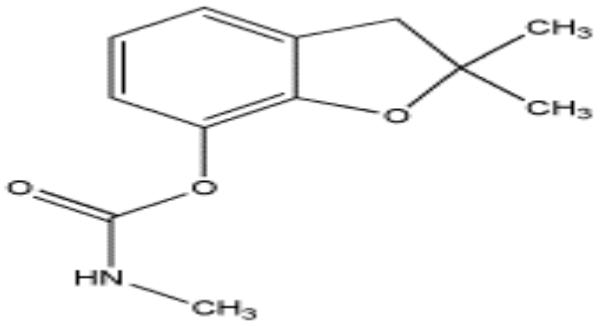


Figure 4: Chemical structure of carbofuran (Extoxnet, 1996)

Chaudhry (2002) also reported microbial metabolites of carbofuran which include 2, 3-dihydro-2, 2-dimethyl-7-hydroxybenzofuran, 3-hydroxycarbofuran, 3-hydroxycarbofuran-7-phenol, 3-ketocarbofuran and 3-ketocarbofuran-7-phenol. Photometabolites of carbofuran include 2, 3-dihydro-2, 2-dimethyl benzofuran-4, 7-diol, and 2, 3-dihydro-3 keto-2, 2-dimethyl benzofuran-7-yl carbamate (or 3-ketocarbofuran) (Raha and Das, 1990).

Hydrolysis and oxidation are the major pathways of microbial degradation of carbofuran, however, hydrolysis aided by microbial esterase, is the main degradation pathway of carbofuran under alkaline conditions (Chiron *et al.,* 1996).

Carbofuran hydrolysis rate is highest under alkaline conditions, being 700 times faster in aqueous solution with pH 10 than in water with pH 7 (Bailey *et al.,* 1996).

Enhanced carbofuran degradation has been reported in farms with history of carbofuran treatments (Singh and Sethunathan, 1999). Repeated application of carbofuran in soil results in built-up of microbial community capable of degrading carbofuran (Parkin and Shelton, 1994). Singh and Sethunathan (1999) compared the recovery of applied carbofuran between previously carbofuran treated and untreated soils and found lower recovery rate in previously treated soils citing advance adaptability and build up of carbofuran utilizing microbes in pretreated soil. Persistence of carbofuran in soil has also been reported. Szeto and Price (1991) found 78 µg/g of carbofuran in Canadian silt loam soils nearly a year after the application of granular material.

2.5 Molecular aspects of Pesticide degradation

Microorganisms are able to degrade a large variety of compounds, including pesticides. Many bacteria which metabolize xenobiotics have been found to possess DNA plasmids which genetically specify their biodegradation capacity (Don and Pemberton, 1981).

The pesticide degradation genes (PDG) present in soil bacteria have been shown to reside on plasmids, transposons, and/or on chromosomes, a common location for degradation genes (Chung and Ka, 1998; Laemmli *et al*., 2000 ; Kumar *et al*., 1996).

Plasmids that bear genes encoding for enzymes capable of degradation have been of great attraction where these plasmids, known as catabolic plasmids, can give the organism containing them the ability to degrade certain compounds.

It has recently become apparent that the evolution and spread of the pesticide-degrading plasmids (PDP) plays a significant role in the occurrence of microbial populations which are able to metabolize various types of xenobiotics (Don and Pemberton, 1981). These genetic elements often have the ability to transfer, sometimes with high efficiencies, to other bacterial species, related as well as unrelated, and thereby propagate the unique metabolic capacity into new species.

Catabolic genes responsible for the degradation of several xenobiotics, including pesticides, have been identified, isolated, and cloned into various other organisms (Kumar *et al*., 1996). Cross-adaptation of bacteria to the degradation of most N-methylcarbamate insecticides has also been reported (Morel-Chevillet *et al.*, 1996).Metabolism of carbofuran is mainly by hydrolysis of the methylcarbamate linkage, yielding carbofuran 7-phenol and methylamine. The methylcarbamate-degrading (mcd) gene, which encodes a carbofuran hydrolase, is located on a 100-kb plasmid called pPDL11 and has been cloned from *Achromobacter sp*. WM111 (Tomasek and Karns, 1989). This gene is present and expressed in many Gram-negative bacteria (Parekh *et al.,* 1995). The hydrolytic enzymes encoded for by mcd gene includes esterase and phosphatase.These genes are tranferable thus can be used to transform strains without biodegradative capability.

Two other classes of enzymes, mono- and di-oxygenases, are also commonly associated with pesticide degradation. These enzymes introduce one or two oxygen atoms, respectively; into the structure of a pesticide.

This oxidation process often makes the pesticide more amenable to further degradation by increasing its water solubility, thereby increasing its bioavailability (http://www.ciwmb.ca.gov/publications/Organics/44200015.doc).

The gene responsible for the degradation process of methomyl is encoded by the plasmid, PMb (5Kb) which has since been isolated from *Stenotrophomonas maltophilia* strain (M1) (Mervat, 2009). Transformed strains with plasmid PMb (5kb) have the ability to grow in the presence of methomyl upto a concentration of 1000 ppm with 0.05% glucose supplement (Mervat, 2009). It is therefore evident that the absence of pesticide degrading plasmids in a degrading strain is an indication of the presence of alternative catabolic pathway for cleavage of pesticide structure.

2.6 Molecular-based techniques used to study microbial diversity

A number of molecular approaches have been developed to study microbial diversity. These include DNA re-association, DNA–DNA and mRNA:DNA hybridization, DNA cloning and sequencing, and other PCR-based methods such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), ribosomal intergenic spacer analysis (RISA) and automated ribosomal intergenic spacer analysis (ARISA) (Kirk *et al.*, 2004).

Molecular techniques based on PCR have been used in analysis of microbial diversity to overcome the limitations of culture-based methods (Kirk *et al.,* 2004). Differences in the guanine plus cytosine (G+C) content of DNA have been used to study the bacterial diversity of soil communities (Nusslein and Tiedje, 1999). G+C content together with amplified ribosomal DNA restriction analysis (ARDRA) abundance patterns and rDNA sequence analysis have been used to study the changes in microbial diversity (Nusslein and Tiedje, 1999).

DNA re-association is a measure of genetic complexity of the microbial community and has been used to estimate diversity (Torsvik *et al*., 1990). Under specific conditions, the time needed for half of the DNA to re-associate (the half association value $C0t_{1/2}$) can be used as a diversity index, as it takes into account both the amount and distribution of DNA re-association (Torsvik *et al*., 1998). This can also be studied by measuring the degree of similarity of DNA through hybridization kinetics (Griffiths *et al*., 2001).

DNA–DNA hybridization has been used together with DNA microarrays to detect and identify bacterial species (Cho and Tiedje, 2001) or to assess microbial diversity. This tool is valuable in bacterial diversity studies since a single array can contain thousands of DNA sequences (Cho and Tiedje, 2001) with high specificity. PCR targeting the 16S rDNA has been used extensively to study prokaryote diversity and allow identification of prokaryotes as well as the prediction of phylogenetic relationships (Pace, 1999).

In these methods, DNA is extracted from the environmental sample and target DNA (16S, 18S or ITS) is amplified using universal or specific primers and the resulting products are separated in different DNA-based characterization techniques.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Determination of pesticide usage and soil sampling

The information regarding the pesticide usage on each farm were obtained through a pre-designed questionnaire sheet which addressed the used pesticide, the period of usage of a particular pesticide as well as their effect on the crop and the type of crop grown. Four regions of Central and Rift-valley Kenya namely Gatanga and Juja in Central Kenya as well as Naivasha and Gilgil in Rift Valley formed areas of study. Gatanga is located at 1^{o} 3′ 0″ south, 37^{o} 5′ 0″ East with a variety of soil type that included aerosols, alisols, lixisols and luvisols. Juja region is located 1^{o} 3′ 0″ south, 37^{o} 5′ 0″ East and two soil types' planosols and vertisols were dominant. Gilgil is situated between Nakuru and Naivasha with the following geographical coordinates 0^{0} 13′ 0″ South 36^{0} 16′ 0″ East. Soil types in the region were mainly andosols and fluvisols. Naivasha is northwest of Nairobi in the scenic Rift Valley and flower farming's epicenter. It is located 0^{0} 43′ 0″ South 36^{o} 26′ 0″ East with cambisols and arenosols types of soils. Samples were then transferred in glass jars and stored at 25^{0}C until used. Sampling was done using stratified random sampling method with the geographical regions forming the four strata. From each region, plots (farms) were identified by simple randomization. Cross-sectional sampling was done in which the upper (5-20 cm deep) mineral soil was collected from five locations within a given plot of ≈100 m^{2}. Aerobic conditions for sampling, medium preparation and culture handling was followed throughout the subsequent enrichment process.

3.2 Isolation of carbofuran and methomyl degrading bacteria

Isolation was done by suspending 1g of each soil sample in 10ml of mineral salt medium (MSM) containing 2mM of furadan® and methomex® as the sole carbon source. MSM contained the following constituents, in grams per liter: K_2HPO_4, 4.8; KH_2PO_4, 1.2; NH_4NO_3, 1.0; $MgSO_4\ 7H_2O$, 0.2; Ca $(NO_3)_2$ $.4H_20$, 0.4, and Fe_2 $(SO_4)_3$, 0.001. The pH was then adjusted to 7.2 using potassium hydroxide solution and the medium autoclaved (121^0C, 15minutes) and supplemented with furadan or methomyl as the sole carbon source just before inoculation (Chaudhry and Ali, 1988).

Cultures were grown in 100ml- culture flasks under aseptic conditions at 30°C with shaking in a rotary shaker at 100 rpm for 18 days. Using wire loop, cultures were then streaked into agar plates containing mineral salt medium supplemented with 2mM of carbofuran or methomyl. Single colonies obtained were re-suspended in basal medium containing 2mM of methomyl or carbofuran for 14 days to confirm the ability of the isolates to utilize the pesticides. Using wire loop, cultures were then streaked into plates containing Luria-Bertoli medium (LB) with the following components, in grams per liter: tryptone, 10; yeast extract, 5; NaCl, 5 and mineral agar 15. Sub-culturing was done periodically on pesticide supplemented medium (agar) until pure colonies were obtained (Chaundry and Ali, 1988).All solid media contained 0.1g/liter cycloheximide (Sigma-Aldrich, Steinheim, Germany) to suppress fungal growth (Maarten *et al*., 2007).

3.3 Growth curves for Isolates on enrichment cultures

The bacterial growth curve for each isolate was determined using 2mM concentrations of either methomyl or carbofuran with which they were initially enriched. From 1-litre conical flasks containing 500ml mineral salt media supplemented with either methomyl (2mM) or cabofuran (2mM), 10ml was obtained into which inoculation was done with 24-hour culture. The initial $O.D_{600}$ at (t_0) was then recorded from the log-phase cultures. Each culture was then placed in a shaker incubator (100rpm) at 27^0C.

At intervals of 24 hours, 4-ml aliquot of the culture was aseptically transferred to a cuvette and its optical density determined using mineral salt medium with 2mM of methomyl or carbofuran but lacking inoculums as a blank. Similarly, 1-ml aliquot of the culture was also aseptically transferred into a 1.5-ml eppendorf tube and kept at -20^0C for further chromatographic analysis of decline in pesticide concentration. Mineral salt medium alone with the inoculum was used as the control for the experiment.

3.4 Growth at various temperatures

LB broth was prepared in two batches. The medium was then dispensed in tubes and autoclaved. Each batch was inoculated with the isolates and incubated at temperatures 10^0C, 20^0C, 30^0C, 40^0C and 50^0C and 60^0C. Observations were made at intervals of 24 hours for 7 days. The presence of growth was determined by optical densities for each isolate using spectrophotometer at 660nm (Cappuccino and Sherman, 2002).

3.5 HPLC analysis of biodegradation products

A standard curve was made using reference standards for both carbofuran and methomyl into a final concentration range of 0.1mM-2mM. The standards were prepared by dissolving the standard pesticides in acetonitrile to the final concentrations (Appendix 3). The dilutions were run at different conditions for each pesticide. Carbofuran was detected at 254nm at a run time of 5 minutes and flow rate of 1ml min^{-1} using HPLC grade acetonitrile (70%) and degassed water (30%) as the mobile phases. Sample of 20µL were injected at an oven temperature of 27^0C. Sample of 20µL were injected and an oven temperature of 27^0C. Methomyl diluted samples were run at the same condition as carbofuran except for the detection wavelength of 235nm, acetonitrile to water ratio of 0.3:0.7 and run time of 10minutes.

The decline in methomyl or carbofuran concentration upon degradation was monitored by high performance liquid chromatography (HPLC) (VP shimadzu) on a reverse phase C-18 column, 25cm x 4.6mm, fitted with a C-18 silica reverse phase guard column and equipped with a UV detector and their retention times identified using reference standards. The analytes were non-polar and therefore were able to be retained and metabolites separated in the reverse phase column. The mobile phase was prepared by first washing the 1000ml- bottles and oven drying for an hour before filling them with 800ml of either acetonirile (HPLC grade) or double distilled water. The double distilled water or acetonitrile was then de-gassed for 1 hour before connecting them to the pumps (LC-10AT VP-shimadzu A and B for water and acetonitrile respectively).

For the decline in the concentration of methomyl, mobile phase of acetonitrile (30%) in 70% demineralized water was used. Chromatography was carried out in ambient temperature at a flow rate of 1ml min^{-1} at wavelength of 235nm and a run time 10 minutes. Methomyl was detected at a retention time of 4.7 minutes while its metabolite detected at a retention time of 4.1 minutes. Decline in carbofuran concentration was also monitored using a mobile phase of acetonitrile (70%) in demineralised water (30%). This was done at a wavelength of 254nm at a run time of 5 minutes. With the mentioned conditions, carbofuran was detected at a retention time of 4.0 minutes. The retention time drifted by ±0.2minutes depending on the level of drift of mobile phase pressure and / or injection intervals. An injection volume of 20µl was used.

3.6 Morphological characterization

This was done to determine the shape of the bacterial cell. Cell morphology was determined by microscopic examination of safranin-stained bacterial isolates using phase contrast Zeiss Axiophot microscope (Carl Zeiss, Oberkochem, Germany). This was supplemented by the classical gram staining method (Bathlomew, 1962) and then 3.0% (w/v) KOH test (Gregersen, 1978).

3.8 Biochemical characterization

Biochemical tests were done to give an understanding to the metabolic properties of different bacterial strains isolated and thus the utilization of pesticides in relation to their structure. The properties were also significant in phylogenetic grouping of the isolated strains. The biochemical tests done were as follows;

3.8.1 Urease test

The urease test was used to determine the ability of an organism to split urea into two units of ammonia by the enzyme urease produced by the isolates (Harold, 2002). Two units of ammonia were formed resulting into the increased alkalinity of the media.The increased salinity was detected by a pH indicator, phenol red, in the Christensen's urea which under acidic conditions (pH 6.8) is yellow and rose pink in alkaline conditions (pH 8.4). Positive reactions were indicated by development of a deep pink color (Cappuccino and Sherman, 2002).

3.8.2 Nitrate reduction test

The ability of the isolates to reduce nitrates to nitrites or beyond was carried out using nitrate reduction broth. Following inoculation and incubation, the addition of sulfanilic acid and alpha- naphthylamine. These two compounds react with nitrite and turn red in color, indicating a positive nitrate reduction test (Cappuccino and Sherman, 2002).

3.8.3 Oxidase test

Oxidase positive bacteria possess cytochrome oxidase or indophenol oxidase (an iron containing haemoprotein). They both catalyze the transport of electrons from donor compounds (NADH) to electron acceptors (usually oxygen). The test reagent, N, N, N', N'-tetra-methyl-p-phenylenediamine dihydrochloride acts as an artificial electron acceptor for the enzyme oxidase. The oxidised reagent forms the coloured compound indophenol blue. In this case, impregnated oxidase test strip method was used. Some 24-hour old cultures were scrapped using the oxidase strip and a blue color developed after 10 seconds indicating a positive result for oxidase test (Cappuccino and Sherman, 2002).

