ENRICHMENT, ISOLATION AND CHARACTERIZATION OF DICHLORODIPHENYLTRICHLOROETHANE (DDT) MICROBIAL DEGRADERS FROM SOILS IN KENYA

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Enrichment, isolation and characterization of dichlorodiphenyltrichloroethane (DDT) microbial degraders from soils in Kenya

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DECLARATION

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

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DEDICATION

To my parents, Mr. and Mrs. Mwangi Kariuki. To Lucy, Mr. Julius Muthuri (SSP) and Mr. Hamisi Mambeya (SSP). Thank you all for your support.

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ABBREVIATIONS

bp	Base pairs
DDT	dichlorodiphenyltrichloroethane
DDTr	Dichlorodiphenyltrichloroethane residues
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DNA	Deoxyribonucleic acid
DDMU	l-chloro-2,2-bis(p-chlorophenyl) ethylene
DDMS	1 -chloro-2,2-bis(p-chlorophenyl)ethane
DDNU	2,2-bis(p-chlorophenyl)ethylene
DDOH	2, 2-bis (p-chlorophenyl) ethanol
DDA	bis(p-chlorophenyl)-acetic acid
DDM	bis(p-chlorophenyl) methane
DBH	4,4'-dichlorobenzhydrol
DBP	4,4' dichlorobenzophenone
DGGE	Denaturant Gradient Gel Electrophoresis
EHIB	Environmental Health Investigation Branch
EDTA	Ethylenediaminetetra acetic acid
ICIPE	International Center of Insect Physiology and Ecology
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KMM1	Kenya Minimal media
OD	Optical density
PCR	Polymerase Chain Reaction
PCBs	Polychlorinated Biphenyl

PCPA	pchlorophenylacetic acid
RPM	Revolution per minute
rRNA	Ribosomal ribonucleic acid
SSP	Senior Superintendent of Police
SD	Standard deviation
WHO	World Health Organisation

ABSTRACT

Dichlorodiphenyltrichloroethane (DDT) has been in use as an agricultural insecticide and as a disease vector control since 1939. Due to its persistence in the environment, its use has been surrounded by controversies regarding its safety to humans and the environment. There has been no reliable and replicable scientific evidence proving its harmful effects to humans though DDT was banned in many developed nations by 1970s. The DDT ban has seen to the reemergence of malaria and other insect borne diseases in the tropics. There being no better alternatives to DDT, its use to control malaria vector has been recommended by the World Health Organization. Responsible use of DDT should include research to determine possible ecotoxicalogical effects due to its accumulation and biomagnifications potential. In this study, the potential for biodegradation of DDT by soil microorganisms through enrichment and isolation of DDT biodegraders in uncontaminated tropical soils has been done. Microorganisms from both cultivated and uncultivated soils were found to grow in MM4 media with DDT (100 ppm) as the only carbon source. DDT degradation over a period of 29 days was higher in uncultivated soils (60.20%) than in cultivated soils (38.58%). Six isolates coded as isolate **101,** isolate **102,** isolate **103,** isolate **104,** isolate **105** and isolate **110** degraded DDT into l, l-dichloro-2, 2-bis