3.8.4 Gelatin liquefaction test

Nutrient broth supplemented with 12% gelatin was used to demonstrate hydrolytic activity of gelatinase. The psence of gelatinase enzyme breaksdown gelatine which liquefies the media. After incubation, cultures that remained liquefied when placed in refrigerator at 4^0C for 30 minutes were considered positive for gelatin hydrolysis (Cappuccino and Sherman, 2002).

3.8.5 Indole and Hydrogen Sulfide production

Sulfur-Indole Mortility (SIM) agar media was used to demonstrate the production of tryptophanase and cysteine desulfurase by the isolates. Tryptophanase utilises the amino acid tryptophan as a carbon and energy source producing indole, pyruvic acid and ammonia. The indole produced was detected by addition of Kovac's reagent (*p*-dimethylamino-benzaldehyde) (Harold, 2002). Positive results for indole were indicated by production of a cherry red layer. Cysteine desulfurase breaks down sulfur containing amino acids producing pyruvate, ammonia and hydrogen sulfide. Iron in the medium reacts with hydrogen sulfide producing the characteristic black precipitate which was a positive test for hydrogen sulfide production by the isolates (Cappuccino and Sherman, 2002).

3.8.6 Esterase activity

The media used was as described by Sierra (1957), briefly in (g l^{-1}): peptone 10.0, NaCl 5.0, $CaCl_2.2H_2O$ 0.1, agar 18.0, pH 7.4.These components were mixed together using distilled water in a 250ml conical flask and autoclaved at 121^0C for 45 minutes.

To the autoclaved media, previously sterilized Tween 80 was added in a final concentration of 1% (v/v). This medium was inoculated with the isolates and incubated at 30^0C for 48 hours. The presence of halos (clearance zone) observed indicated the production of esterases by the isolates and halo diameter recorded. The enzymatic index was then calculated by dividing the halo diameter by the colony diameter.

3.8.7. Phosphatase activity

Phosphatase enzymes are involved in the hydrolytic breakdown of carbamates as earlier mentioned (Tomasek and Kerns, 1989; Parekh *et al.,* 1995). Their production by the isolated strains is an indication of the ability of the strains to hydrolyse amide bonds within the carbabate structures for effective biodegradation. Phosphatase activity was estimated in duplicate samples by the liberation of p-nitrophenol from p-nitrophenyl phosphate (disodium salt; BDH) using a modified procedure described by Bolton and Dean (1972). The isolates were first grown in a low phosphate media suitable for formation of both repressible and constitutive alkaline phosphatase from bacteria. The composition of the media includes peptone water 3g/L,Yeast extract 1 g/l, NaCl 31.2 g/L, MgCl:$6H_2O$ 5.39g/l, $Fe(NH4)_2SO_4$, Trizima base 12.1 g/L pH 7.4. Each isolate was cultured in 10ml of the medium in universal bottles with reciprocal shaking at 27^0C for 24 hours. Cultured medium (1 ml) was incubated with 1 ml of 50mM p-nitrophenol phosphate solution, 1 ml of 0.5M Tris-HCl buffer, pH 8.2 and 1 ml of mineral salts media composed of; 0.4M NaCl, 0.05M $MgCl_2$,0.01M KCl and 0.01M $CaCl_2$.

The mixture was incubated for 30 minutes at 28^0C and the p-nitrophenolate liberated measured using spectrophotometer at 405nm. P-nitrophenolate standard curve was made in triplicate using p-nitrophenol in a concentration range of 0.1-1mM (Appendix 3).

3.8.8. Citrate utilization test

Simmons' Citrate agar slants were used to determine the capability of the isolates to use citrate as a carbon source for their energy (Harold, 2002). Bromothymol blue indicator incorporated in the media turned from green to Prussian blue indicating positive test for citrate utilization (Cappuccino and Sherman, 2002).

3.8.9 Catalase test

Catalase test detects the catalase enzyme present in most cytochrome containing aerobic bacteria which form hydrogen peroxide as an oxidative end product of the aerobic breakdown of sugars. Catalase decomposes hydrogen peroxide to water and oxygen. Catalase production was determined by addition of 3% hydrogen peroxide to Tryptic Soy Agar (TSA), 24-hour cultures of isolates. A positive reaction was indicated by the formation of bubbles (Cappuccino and Sherman, 2002).

3.8.10 Methyl Red-Voges-Proskauer test (MR-VP)

MR-VP test was done to determine the ability of the isolates to oxidize glucose with the production and stabilization of high concentration of acids as end products. MR-VP broth was inoculated and incubated with the isolates for 48 hours at 27^0C.

Aliquots of each culture were added with either methyl red indicator or Barritts reagent. For positive cultures the methyl red indicator appeared red and absence of red coloration was an indication of a negative test for MR-VP (Cappuccino and Sherman, 2002).

3.9 Total protein determination

Total proteins in the bacterial cells were determined to establish the relative phospatase enzyme to the total proteins in the strains. Protein determination was thus done using modified Lowry method. Lowry reagents were prepared as follows: 20 grams of sodium carbonate were dissolved in 260 ml distilled water, 0.4 gram cupric sulfate (5x hydrated) in 20 ml of water, and 0.2 gm sodium potassium tartrate in 20 ml of water. The three solutions were mixed to prepare the copper reagent. 2x Lowry reagent was prepared by mixing 3 parts of copper reagent, 1 part of sodium dodecyl sulphate (1% w/v) and 1 part of 1M sodium hydroxide. Folin reagent (0.2N) was prepared by mixing 10ml of 2N Folin reagent with 90ml of water. Assay was done by diluting the samples to an estimated 0.025-0.25mg/ml with water and made to a final volume of 1 ml for each dilution.

A reference of 1ml water was prepared and a 3 dilutions (for standard) spanning in an order of magnitudes were made from 0.25 mg/ml bovine serum albumin to a final volume of 1 ml. 5ml of 2x Lowry reagent was then added to each dilution, mixed thoroughly and incubated at room temperature for 10 minutes. Thereafter, 0.5 ml of Folin reagent (0.2 N) was added to each dilution and quickly vortexed and further incubated for 30 minutes at room temperature (Lowry *et al.*, 1951).

Using clean polystyrene cuvettes, absorbance at 660 nm was taken for both the standard curve (Appendix 3) and samples using water as reference (Stoscheck, 1990).

3.10 Molecular characterization

3.10.1 DNA extraction

Genomic DNA was extracted from carbofuran and methomyl degrading isolates at exponential growth phase grown aerobically in nutrient broth. Prior to extraction, bacterial cells were harvested from broth by centrifuging (13000g, 5min.) 1ml of culture in a 1.5ml eppendorf tubes with supernatant poured out. The medium (nutrient broth) was washed by re-suspending the cells in equal volumes of TE buffer, centrifuged for 5 minutes at 13000g and the supernatant discarded. The cells were then re-suspended in 200 µl of solution 1 [50mM Tris (pH 8.5), 50mM EDTA pH (8.0) and 25% sucrose solution], 5µl of lysozyme (20mg/ml) and 5µl of RNase A (20mg/ml) then mixed gently (Sambrook *et al*., 1989). The mixture was then incubated at 37^0C for 1 hour 600ul of solution 2 [10mM Tris (pH 8.7), 5mM EDTA (pH 8.0) and 1% sodium dodecyl sulphate] and 10ul of proteinase K (20mg/ml) were added and mixed gently. The mixture was then incubated at 55^0C for 30 minutes (Sambrook *et al.*, 1989). Equal volumes of phenol-chloroform was added and spun for 5minutes at 13000g. Carefully, the upper aqueous layer was transferred into a separate 1.5ml eppendorf tube. This step was repeated before adding an equal volume of diethyl ether to wash off the phenol. This mixture was then spun at 13000g for 5 minutes and the supernatant carefully discarded (Sambrook *et al*., 1989). The procedure was repeated twice.

The DNA was then precipitated by adding an equal volume of ice cold absolute ethanol and 0.1 volumes of 3M potassium acetate and left overnight at -20^{0}C. The pellet was concentrated by centrifuged at 13000g for 30 minutes and the supernatant discarded. Equal volumes of 70% ethanol was added and centrifuged at 13000g for 5 minutes. Supernatant was discarded carefully not to discard the pellet. This procedure was repeated twice before leaving the pellet on the bench to air dry completely at room temperature (this step eliminates residual ethanol) (Sambrook *et al.*, 1989). The dry pellet was then re-suspended in 45ul of TE buffer (the advantage of TE is that EDTA chelates magnesium ions which makes it more difficult for residual DNases to degrade the DNA) and then kept at -20^{0}c for future applications (Magarvey *et al*., 2004). The DNA was visualised on a 1% agarose gel in 1xTAE buffer under UV by staining with ethidium bromide (Sambrook *et al*., 1989).

The DNA quantification was done using spectrophotometer with the absorbance at 260nm and 280nm used to determine the purity of the DNA. The ratio $\geq$ 1.8 was used in the subsequent polymerase chain reaction.

3.10.2 Polymerase Chain Reaction

For the amplification of the 16S rDNA, 1µl of 600µg/ml DNA from each extract was amplified using genescipt Taq polymerase and 10x buffer according to manufacturer's instructions.

Nearly full-length 16S rRNA gene sequences were PCR-amplified using bacterial primer pair 8F forward 5'-AG (A/G) GTTTGATCCTGGCT-3') and 1492R reverse, 5'-CGGCTACCTTGTTACGACTT-3' (Sigma) according to the position in relation to *Escherichia coli* gene sequence (Embley and Stackebrandt, 1994; Lane, 1991).Amplification was performed using a model PTC-100 thermal cycler (MJ research inc., USA). Amplification was carried out in a 40 µl mixture containing 0.25 µl of genescript Taq, 1.0 µl (5-pmol) of 8F forward primer, 1.0 µl (5-pmol) of 1492R reverse primer, 1 µl of template DNA, 2.5 µl of dNTPs mix (2.5mM), 4.0µl PCR 10x buffer (genescript) and 30.25 µl of PCR water. The control contained all the above except the DNA template (Sambrook *et al.,* 1989).

Reaction mixtures were subjected to the following temperature cycling profiles repeated for 35 cycles: Initial denaturation of the template at 94^{o}C for 5 minutes, denaturation at 94^{o}C for 45 seconds, primer annealing at 52^{o}C for 30 seconds, chain extension at 72^{o}C for 1.5 minutes and a final extension at 72^{o}C for 5 minutes (Roux, 1995).

Amplification products (7.0 µl) were separated on a 1% agarose gel in 1X TAE buffer and visualized by ethidium bromide staining (Sambrook *et al.,* 1989).

3.11.3 Restriction analysis of the PCR products

The preliminary genetic diversity of the carbofuran and methomyl degrading bacterial strains was determined by amplified ribosomal DNA restriction analysis (ARDRA) of 16S rDNA using a modified procedure (Desaint *et al* 2000).

Aliquota of 8-µl of the PCR product was digested in a final volume of 30 µl for 12 h at 37°C with 0.5 µl of a restriction endonuclease (*RsaI*) according to the manufacturer's specifications (Appligene, Illkirch, France). Digested DNA fragments were separated by electrophoresis in 1.5 (w/v) agarose gels for 2 hrs at 80 V. The gel was stained with ethidium bromide and DNA fragments visualized under UV illumination. Similarity among strains was estimated from the proportion of shared restriction fragments bands generated by *RsaI* digestion.

3.12.4 Agarose gel electrophoresis

1.0% Agarose gel (w/v) was prepared by dissolving 1.0 g of agarose powder into 100 ml of 1X TAE buffer. The gel solution was stirred, brought to boil in a microwave for 3 minutes to completely dissolve the powder then the cooled gel solution was poured in a casting tray having combs and left for sometime to gel (polymerise).

Ethidium bromide (3µl) was incorporated in the gel to facilitate visualization of DNA under UV light. The PCR products (7µl) was mixed with 3µl of loading dye (Bromophenol blue) and loaded into the well and subjected to electrophoresis at 80 V for 45 minutes (modified) (Sambrook *et al.*, 1989).

3.13.5 Purification of PCR products

The PCR products were purified using the QIAquick PCR purification Kit protocol (Qiagen, Germany) according to manufacturer's instructions. Five volumes of buffer PB (binding buffer) (Qiagen, Germany) was added to 1 volume of the PCR sample and thoroughly mixed.

The QIAquick spin column was placed in a 2 ml collection tube, the sample applied to the QIAquick column to bind the DNA, and then centrifuged for 30-60 seconds at 13000 rpm. The flow-through was discarded, and the QIAquick column placed back into the same tubes. To wash the DNA, 0.75 ml buffer PE (washing buffer) was added to the QIAquick column and centrifuged for 1 minute. The flow-through was discarded and the column centrifuged again for an additional 1 minute at 13000 rpm to remove residual ethanol from buffer PE. The Qiaquick column was placed in a 1.5 ml microcentrifuge and 30 μl of buffer EB (elution buffer) (10mM Tris-Cl, pH 8.5) added to elute DNA. The tubes were then centrifuged for 1-minute at 13000rpm, the spin column removed and DNA stored at -20^0C(Sambrook et al., 1989).

3.14.6 Sequencing and sequence analysis

Sequencing of purified PCR products was done without cloning, using a commercial service provider. Purified PCR products were cycle- sequenced with an ABI prism big dye ^{TM} terminator, cycle sequencing ready reaction kit (Perkin- Elmer) 60 ng template DNA, 2 pmol primer 27F, 2 ul of nucleotides, enzymes and cofactors in a reaction tube (10 ul). Unincorporated dye terminator was removed using QIGEN DYE EX ^{TM} spin kit. This technique utilized labelled chain terminator ddNTPs, which permited sequencing in a single reaction. Each of the four dideoxynucleotide chain terminators were labelled with fluorescent dyes, each of which with different wavelengths of fluorescence and emission.

The DNA sequencers carried out capillary electrophoresis for size separation, detection and recording of dye fluorescence, and data output as fluorescent peak trace chromatograms. Reverse sequencing (1492r) was done for all the isolates. The sequencing was done at ILRI (commercial service provider).

The CHECK-CHIMERA program (http://rdp.cme.msu.edu/html/) of the Ribosomal Database Project (Maidak *et al*., 2001) was used to check for the presence of possible chimeric artifacts (Janssen *et al*., 2002). Sequenced data was analyzed with ARB software package [version 2.5b; O.Strunk and Ludwig, Technishe Universitat Munchem (http://www.arb-home.de). The new sequences were added to the ARB database and aligned with the Fast Aligner Tool (version 1.03). Alignments were checked and corrected manually where necessary, based on conserved regions.

The 16S rRNA gene sequences were compared to sequences in the public database using Basic Local Alignment Search Tool (BLAST) on the National Center for biotechnology Information (NCBI) website (http://www.ncbi.nih.gov) in order to determine similarity to sequences in the Genebank database (Altschul *et al*., 1990; Shayne *et al*., 2003). The 16S rDNA gene sequences with high similarities to those determined in the study were retrieved and added to the alignment based on BLAST results. Phylogenetic trees were constructed by Maximum likelihood method. Bootstrap analysis-using PHYLIP for 100 replicates was performed to attach confidence estimates for the tree topologies (Saitou and Nei, 1987).

3.15 Statistical data analysis

Means and standard errors within and between the different concentrations of Methomyl and carbofuran at different days were obtained by statistical software SPSS using one-way ANOVA. Data on total protein, esterase and phosphatase activity were also subjected to the same analysis.

CHAPTER FOUR

4.0 RESULTS

4.1 Survey on pesticide usage and sampling

The farmers interviewed use atleast two different combinations of pesticides with over 30 different formulations found to be used by farmers in the three geographically distinct regions sampled (Figure 5). The study showed that of the different pesticide formulation types (based on the target pest) used by farmers in these areas most were insecticides (44%), herbicides (34%), fungicides (13%) and bactericides (5.7%) with the remaining 3.3% being nematicides (Figure 7). Insect pests such as thrips (19%), aphids (23%) and mealybugs (23%) were found to be common in the sampled areas with minimal nematodes (3%) recorded (Appendix 1). Diazole 60EC and Methomex 90SP were the pesticide formulations that had high percentage usage compared to other formulations (Figure 5). Classes of pesticide used by farmers included pyrethroids (Lambdex 5EC and Karate Zeon) Carbamates, (methomyl and Furadan), Organophosphates (Diazol 60EC and dimekil 40EC) Triazoles, Phenyl urea and Neonicotinoids (Figure 10). Carbamates were found to have the highest proportion of usage (28%) followed by organophosphates (26%). Low usage percentage was recorded on other classes of pesticides such as deltamethins, dithiocarbamates, pyrimidines and sulfosates tus were grouped as others (Figure 6). Farmers were found to use a combination of different pesticide formulations on the same piece of land with the choice of pesticide used being influenced by the cost, technical knowledge required for application and availability of the pesticides.

Most of the farms sampled were under flower production (flouriculture) with Mobydick, Arabicum and Eryngium being the dominant flowers grown by farmers. French beans and kales were also gown by some farmers but to a small scale (Appendix 1).

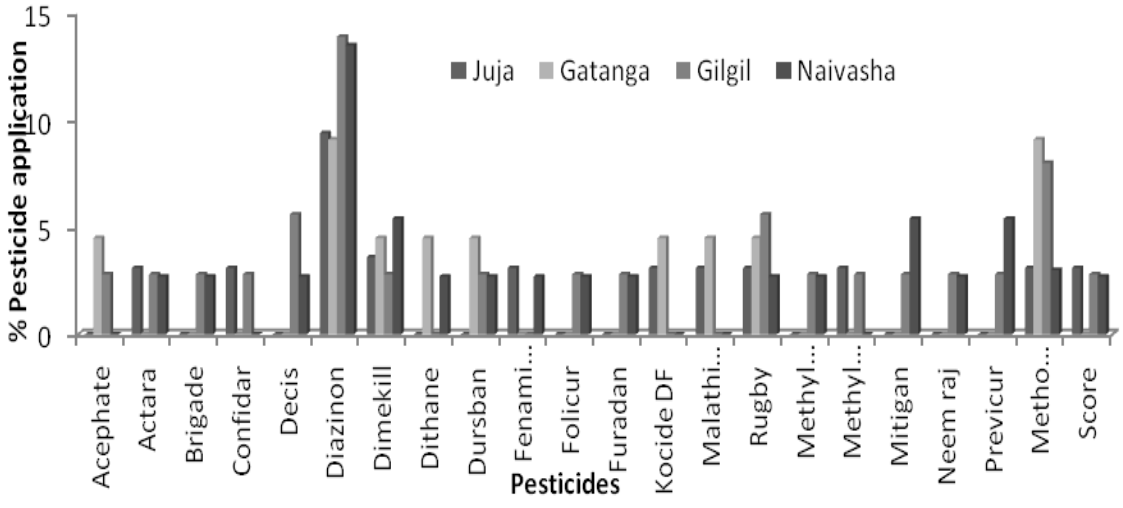


Figure 5. Pesticide formulations used by farmers in the four sampling regions

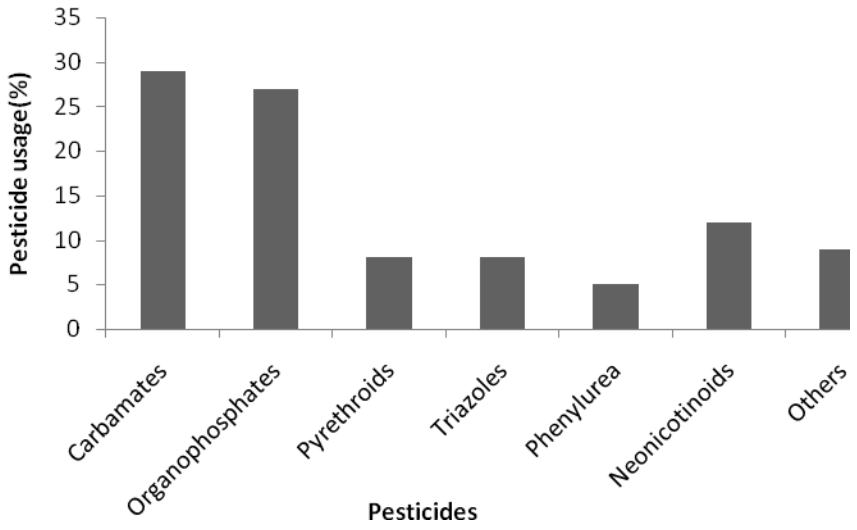


Figure 6. Proportions of classes of pesticides used by farmers in Rift Valley and Central Kenya

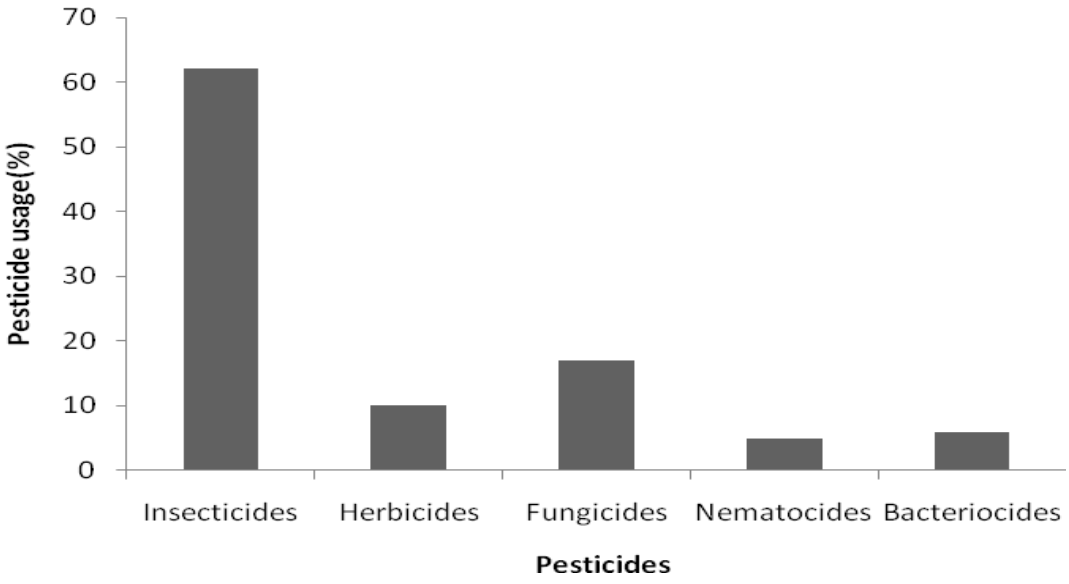


Figure 7. Proportion of types of pesticides used by farmers in Rift Valley and Central Kenya

4.2 Isolation and growth of carbofuran- degrading bacteria

Using independent enrichment step with carbofuran as the sole carbon and energy, fourteen carbofuran-degrading bacteria were isolated. The ability of the isolates to utilize carbofuran as sole carbon source was assessed using HPLC over a period of 120 days (Figure 8 and 9) with the reduction in the concentration of carbofuran determined against carbofuran standard curve. The extent of degradation varied among the isolates. Growth of isolates was also monitored against change in concentration of carbofuran. Change in cell biomass (OD_{600}) was recorded with maximum turbidity of 0.15 with 1% glucose supplementation (Figure 8 and 9). The growth of isolates in the enriched culture terminated after 90 days and eventually declined with the depletion of the carbon source (carbofuran).

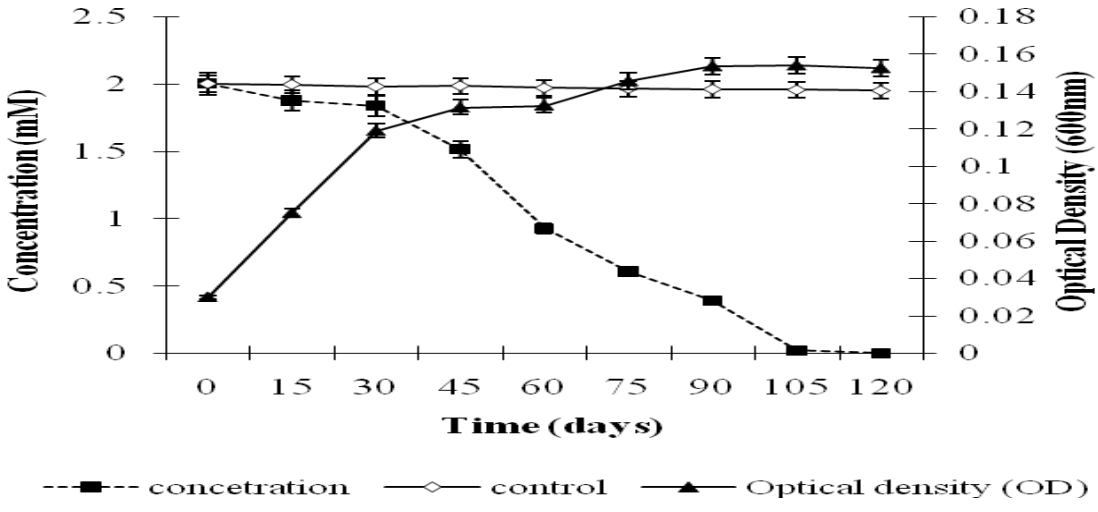


Figure 8. The increase in cell biomass with decrease in the carbofuran concentration for isolate C-9.

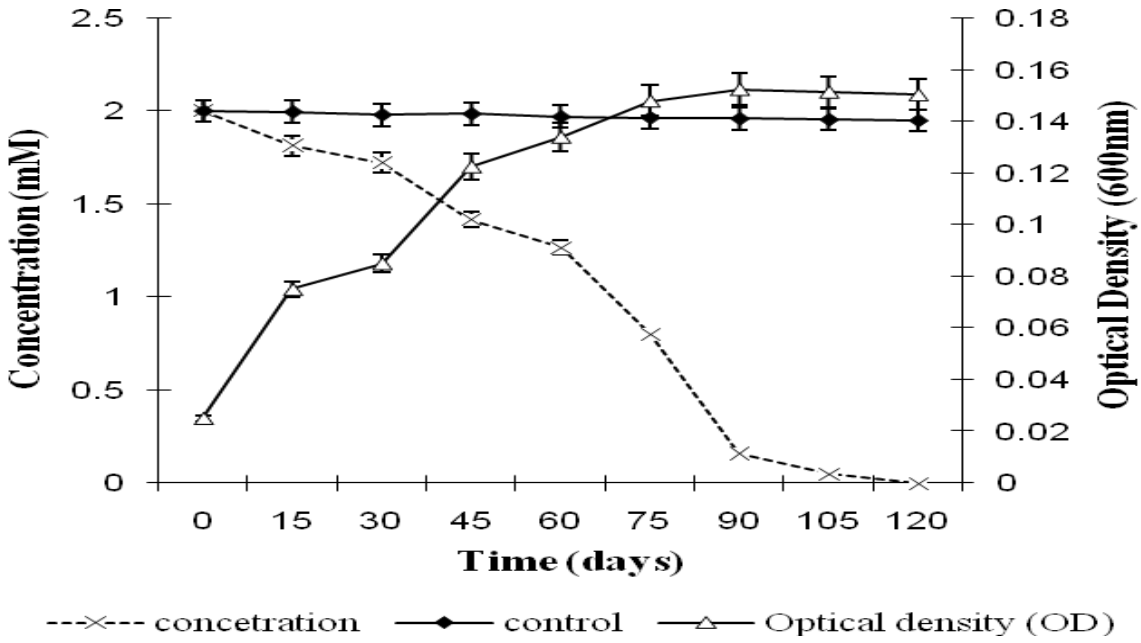


Figure 9. Change in cell biomass with decrease in carbofuran concentration for isolate C-10

4.3 Isolation and growth of methomyl degrading isolates

In an enrichment culture using methomyl as the sole nutrient and carbon source, twenty three bacterial strains were isolated. The ability of the isolates to utilize methomyl as sole carbon source was assessed using HPLC over a period of 40 days with the reduction in the concentration of methomyl determined against methomyl standard curve. The extent of degradation varied among the isolates (Figure 10 and 11). The growth of isolates on methomyl as a carbon source was monitored for 40days in which increase in cell-biomass was recorded (OD_{600}) with maximum turbidity of 0.088 noted. There was a decrease in methomyl concentration with change in the turbidity of isolates (Figure 10 and 11). The growth of isolates on methomyl gradually terminated and eventually started to decline with further decrease in the concentration of methomyl.

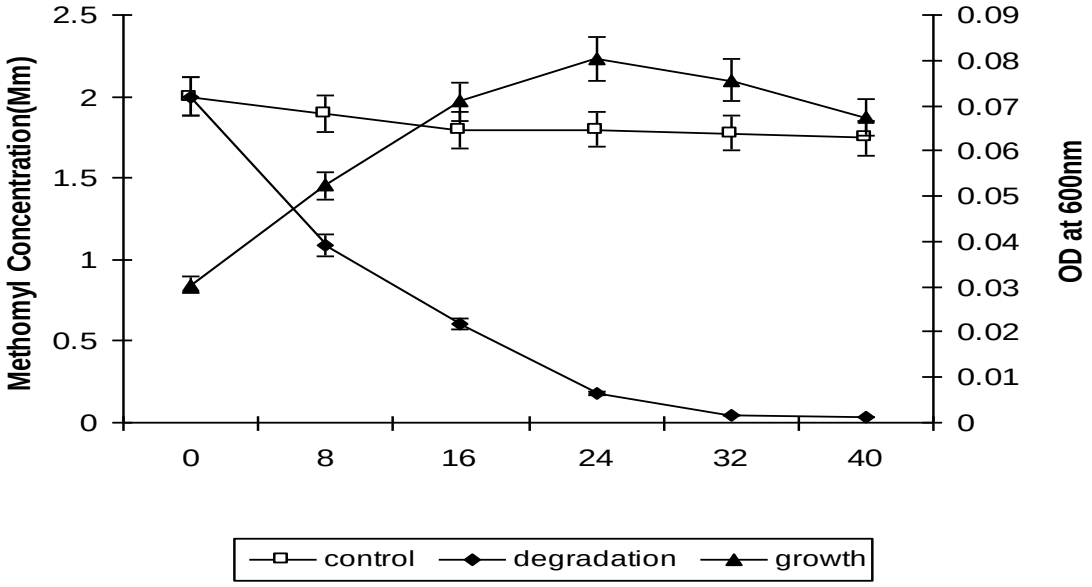


Figure 10. Change in cell biomass with decrease in methomyl concentration for isolate M-9.

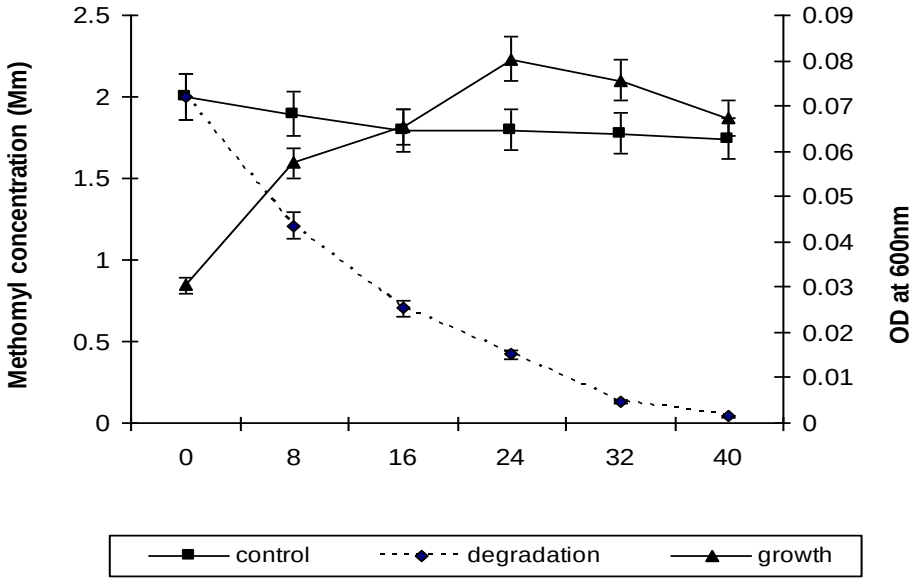
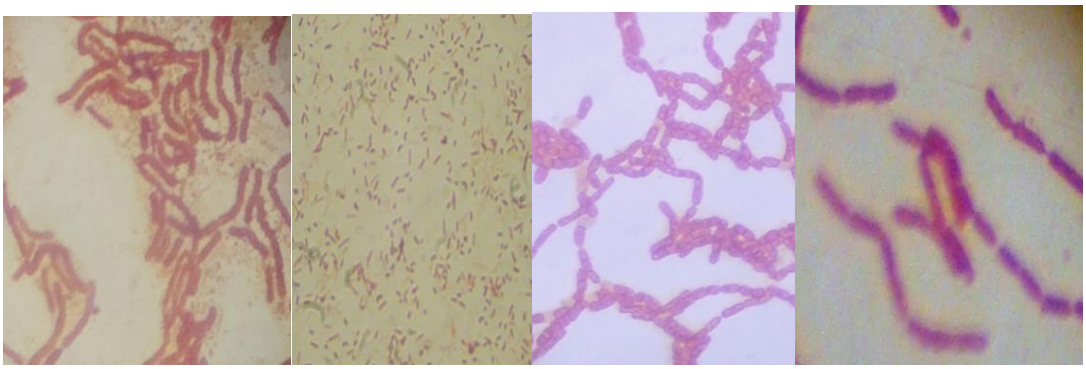


Figure 11. Change in cell biomass with decrease in methomy concentration for isolate M-14.

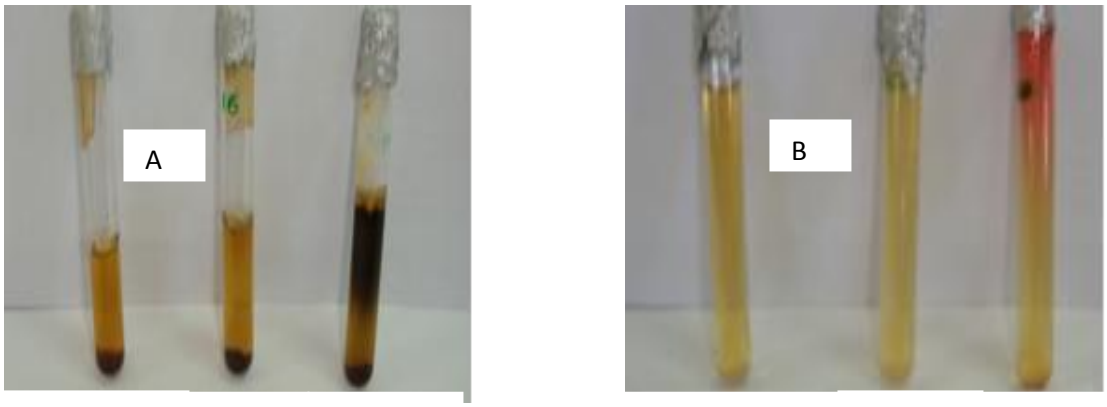
4.4 Microscopic and biochemical characterisation.

Morphological characterization of the thirty seven isolates placed the isolates into two major groups based on gram reaction, mortility and shape. Gram staining and 3% KOH test revealed that thirteen isolates were Gram positive and twenty four isolates were gram negative. Among the Gram positive isolates, three were rod shaped, motile while the other ten isolates were cocoid or coccus. All the Gram negative isolates were rod shaped and motile except eight which were cocoid in shape (Plate 1).



Isolate C-2 Isolate C-3 Isolate M- 23 Isolate M-14

Plate 1. Photograph showing the shapes of isolates C-2, C-3, M-23 and M-14 as viewed under light microscope



Control -ve + ve Control -ve +ve

Plate 2. Photograph showing SIM test (A) and methyl red test (B) as part of biochemical tests carried out.

Table 1: Biochemical characteristics of ten isolates representing methomyl and carbofuran degrading isolates

	Isolates									
	M-9	M-14	M-23	M-17	C-9	C-3	C-4	C-14	C-10	C-2
Morphology										
Shape	Coccoid	Rod	Rod	Coccoid	Rod	Coccoid	Rod	Rod	Rod	Rod
Gram reaction	+	-	-	+	-	+	-	-	-	+
Mortility	+	+	+	+	+	+	+	+	+	-
Biochemical tests										
Gelatinase	+	+	+	+	+	+	+	-	+	+
Nitrate reduction	+	+	+	+	+	+	+	+	+	+
Methy red	-	+	+	-	+	-	+	+	-	+
Voges-Proskeaur	-	+	+	-	-	-	+	+	+	-
Catalase	+	+	+	-	+	-	+	+	+	+
Oxidase	+	+	+	-	+	-	+	-	+	+
Urease	-	+	+	-	+	-	+	-	+	+
Citrate		+	+	+	-	+	+	+	+	-
H_2S	+	+	-	+	-	+	-	-	+	-
Indole	-	+	-		-	-	-	+	+	-

4.5 Determination of phosphatase activity and total protein

The phosphatase activity was estimated using the amount of p-Nitrophenolate produced which was determined against a p-nitrophenolate standard curve. All the isolates had phosphatase activity which varied among individual isolates (Figure 12). The total protein was also estimated against a standard curve prepared using Bovine Serum Albumin (BSA).The protein content also varied among the isolates (Figure 13 and 14).

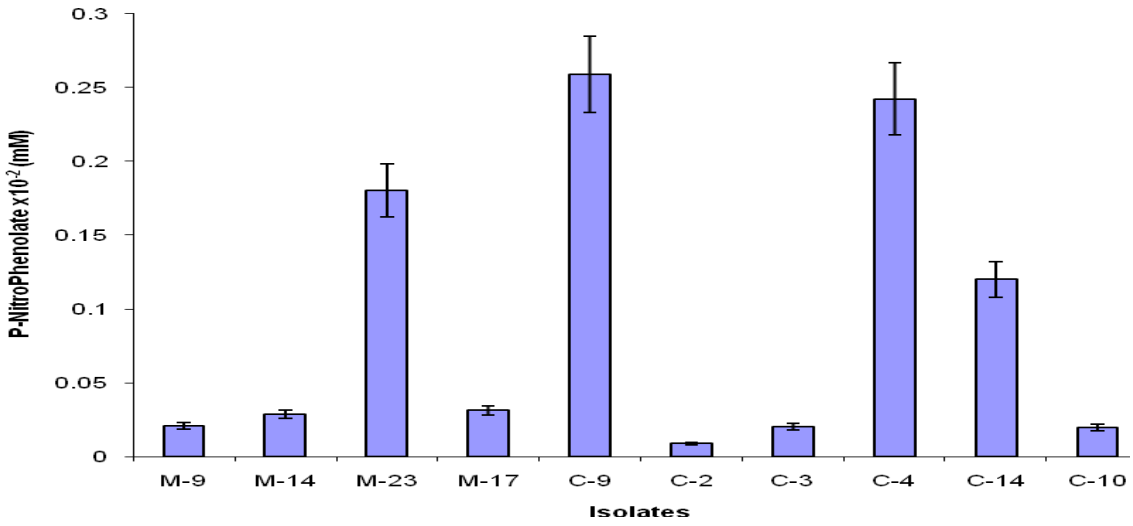


Figure 12: The concentration of p-nitrophenolate produced by isolates from 50mM p-nitrophenyl phosphate in 30 minutes.

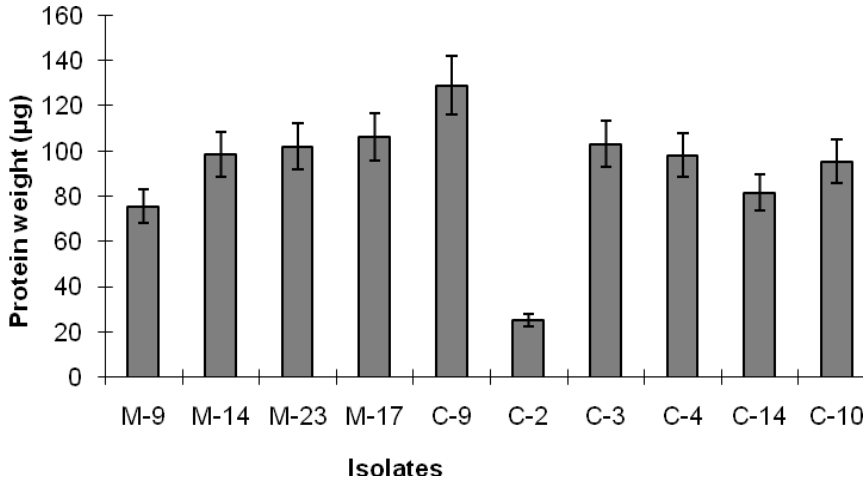


Figure 13. The total protein produced by different isolate cultures after 24 hours in a low phosphate media

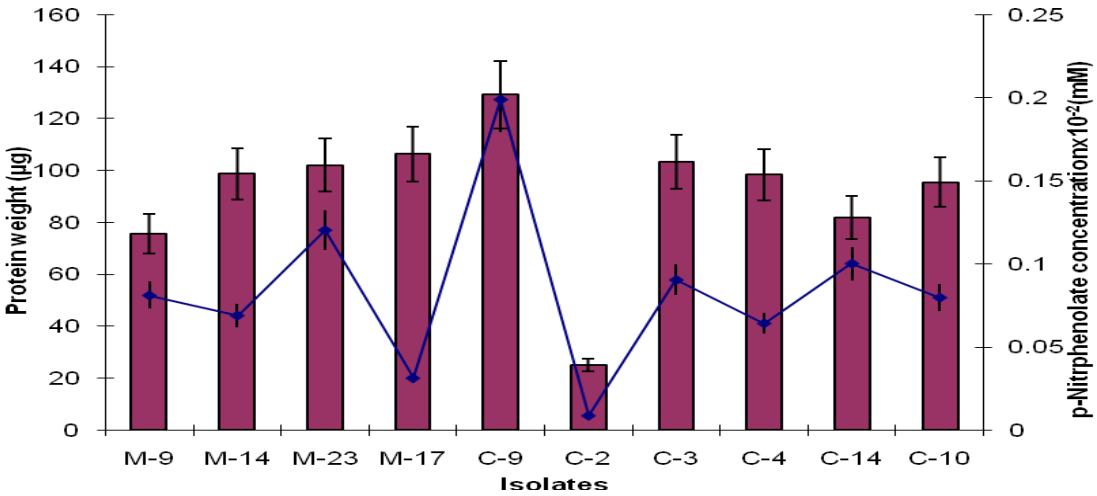


Figure 14. Relationship between the total protein produced by different isolates and phosphatase activity determined by the amount of p-nitrophenolate produced

4.6 Determination of esterase activity

The esterase activity was estimated by calculation of enzymatic index from the diameter of the colony and the halo (clearance zone) in a media supplemented with Tween 80 to final concentration of 1% (v/v). Enzymatic index (EI) = Halo diameter (mm)/Colony diameter (mm) (Figure 15).

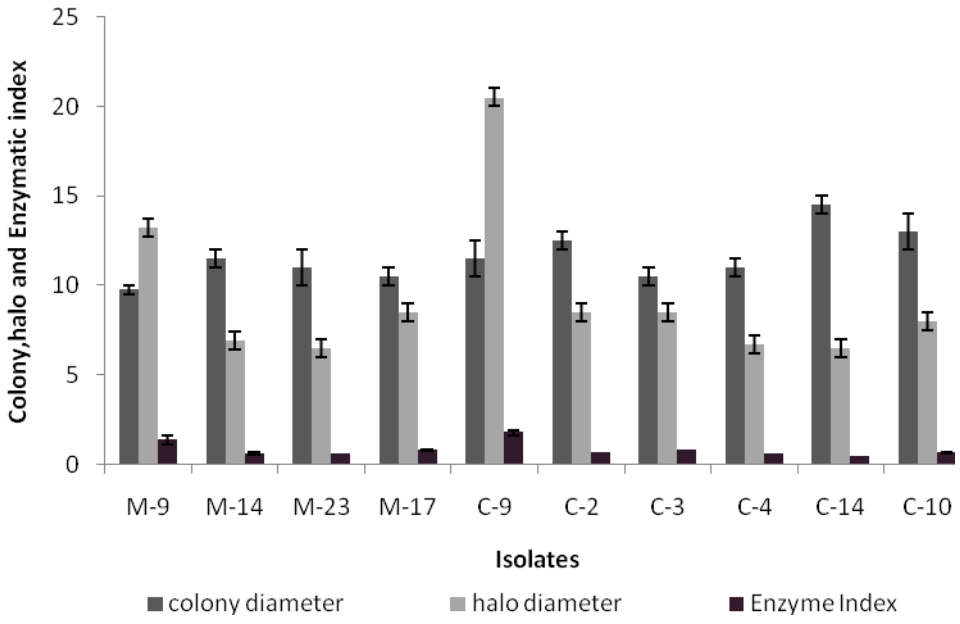


Figure 15: Graph of enzymatic indices for various isolates

4.7 Growth of isolates in various temperatures

The isolates were able to grow between the temperatures 25-37^{0}C with optimal growth for all the isolates observed between 30-35^{0}C (Figure 16).

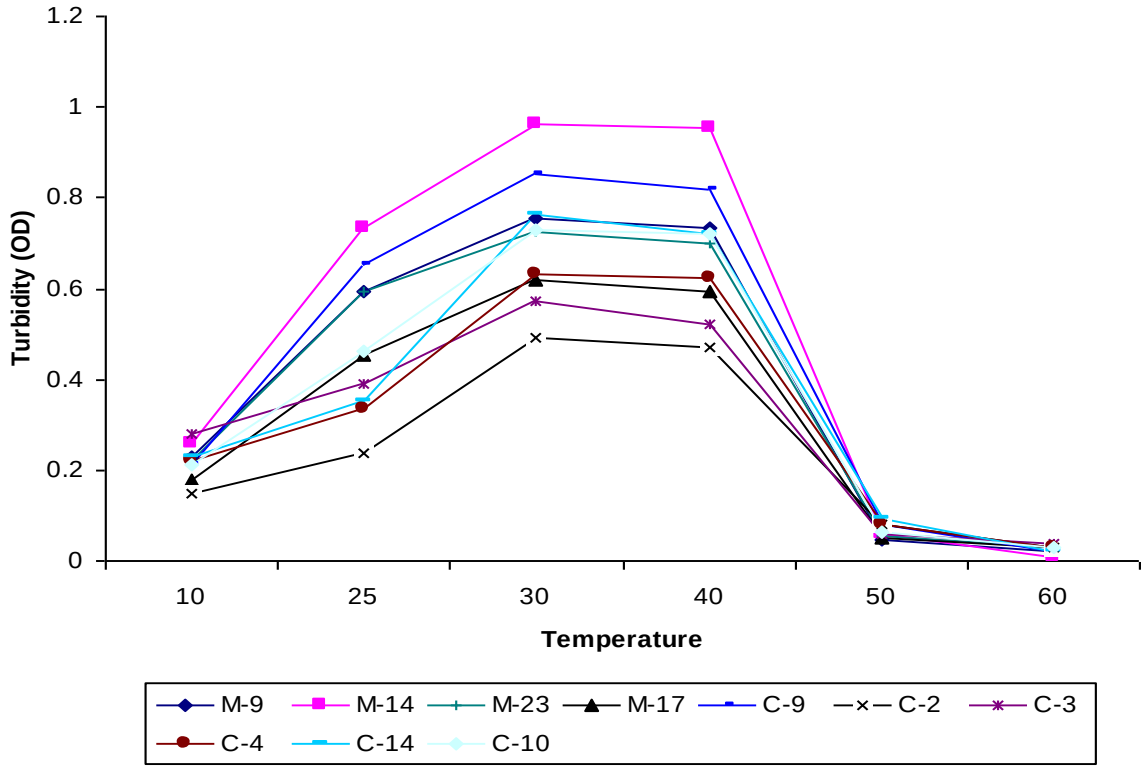


Figure 16: Growth of isolates on different temperature regimes in nutrient broth. The optical density was determined after 24 hours of incubation.

4.8 Chromatographic profiles for degradation of Carbofuran

Carbofuran was biodegraded forming three key metabolites identified at the retention times 1.7minutes, 2.0 minutes and 2.7 minutes (Figure 17 and 18). The concentration of carbofuran declined with time with subsequent increase in the concentration of metabolites. At 120 days, carbofuran was completely degraded with metabolites showing considerable decline in concentration. The decrease in metabolites concentration was followed by an emergence of a metabolite at the retention time 3.5 minutes (Figure 17 and 18).

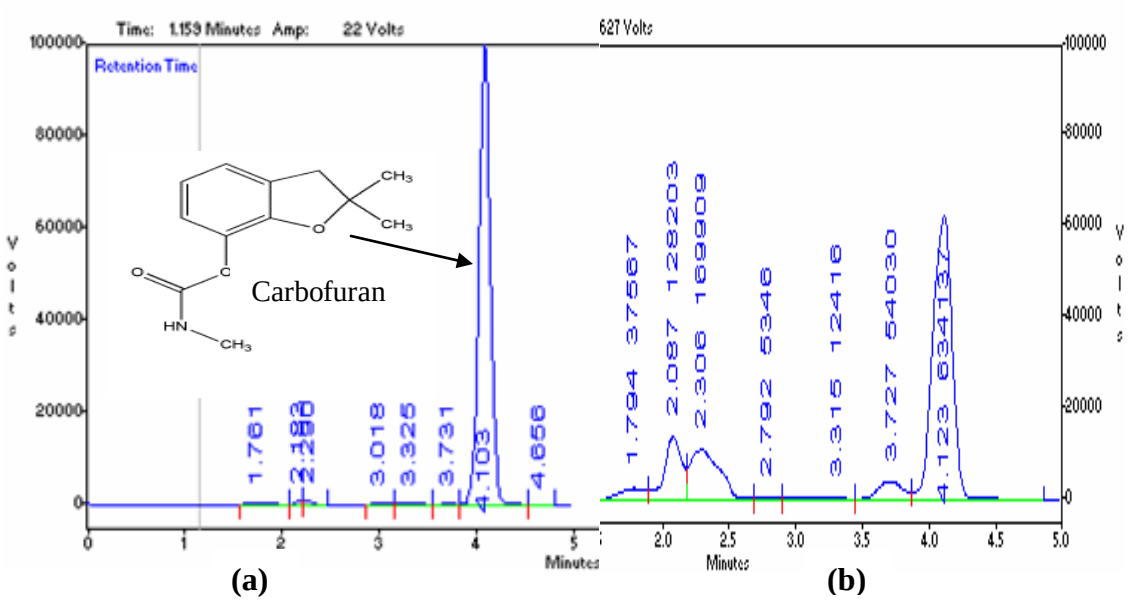


Figure 17. Chromatographs for carbofuran and its metabolites at (**a**) day 1 and **(b)** 28 days.

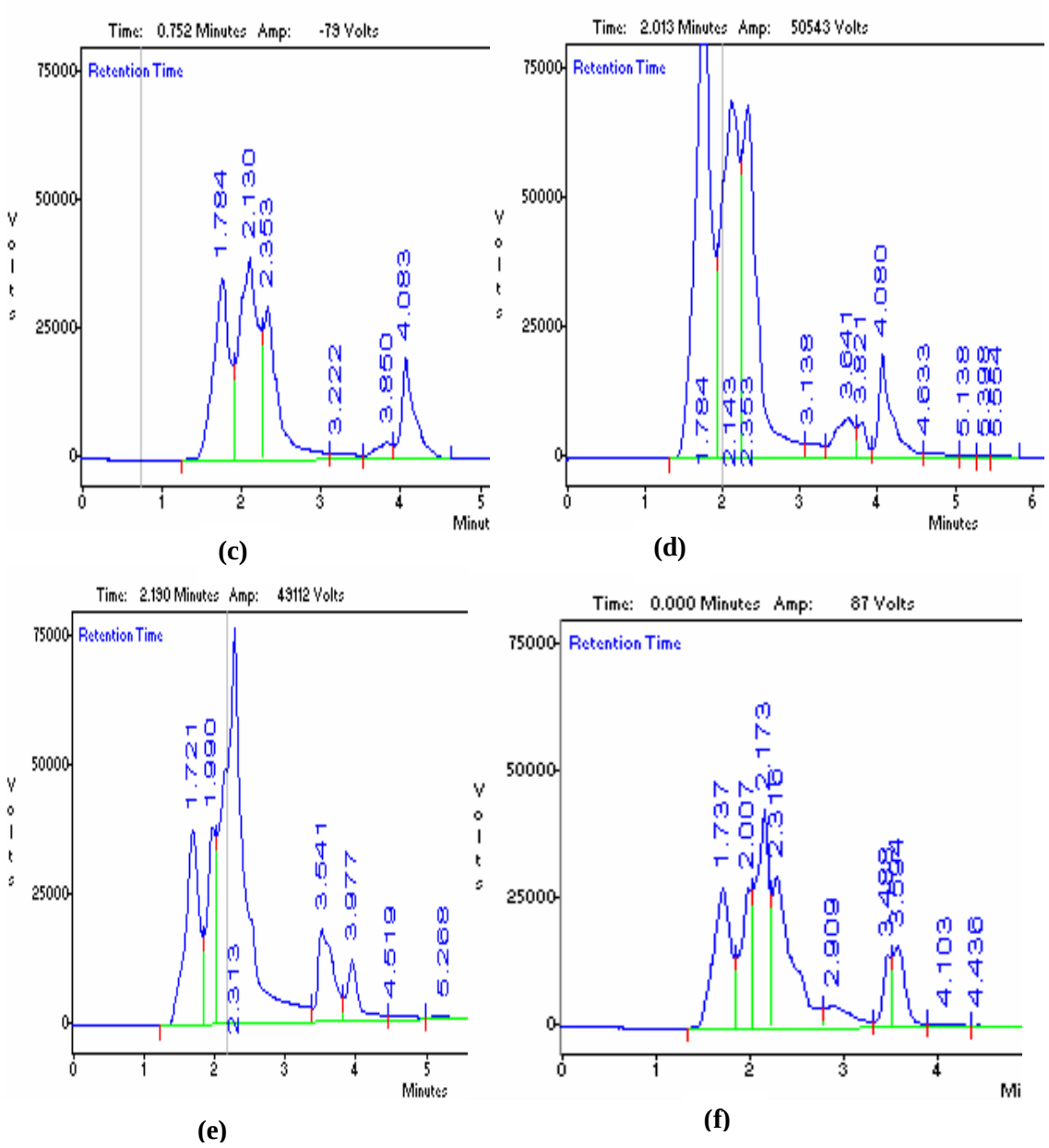


Figure 18. Chromatographs showing the decline in the concentration of carbofuran and its metabolites at **(c)** 30 days **(d)** 60 days **(e)** 90 days and **(f)** 120 days

4.9 Chromatographic profiles for degradation of Methomyl

Methomyl was completely depleted with 40 days (Figure 19 and 20). Methomyl initially degraded into one metabolite observed at the retention time 4.1 minutes. The metabolite was not stable to be monitored through the study. At 35 days, the metabolite depleted and two other metabolites were observed at retention times 1.4 minutes and 6.1 minutes.The two metabolites depleted five days later (Figure 19 and 20).

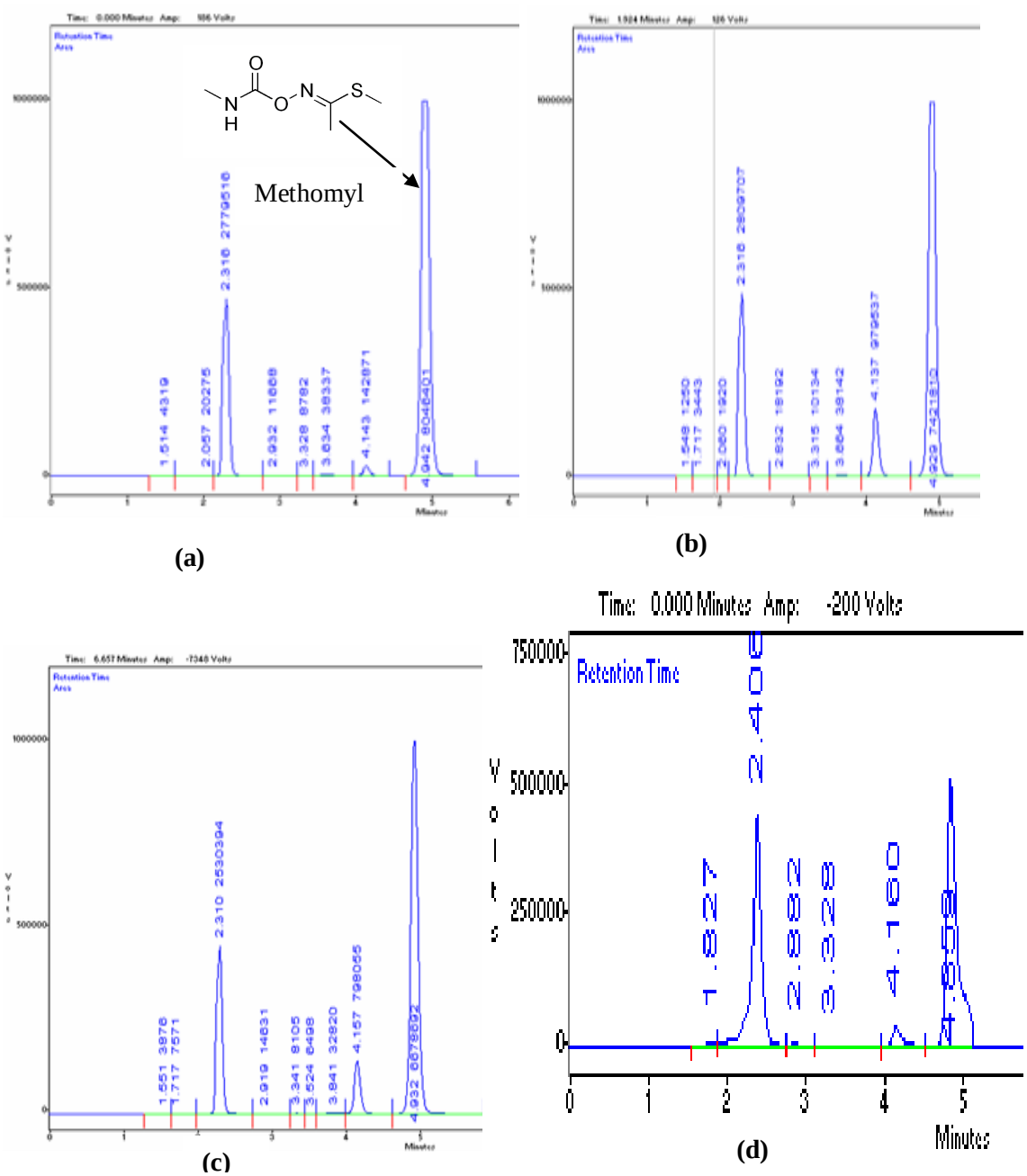


Figure 19: Chromatographs showing the decline in the concentration ofmethomyl and its metabolites at **(a)** 3 days **(b)** 12 days **(c)** 18 days and **(d)** 25 days

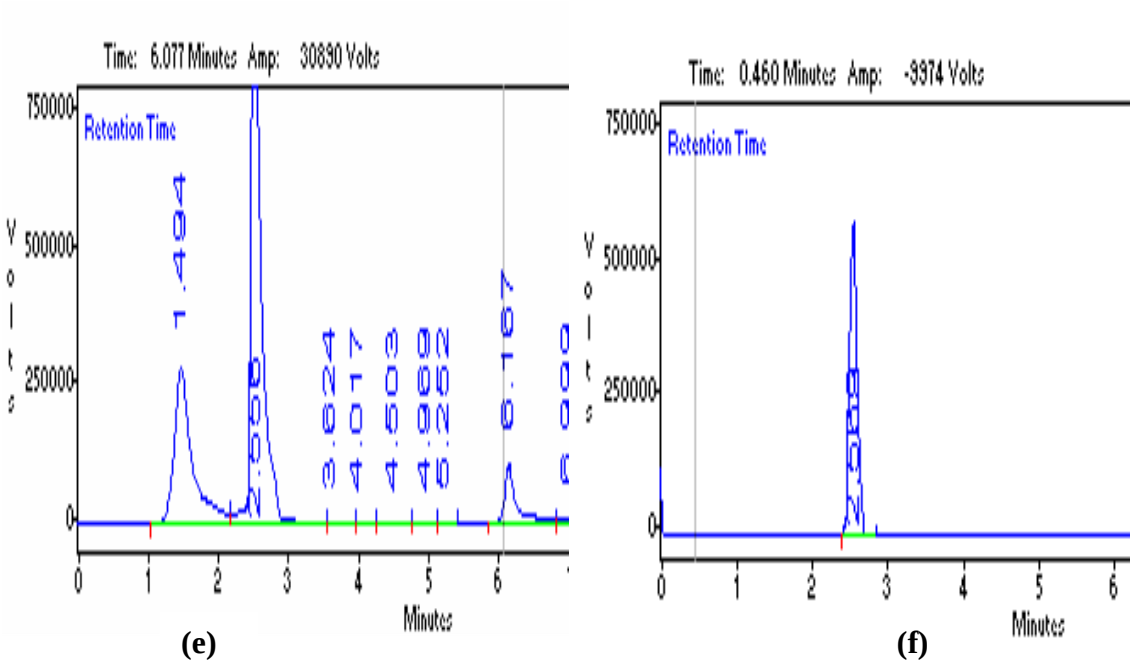


Figure 20: The chromatographs showing the decline of methomyl metabolites at **(e)** 35 days and **(f)** complete depletion of methomyl metabolites at 40 days.

4.10 Molecular analysis

Genomic DNA from all the isolates was successfully isolated and amplified using specific primers (1492R and 27F) (Figure 21). Restrictriction analysis of the PCR products clustered the isolates into three groups according to the restriction profiles (Figure 22 and Table 2). The 16S rDNA gene sequence for the isolates comprising of 1200 nucleotide of *E. coli* sequence were successfully determined in this study. The 23 methomyl degrading isolates clustered into four genera; *Flavobacterium, Vagococcus, Paracocus* and *Alcaligenes* with 95-99% sequences similarity (Figure 27).

The 14 carbofuran degrading isolates were more genetically diverse and clustured into six genera; *Flavobacterium, Vagococcus, Alcaligenes, Psedomonas, Providencia* and *Bacillus* with 94-99% sequence similarity (Figure 28).

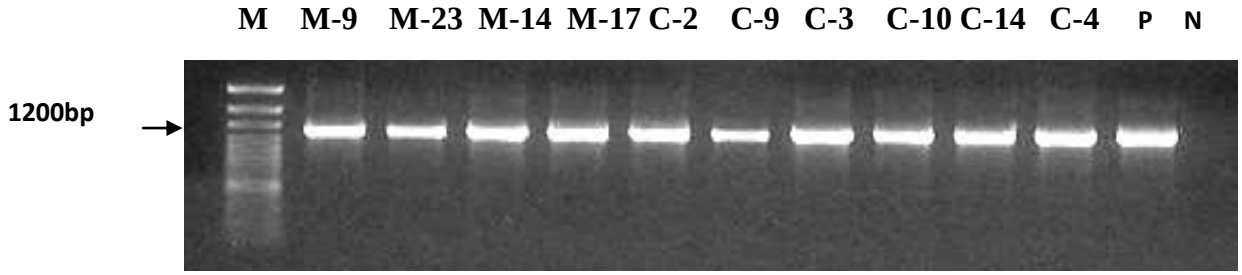


Figure 21. PCR products for ten isolates run in 0.8% (W/V) agarose gel. M-Marker, P-positive control and N-negative control

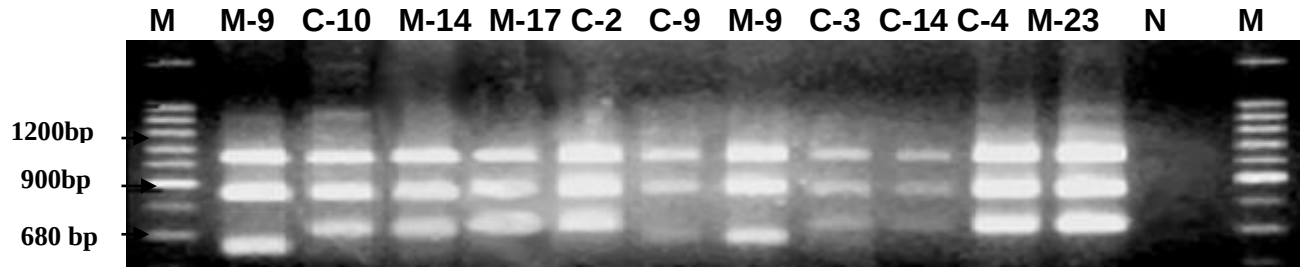


Figure 22. Restriction products as generated by *RsaI* digestion run in 1.5% (w/v) agarose gel. M-marker and N-negative control.

Table 2: Restriction fragments of different isolates as generated by *Rsa I* digestion

	Isolates									
	M-9	M23	M-14	M-17	C-2	C-9	C-3	C-10	C-14	C-4
Fragments										
950/1000bp	+	+	+	+	+	+	+	+	+	+
850/900bp	+	+	+	+	+	+	+	+	+	+
750bp	-	+	+	+	+	-	+	+	-	+
640/700bp	+	-	-	-	-	+	-	-	-	-

Key: (+) - Presence of fragment (-)-Absence of fragment

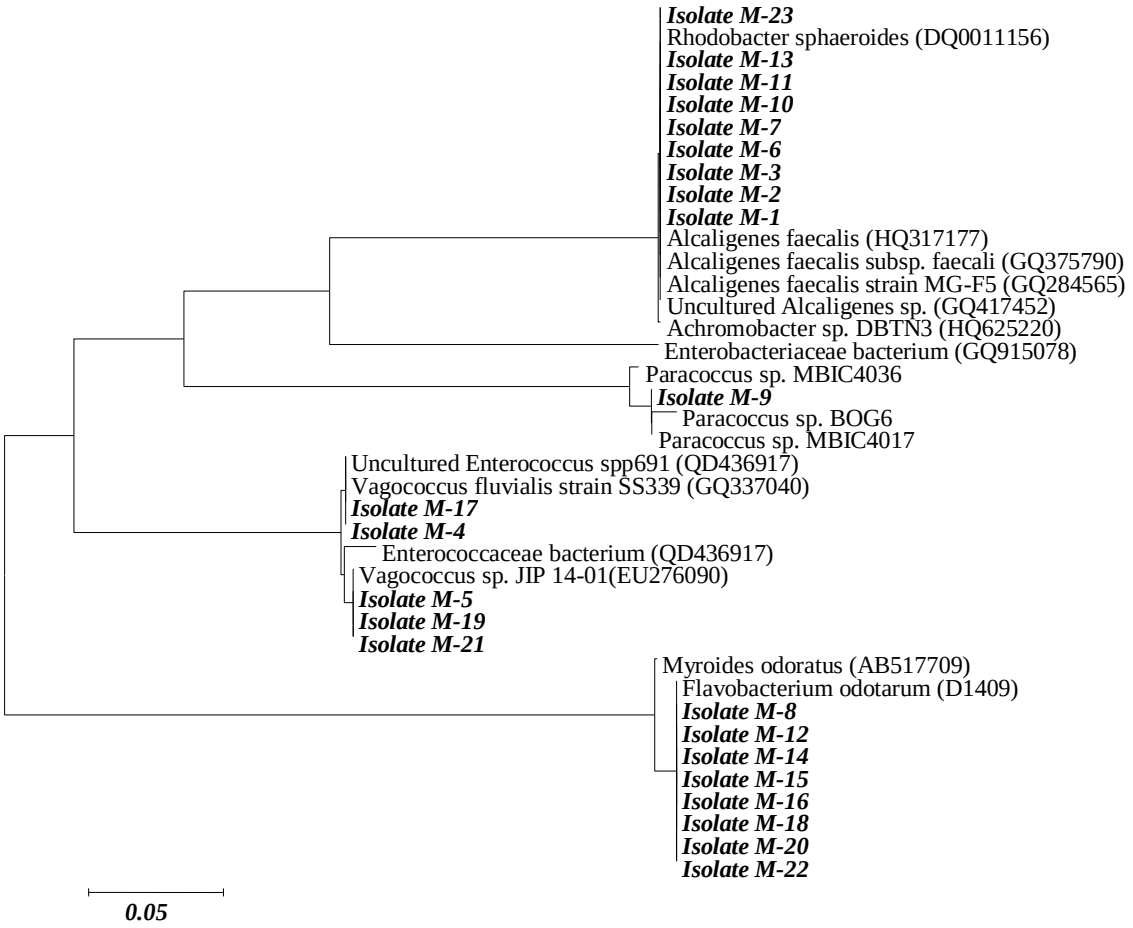


Figure 23. Neighbour-joining phylogenetic tree showing the position of methomyl degrading isolate. The bar indicates the estimated substitution per nucleotide position

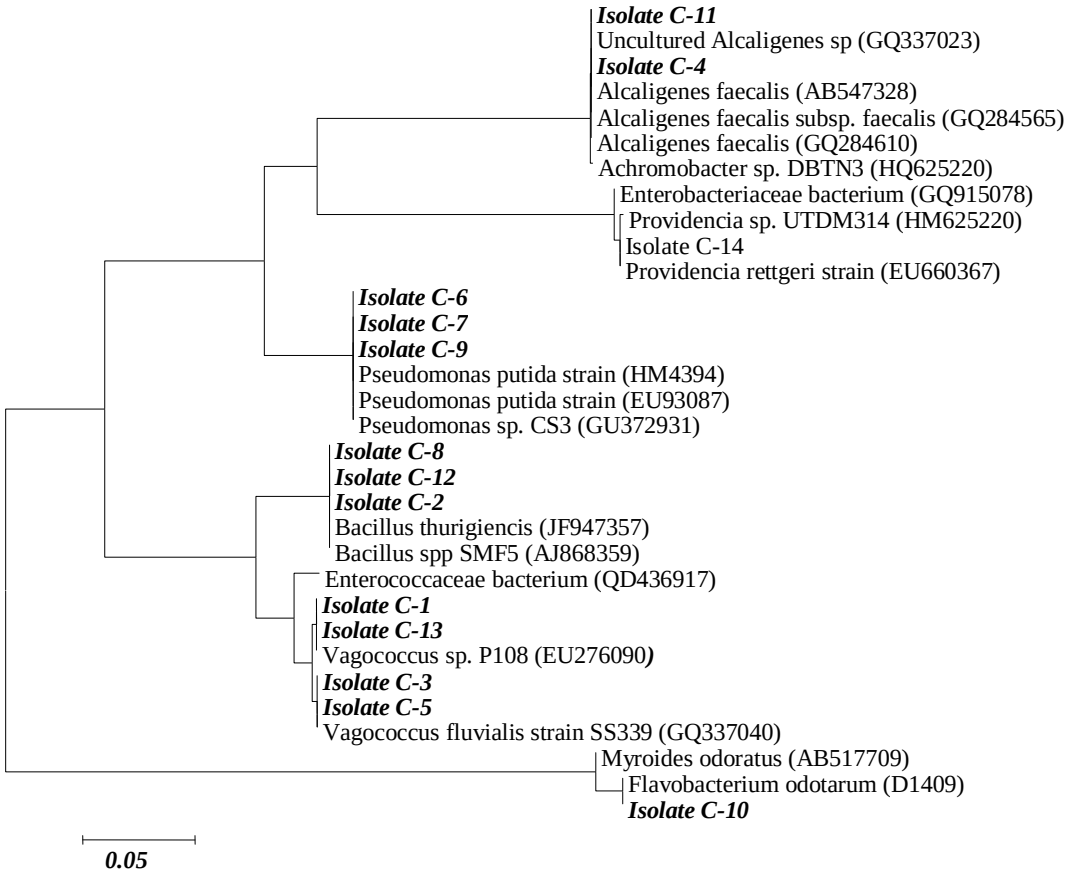


Figure 24. Neighbour-joining phylogenetic tree showing the position of carbofuran degrading isolates. The bar indicates the estimated substitution per nucleotide position.

4.11 Geographical distribution of carbamate-degrading bacteria

The isolated strains were found to be present in all the sampling regions displaying their wide distribution across different ecological regions (Table 3 and 4).Thika region had more species distribution for both methomyl- and carbofuran-degrading bacteria (Table 3 and 4). Some bacterial strains were able to utilize both methomyl and carbofuran as carbon source showing their multidegradative capability (Table 5)

Table 3. Geographical distribution of carbofuran degrading isolates as identified by 16S rDNA sequence analysis

	Region		
	Thika (Juja and Gatanga)	Naivasha	Gilgil
Isolates			
Bacillus sp	++	-	+
Vagococcus fluvialis	++	+	+
Pseudomonas pituda	-	+++	-
Alcaligenes faecalis	++	-	-
Flavobacterium odotarum	+	-	-
Providencia rettgeri	-	-	+

Key: (+) - Presence of one isolate (-) - No isolate

Table 4. Geographical distribution of methomyl-degrading isolates as identified by 16S rDNA sequence analysis

	Region		
	Thika (Juja and Gatanga)	Naivasha	Gilgil
Isolates			
Flavobacterium odoterum	+++++	+++	-
Alcaligenes faecalis	+++	-	++++++
Vagococcus fluvialis	+	+++	+
Paracoccus spp	+	-	-

Key: (+) - Presence of one isolate (-) - No isolate

Table 5. Cross-utilization of methomyl and carbofuran by isolates as identified by 16S sequence analysis

	Isolates						
	Bacillus sp.	*V. fluvialuis*	*P. pituda*	*A. faecalis*	*F. odotarum*	*P. rettgeri*	*Providencia sp.*
Pesticides							
Methomyl	+	+	+	+	+	+	-
Carbofuran	-	+	-	+	+	-	+

Key: (+) - degradation (-) - No degradation

CHAPTER FIVE

5.0 DISCUSSION

5.1 Survey of pesticide usage

The use of pesticides was observed to be common among farmers with over 30 different formulations being used in the sampling regions. This is attributed by an assumption by farmers that the only solution to pest problems is to apply pesticides more frequently and use different types and classes of pesticides. This view has also been reported earlier (Dinham, 2003). The pesticides usage in the study areas seemed to be highly influenced by manufacturers and pesticide vendors who were carrying out their business right in the farming communities and very interested in achieving large sales of their pesticides. Moreover, regional agronomists hired by firms dealing with horticultural products had a great influence on farmers' choice of pesticides to use. These firms contracted farmers to grow flowers and in return offer extension services and market to the farmers produce.

Insecticides were the most used type of pesticides because insect pests were the most serious problem in vegetable and flower production in the study area. This was followed by fungicides usage indicating that fungal attacks ranked second to insect pests. The fungal infection may be due to the high irrigation frequency on the horticultural crops creating dampness which favours the fungal infection.

Carbamates were among the class of pesticides that farmer preferred because, in farmers' perception, most carbamate pesticides are available in powder/granular forms and thus can just be applied directly to the farm compared to liquid formulations that requires technical skills on dilution which most farmers are not comfortable with.

Lack of dilution knowledge by farmers may result into use of concentrations which could be more or less concentrated thus harmful or less effective for the target purpose.

5.2 Biodegradation, ecological and metabolic diversity of the isolates

The microbial world is characterized by an incredible metabolic and physiological versatility that permits microorganisms to inhabit hostile ecological niches and to exploit as carbon and energy sources, compounds unpalatable to higher organisms (Timmis *et al*., 1994). The metabolic versatility and adaptability of bacteria is based on the existence of catabolic plasmids most of which are self-transmissible and have a broad host range, related as well as unrelated, and thereby propagate the unique metabolic capacity into new species (Ramon, 1984). In this study, different bacterial communities were isolated with capability of degrading methomyl and Carbofuran.

Some members notably *Vagococcus, Alcaligenes* and *flavobacterium* were able to utilize both the pesticides showing metabolic versatility of the isolates. Members of genera *Flavobacterium, Pseudomonas* and *Achrobacterium* have been isolated and shown to degrade carbofuran to carbofuraphenol, 3-ketocarbofuran and 3-hydroxycarbofuran as major carbofuran metabolites (Seo *et al*., 2007; Chaudhry and Ali, 1988).

In this study, carbofuran was degraded to three key metabolites noted at retention time 1.7 minutes, 2.1 minutes and 2.3 minutes. The metabolites were suspected to be carbofuranphenol, 3-ketocarbofuran and 3-hydrox1ycarbofuran; the major carbofuran metabolites described earlier (Seo *et al*., 2007; Chaudhry and Ali, 1988).

The biodegradation trend of carbofuran was similar in all the carbofuran degrading isolates with a notably decrease in the concentration of the metabolites after the depletion of the carbofuran. This indicated the ability of the isolates to also utilize the intermediates of the carbofuran as carbon source as confirmed by their high biomass even after the depletion of the carbofuran. Methomyl took shorter time for its complete degradation with one key metabolite noted at retention time 4.1 minutes. The metabolite was suspected to be *S*-methyl- *N*-hydroxythioacetimidate (MHTA), principal biodegradation product of methomyl which further breakdown to carbon dioxide as the end-product (WHO, 1996). *S*-methyl- *N*-hydroxythioacetimidate (MHTA) was highly unstable thus could not be followed further in the study since it did not sustain for long to be detected in the test samples.

Isolates M-8, M-12, M-14, M-15, M-16, M-18, M-20, M-22 and C-10 phylogenetically clustered with *Flavobacterium odotarum* and were capable of degrading both methomyl and carbofuran . *Flavobacterium* has a wide metabolic versatility capable of degrading short carbon chain (C8–C16) to very long carbon chain (C44) hydrocarbon of aliphatic fractions of petroleum hydrocarbons (Mishra *et al*., 2001).

Some species have been isolated from sediment and water of a crude oil polluted river and assessed in terms of ability or inability to grow in the presence of 0.5% (v/v) of diesel oil and potential to degrade the diesel oil (Kayode-Isola *et al.*,2008). *Flavobacterium* has also been used in bioremediation due to its effectiveness in cleaning up petrochemical wastewaters (Shokrollahzadeh *et al*., 2008).

The biodegradability of *Flavobacterium sp* is not limited to oil contaminated environments but has been found to degrade various pesticides including carbamates as noted in this research and other studies (Seo *et al*., 2007; Chaudhry and Ali, 1988).

Many *Flavobacterum* species have been described including *Flavobacterium psychrophilum* which causes the Bacterial Cold Water Disease (BCWD) on salmonids and the Rainbow Trout Fry Disease (RTFS) on rainbow trouts. *Flavobacterium columnare* which causes the cotton-wool disease on freshwater fishes. *Flavobacterium branchiophilum* causes the Bacterial Gill Disease (BGD) on trouts.

Yağcı *et al.*, (2000) reported that *Flavobacterium odoratum* is associated with urinary tract infections and highly resistant to a wide range of antimicrobial agents (Holmes et *al* 1979). The biodegradative capability of *F. odoratum* has since not been documented thus further research on its metabolic capability can place it to the list of other metabolically versatile members of the genus *Flavobacterium* having been isolated in this study to utilize both carbofuran and methomyl.

The metabolic capability of members of the genus *Pseudomonas* has been widely explored with a number of *Pseudomonas* strains known to utilize a variety of organic to inorganic compounds. They have been shown to degrade various di-aromatic compounds. *P. putida* utilizes 1- and 2-methylnaphthalene as the sole source of carbon and energy (Mahajan *et al*., 1994).The complete pathway for carbaryl, (1-naphthyl-*N* methylcarbamate) degradation has been elucidated for *Pseudomonas* sp.

These strains were found to utilize carbaryl via the 1-naphthol-1, 2-dihydroxynaphthalene, salicylate, gentisate pathway to the central carbon pathway (Swetha and Phale, 2005). *Pseudomonades* have also been found to degrade tri-aromatic compounds such as anthracenes and phenanthrenes.

A broad substrate-specific salicylate hydroxylase in *P. putida* catalyze the conversion of 1-hydroxy-2-naphthoic acid to 1, 2-dihydroxynaphthalene (Balashova *et al.,* 2001). *P. aeruginosa* degrades anthracene via *cis*-1,2-dihydrodiol-1,2-dihydroxyanthracene, *cis*-4-(2-hydroxynaphth-3-yl)-2-oxo-but-3-enoic acid, 2-hydroxy-3-naphthaldehyde, 2-hydroxy-3-naphthoic acid, and 2,3-dihydroxynaphthalene (Evans *et al.,* 1965).

Different *Pseudomonas* species have also been patented for production of various biosurfactants and bioemulsifiers that are useful in bioremediation process (Shete *et al.,* 2006).The wide ecological and metabolic versatility cut across various species of *Pseudomonas*; *P. alcaligenes*, which can degrade polycyclic aromatic hydrocarbons (Mahony *et al.,* 2006) *P. mendocina*, which is able to degrade toluene (Yen *et al.,* 1991). *P. pseudoalcaligenes* are able to use cyanide as a nitrogen source (Huertas *et al*., 2006). *P. resinovorans* can degrade carbazole (Nojiri *et al.,* 2002). *P. veronii* has been shown to degrade a variety of simple aromatic organic compounds (Nam, *et al.,* 2003*)*. *P. putida* has the ability to degrade organic solvents such as toluene (Zylstra and Gibson, 1989**)**. At least one strain of this bacterium is able to convert morphine in aqueous solution into the stronger and somewhat expensive to manufacture drug hydromorphone.

Strain KC of *P. stutzeri* is able to degrade carbon tetrachloride (Sepulveda-Torres, *et al.*, 1999). *Pseudomonads* have been documented to biodegrade various organophosphates and carbamate pesticides including carbofuran. The isolates C-6, C-7 and C-9 are phylogenetically closely related to *P. pituda* and were able to completely degrade carbofuran. They can thus be used in cleaning up of carbamate contaminated environment owing to their underlining geographical and metabolic diversity.

Alcaligenes are reported to have wide ecological distribution contributed by their metabolic diversity. The bacterium *Alcaligenes eutrophus* JMP134 (pJP4) degrades trichloroethylene (TCE) by a chromosomal phenol-dependent pathway and by the plasmid-encoded 2,4-dichlorophenoxyacetic acid pathway. The 2,4-dichlorophenoxyacetic acid (2,4-D) degrading bacterium *Alcaligenes eutrophus* JMP134 expresses two catabolic pathways which degrade phenol and 2,4-dichlorophenol. One is encoded on the chromosome; the other is encoded on the plasmid pJP4. *Alcaligenes* sp. has been known to be excellent hydrocarbon degrader (Krooneman *et al,* 1996).

Some members of this group like the *A. dinifricans, A. odorans,* and *A.eutrophus* are known to be excellent hydrocarbon degraders including the polycyclic aromatic hydrocarbons (Weissenfeis *et al.,* 1990, Harayama *et al.,* 1999). *Alcaligenes faecalis* has been isolated from acclimated activated sludge, collected from municipal gas works and it has been shown that immobilized *Alcaligenes faecalis* are also able to biodegrade phenol (Jiang *et al.,* 2007).

Isolates M-23, M-13, M-11, M-10, M-7, M-3, M-6, M-3, M-1, C-11 and C-4 were found to phylogenetically closely relate to *A. faecalis*. The isolates were able to degrade both carbofuran and methomyl completely confirming the metabolic versatility of *A. faecalis.* The bacterium thus can be employed in the cleaning up of xenobiotics contaminated environments. Geographically, the isolates were isolated from farms in Gilgil, Naivasha, Juja and Gatanga indicating the wide distribution of *A.faecalis* and thus can be applied for clean up purpose in wide geographical areas.

Many different *Paracoccus* strains have been isolated from contaminated sledge and treated waters. They can either use organic energy sources, such as methanol or methylamine, or act as chemolithotrophs, using inorganic energy sources with carbon dioxide as their carbon source. A highly effective dimethoate-degrading *Paracoccus sp.* strain Lgjj-3 has been isolated from treatment waste water. The strain can utilize dimethoate as its sole carbon source for growth (Rong *et al.*, 2010). *Paracoccus* sp. YM3 capable of degrading carbofuran was isolated from carbofuran-contaminated sludge. The strain was shown to metabolize carbofuran to carbofuran-7-phenol (Peng *et al.*, 2008). However, in this study, it was isolated and found to completely degrade 2 mM of methomyl in approximately 40 days. *Paracoccus* sp. M-1 is capable of degrading monocrotophos. The ability of the strain to mineralize monocrotophos has also been investigated under different culture conditions and found to degrade organophosphorus insecticides and amide herbicides (Jia *et al.*, 2006).

Paracoccus kocurii sp., tetramethylammonium-assimilating bacteria has also been isolated from an activated sludge used for the treatment of tetramethylammonium hydroxide contained in the wastewater from semiconductor manufacturing process (Ohara *et al.*, 1990). *Paracoccus* sp. mdw-1 has been found to completely transform methomyl to *S*-methyl-*N*-hydroxythioacetamidate (Xu *et al.,* 2009). Isolate M-9 was able to degrade methomyl completely after approximately 40 days and was found to phylogenetically closely related to *Paracoccus sp* MBIC4036.

Vagococcus was isolated with both methomyl and carbofuran showing its metabolic versatility. The geographical distribution of vagococcus species was wide being isolated in almost all the regions sampled. In this study, the isolates M-17, M-4, M-5, M-19, M-21, C-1, C-13, C-3 and C-5 were found to be phylogenetically closely related to *Vagococcus fluvialis*. *Vagococcus fluvialis* has been found to adsorb and afterwards accumulate Cu^{2+} inside their cells thus has been used in remediation of heavy metals in wastewater (Shaakoori, 2002). Many other species of *Vagococcus* have also been described (Pot *et al.,* 2004).

Isolates C-2, C-12 and C-8 were found to utilize carbofuran as carbon source and phylogenetically closely related to *Bacillus sp*. *Bacillus* species are reported to be capable of utilizing p-cresol as sole source of carbon. The organism also utilizes phenol, o-cresol, m-cresol, 4-hydroxybenzoic acid, and gentisic acid as growth substrates. The organism is reported to degrade p-cresol to 4-hydroxybenzoic acid (Tallur *et al.,* 2006).

B. subtilis has been described to have multidegradative capabilities ranging from hydrocarbons (Christova *et al*., 2004) to Phthalic acid derivatives (Junichi *et al*, 2005). The efficiency of a strain of *B. thuringiensis* MOS-5 (Bt) to degrade organophosphorus insecticide has been established with its ability to degrade Malathion (Kamal *et al.*, 2008).

Isolate C-14 was found to utilize carbofuran and phylogetically closely related to *Providencia rettgeri*. Different strains of *Providencia* have been described with *P. stuartii* having diverse degradative capability. *P. stuartii* has been isolated from patient with purple urine syndrome and found to degrade Urinary Indoxyl Sulfate (Dealler *et al*., 1988). *P. stuartii* has also been isolated from agricultural soils and found to be capable of degrading chlorpyrifos (Rani *et al*, 2008). *P. rettgeri* has been isolated from nitramine explosive-contaminated soils and found to reduce hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) (Zhao *et al* .,2002). The ability of *p. rettgeri* to reduce RDX and HMX is an indication of preference to nitrogen containing compounds thus the ability to utilize carbofuran, an N-methylcarbamate compound.

5.3 Phosphatase and esterase activity

The conversion of p-nitrophenyl phosphate disodium salt to p-nitrophenolate, a compound that was detected at a wavelength of 405nm was an indication of phosphatase activity by the isolates.

A clearance zone by isolates grown on a nutrient agar supplemented with Tween 80 to a final concentration of 1 % (v/v) was an indication of esterase activity. It had been reported that soils that showed enhanced biodegradation of organophosphate insecticides had significantly high phosphatase enzyme activities (Sikora *et al,* 1989). Enhanced degradation of pesticides is aided by hyrolase enzymes (Esterase and/ or phosphatases) which catalyses the hydrolysis of pesticide compounds (Kumar *et al*. 1996). In this study, both the enzymes were detected as all the isolates showed both phosphatase and esterase activity. There was no significant difference ($p>0.05$) in the phosphatase activity among the isolates M-9, M-14, M-17, C-3 and C-10. There was no significant difference in the enzymatic indeces for the isolates M-14, M-23, C-4 and C-14. In addition; there was no significant difference in the enzymatic index for isolates M-17, C-2, C-3 and C-10. The relatively high phosphates activity and enzymatic index in isolate C-9 matched the high biodegradative capability displayed by the isolate. This is an indication of the high relationship between the phosphatase and/or esterase activity and biodegradation. There was no relationship between total protein and phosphatase activity as isolates with high total protein did not necessarily have high phosphatase activity.

5.4 Morphological, biochemical and molecular identification of carbamate-degrading isolates

The taxonomic classification of isolates performed using 16S ribosomal DNA sequences of their genomic DNA placed the isolates to belong to the genera *Paracoccus, Vagococcus, Bacillus, Pseudomonas, Flavococcus, Providencia* and *Alcaligenes.* Biochemical, physiological and morphological characteristics of the isolates also supports these genus assignments.

Paracoccus are described as gram-negative, coccoid **(**La *et al.,* 2005), nitrate reducing bacteria able to reduce nitrates to nitrites and are compounds, positive with oxidase, catalase and hydrogen production tests (Lee *et al.,* 2004). The species of *paracoccus* are diversely distributed and live primarily in soil as either aerobes or anaerobes. Many different *Paracoccus* strains have also been isolated from contaminated sledge and treated waters. They can either use organic energy sources, such as methanol or methylamine, or act as chemolithotrophs, using inorganic energy sources with carbon dioxide as their carbon source. They have been diversely isolated and found to have inherent capability to degrade various pesticides. Several strains of *Paracoccus* have been described with *P. denitrificans*, *Paracoccus sp*. strain Lgj, Paracoccus *sp*. strain HPD-2 j-3, *Paracoccus* sp. 12-A, *Paracoccus* sp. M-1 and *Paracoccus sp*. YM3 are notably isolated with biodegration potentials.

Isolate M-9 displayed the typical characteristics and biochemical properties of members of the gen*us Paracoccus.* They ferment glucose and lactose, negative with urease and citrate tests with an optimum growth at temperatures 30-37^{0}C. Sequence analysis by the BLAST search system on the NCBI website showed that the isolate M-9 was phylogenetically most closely related to *Paracoccus sp.* MBIC4017.

Providencia can be described as aerobic, gram negative, rod-shaped, flagellated motile bacteria (Costas *et al.,* 2008) with a wide ecological diversity found in soil, water and sewage environments. They are also found in multiple animal reservoirs, including flies, birds, cats, dogs, cattle, sheep, guinea pigs, and penguins, and are resident oral flora in reptiles such as pythons, vipers, and boas. Many *Providencia* species have been described including *P stuartii, P alcalifaciens, P rettgeri* and *P heimbachae* which are pathogens of urinary tract (Müller, 1986) and also associated with gastroenteritis and bacteremia. Some of the *Providencia* species have been described to degrade pesticides with *P. stuartii* capable of utilizing chlorpyrifos as sole carbon source while *Providencia rettgeri* described as an oxalate-degrading bacterium (Hokama *et al.,* 2005). Sequence analysis by the BLAST search system on the NCBI website showed that the isolate C-14 was phylogenetically most closely related to *Providencia rettgeri strain* CT15 with 96% rDNA sequence similarity. Isolate C-14 adheres to all the signature phenotypic and biochemical characteristics of *Providencia rettgeri strain* CT15 which is a rod-shaped gram negative nitrate reducing bacteria positive with catalase reaction, indole production and citrate reduction tests (Costas *et al* 2008). They are oxidase and urease negative and unable to reduce gelatine.

They produce acid from aerobic reduction of glucose and reduce P-Nitrophenyl phosphate to p-Nitrophenolate in alkaline condition indicating the production of phosphatase enzyme by the isolate.

Phylogenetic position of methomyl-degrading isolates; M-23, M-13, M-11, M-10, M-7, M-3, M-6, M-3 and M-1 showed that they are closely related to *Alcaligenes faecalis* along with carbofuran degrading isolates C-11 and C-4. *A. faecalis* is a gram-negative, rod-shaped, motile aerobic bacterium commonly found in soil and water environments (Schroll *et al.*, 2001). *A. faecalis* has been described as pathogenic, opportunistic bacteria thus may also be isolated from blood, sputum, and urine, especially in immunocompromised hosts. *A faecalis* has been described to have diverse metabolic capability degrading various organic compounds including pesticides (Jiang *et al.*, 2007). Morphological, physiological and biochemical characteristics distinguished isolates M-23, M-13, M-11, M-10, M-7, M-3, M-6, M-3, M-1, C-11 and C-4 as closely related to *A. faecalis.* They are oxidase and catalase positive with ability to reduce urea and citrate, growing at an optimal temperature of 25-30^{0}C. They are negative with indole test having light cream colony which is irregular with flat elevation and irregular margin. The isolates reduced p-nitrophenyl phosphate to p-nitrophenolate under alkaline conditions indicating the production of phosphatase enzyme. A clearance zone (halo) was also observed in a nutrient agar supplemented with Tween 80 suggesting the production of esterase enzyme.

The 16S rDNA analysis further suggests that the isolates are phylogenetically related to *A. faecalis* with 99% sequence similarity.

Phylogenetic analysis showed that the carbofuran degrading isolates C-6, C-7 and C-9 are closely related to the genus *Pseudomonas* . *Pseudomonades* are described as aerobic, rod-shaped, gram-negative bacteria with one or more polar flagella providing motility. The genus demonstrates great deal of metabolic diversity, and consequently are able to colonise a wide range of niches. The best studied species include *P. aeruginosa* in its role as an opportunistic human pathogen, the plant pathogen *P. syringae*, the plant growth promoting *P. fluorescens* and the soil bacterium *P. putida*. *P. putida* has a very diverse aerobic metabolism thus is able to degrade organic solvents such as toluene and also to convert styrene oil to biodegradable plastic, polyhydroxyalkanoates (PHA). They are found in most soil and water habitats where there is oxygen. The morphological, physiological and biochemical signature for isolates C-6, C-7 and C-9 indicated that they are highly closely related to *Pseudomonas pituda.* They were indole negative, methyl red negative and showed negative result with Voges–Proskauer test. The isolates had multiple polar flagella for motility and grew optimally at a temperature range of 27-35^{0}C. They also reduced p-nitrophenyl phosphate to p-nitrophenolate and formed a zone of clearance with Tween 80 suggesting the production of phosphatase and esterase enzymes respectively. The 16S rDNA sequence analysis suggested that the isolates C-6, C-7 and C-9 are phylogenetically closely related to *Pseudomonas pituda* with 97% sequence similarity.

Vagococcus are described as gram positive, motile, coccus-shaped bacteria related to the genera *Enterococcus* and *Carnobacterium* (Teixeira et *al.*, 1997). These organisms occur singly or in short chains, with cells elongated in the direction of the chain, and some giving the appearance of short, fat rods. The genus was originally monospecific, comprising the species *Vagococcus fluvialis*, isolated from chicken faeces and river water. *V. fluvialis* has since been recovered from diverse sources, including human clinical specimens (blood, peritoneal fluid and wounds; Teixeira *et al.*, 1997) and various domestic animals (chickens, pigs, cattle, horses and cats; Pot *et al*., 1994). Many species of *Vagoccocus* have since been described; *Vagococcus salmoninarum* has been recovered from diseased fish (e.g.Atlantic salmon, rainbow trout and brown trout with peritonitis; Schmidtke & Carson, 1994), *Vagococcus fessus* (Hoyles *et al.*, 2000) has been isolated from a seal and a harbour porpoise, *Vagococcus lutrae* originated from the common otter (Lawson *et al.*, 1999) while *Vagococcus carniphilus* was isolated from ground beef (Shewmaker *et al.*, 2004). *Vagococcus* are facultatively anaerobic and catalase-negative. The phylogenetic analysis of carbofuran and methomyl-degrading isolates, C-1, C-13, C-3, C-5, M-17, M-4, M-5, M-19 and M-21 showed that they are closely related to *Vagococcus fluvialis* with a 95-99% sequence similarity. Morphological, physiological and biochemical characteristics of the isolates also confirm the genus assignment.

Phylogenetic analysis of carbofuran degrading isolates C-8, C-12 and C-2 suggested that they were closely related to members of the genus *Bacillus* with 97% rDNA sequence analysis similarity. *Bacilli* are described as gram-positive rod-shaped obligate aerobic bacteria belonging to the division Firmicutes. They are ubiquitous in nature occurring both as free-living and pathogenic species. Different species of *Bacillus* have been described; *B. anthracis*, which causes anthrax, *B. cereus*, which causes a food-borne illness similar to that of *Staphylococcus*, *B. thuringiensis* is an important insect pathogen, and is sometimes used to control insect pests, *B. subtilis* and *B. coagulans* notable food spoilers, causing ropiness in bread and related food.

Some species of *Bacillus* have also been isolated from agricultural waste waters (Kamal *et al.,* 2008) and petroleum contaminated soils (Christovaa *et al.,* 2004) with capability of biodegrading various xenobiotic and natural compounds. Morphological, physiological and biochemical assignments of the isolates also suggested their close relatedness with members of the genus *Bacillus.* They are negative with indole, hydrogen production and Voges Proskauer tests with a positive reaction for methyl red test.

Fragment restiction using *RsaI*, three clusters of isolates were generated based on fragment sizes. Isolates M-23, M-14, M-17, C-2, C-3, C-10 and C-4 clustered together generating three fragments, 950/1000bp, 850/900 and 750bp, with *RsaI* digestion. The other cluster had isolates M-9 and C-9 having three restriction fragments, 950/1000bp, 850/900bp and 640/700bp.

Isolate C-14 had two restriction fragments of 950/1000 and 850/900bp making it different from other isolates. The rDNA sequence analysis clustered the ten isolates into seven different genera namely: *Vagococcus, Flavobacterium, Bacillus, Providencia, Alcaligenes, Paracoccus* and *Pseudomonas.*

5.5 Conclusion and Recommendations

5.5.1 Conclusion

Survey analyses indicate the use of various types of pesticide and that farmers prefer using a combination of pesticides in the same farm. The isolation of carbamate degrading bacteria from horticultural soils in different geographical regions indicated that horticultural soils harbour pesticide degrading bacteria and that these bacteria have a wide genetical diversity and geographical distribution thus are potentially useful in environmental bioremediation. The phylogenetic analysis of the isolated strains clustered them into seven different genera namely *Vagococcus, Pseudomonas, Paracoccus, Bacillus, Alcaligenes, Providencia and Flavobacterium.*Morphological and biochemical characteristics of the isolated strains also confirmed phylogenetic signatures. Using HPLC, the ability of degradation ability of different stains was monitored and the strains were able to degrade both methomyl and carbofuran.

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5.5.2. Recommendations

5.5.2.1. Recommendations from the findings

From the study findings, I recommend the following:

1. Isolate C-9 and C-4 which were able to efficiently degrade carbofuran can be used in bioremediation of carbofuran contaminated soils.

2. Isolate M-14 and M-23 efficiently degraded methomyl and can be applied in bioremediation of methomyl contaminated soils.

3. Isolate C-4 and M-23 showed multidegradative potential and can be used in a consortium for improved biodegradaion of pesticide contaminated soils.

5.5.2.2. Recomendations for further research

From the study, I recommend the following for further research;

1. Future research should target on regulation of pesticide degrading genes as well as physiological conditions that favour the optimal production of resulting biodgradative enzymes.

2. Further research is needed on members of *Flavobacterium* and *Myroides* since based on the phenotypic and genetical data obtained from this study, the two genera are almost indistinguishable.

3. Chemical/spectral tests should be carried out to determine the structure/form of the metabolites from the pesticides

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APPENDICES

APPENDIX 1: FIGURES

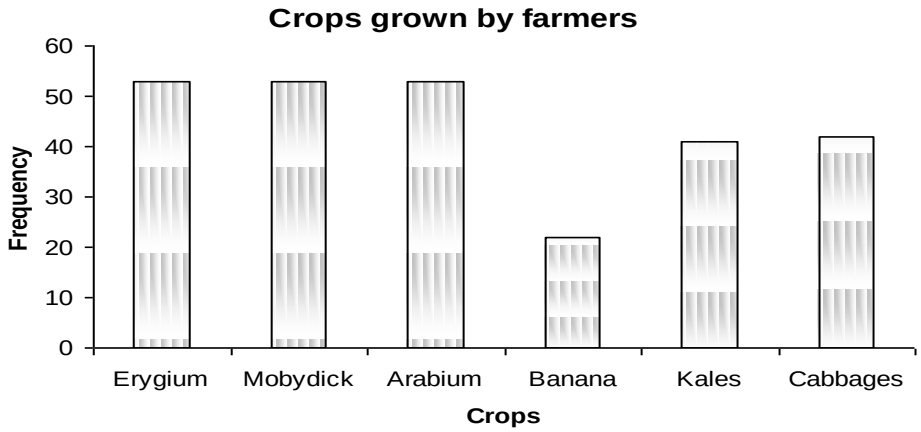

Figure 25: Crops grown by farmers in Rift Valley and Central Kenya

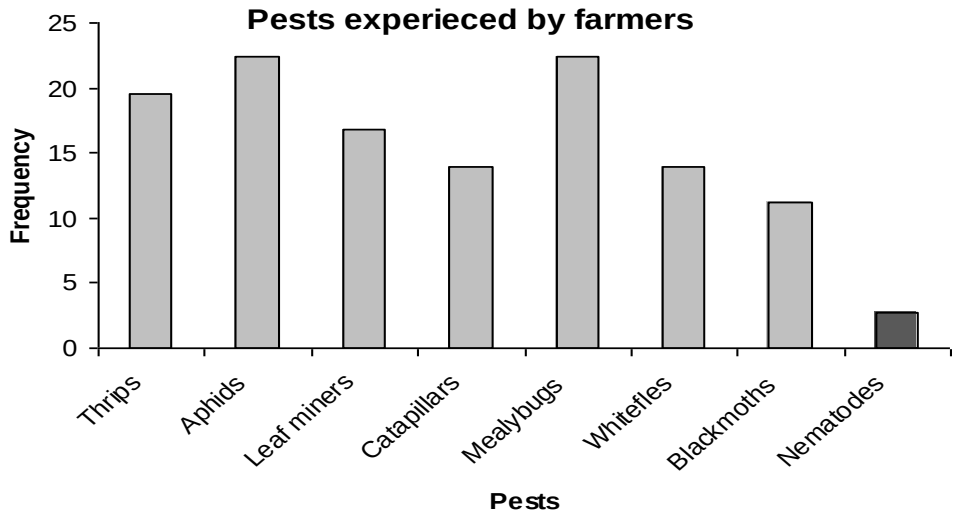

Figure 26: Pest experienced by farmers in Rift valley and central Kenya

APPENDIX 2: QUESTIONNAIRE

JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY

QUESTIONNAIRE FOR AGROCHEMICAL SURVEY

Questionnaire No. _____ **Date** _____

1. Name of farm _____

2. District_____**Division**_____

3. Contact person _____**Title** _____

4. Telephone _____ **Email** _____

5. Address PO BOX _____ **FAX** _____

6. Crops grown a) Fruits b) Vegetables c) Flowers

7. Specific crops _____, _____, _____

8. Agrochemicals used

Target pests for each		Pesticide (Trade name)
i)	_____	_____
ii)	_____	_____
iii)	_____	_____
iv)	_____	_____
v)	_____	_____

9. Class of chemicals

_____
_____
_____
_____
_____
_____

10. Methods of waste water disposal (if any)

a) Wet lands
b) Drain into ditches
c) None
d) Other

11. Any visible Agrochemical effect on the environment

_____

APPENDIX 3: CALIBERATION CURVES

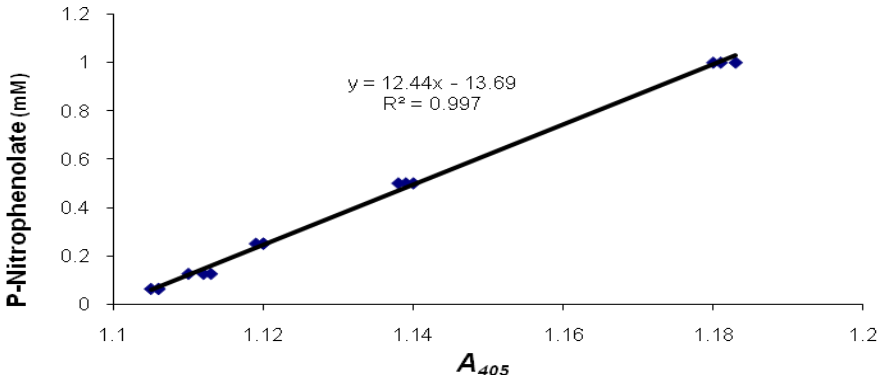

Figure 27. Standard curve for p-Nitrophenolate prepared from p-Nitrophenol at concentration range of 0.1-1mM.

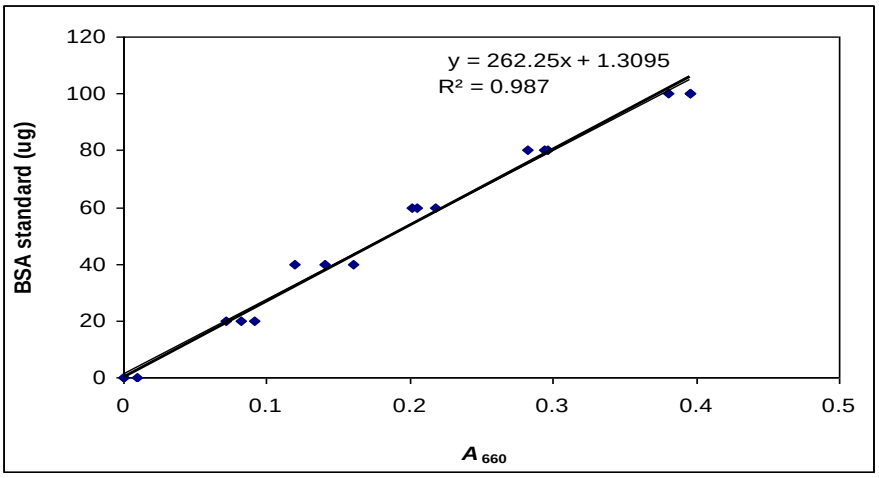

Figure 28. Standard curve for Bovine serum albumin prepared at a concentration range of 0-100µg.

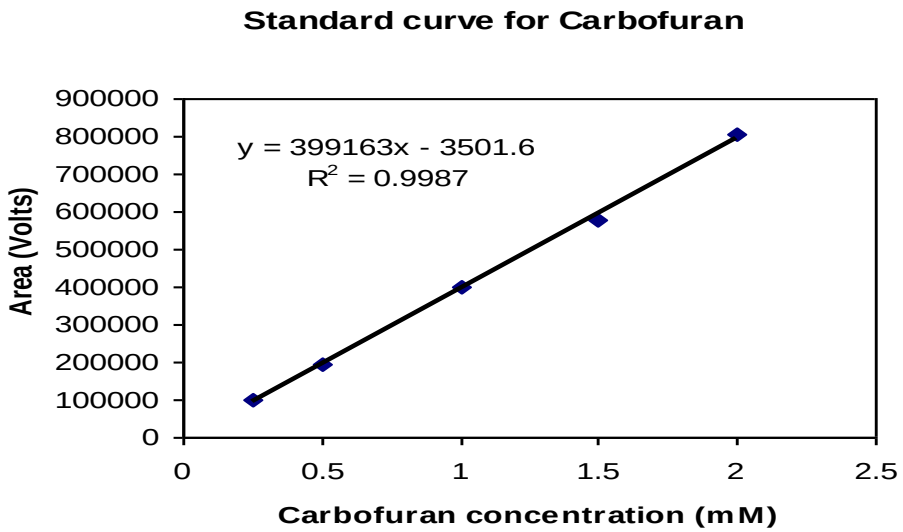

Figure 29. Standard curve for carbofuran prepared with concentration range between 0.25Mm to 2Mm.

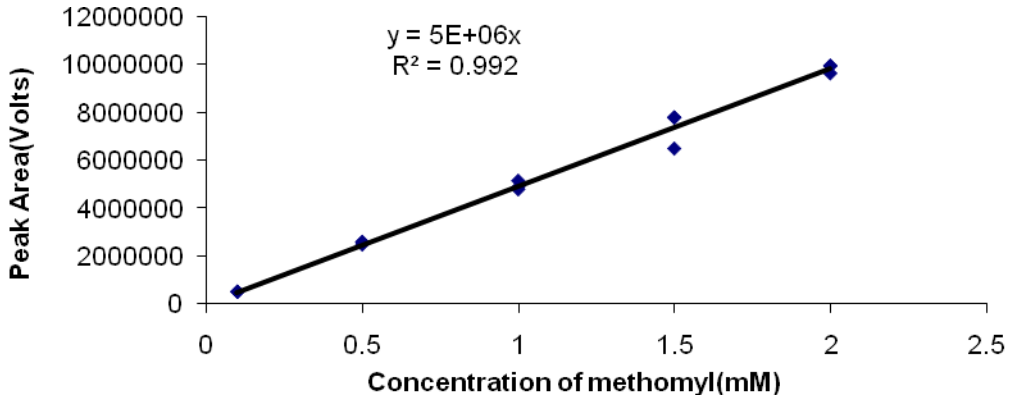

Figure 30. Standard curve for methomyl prepared with a concentration range between 0.25mM and 2mM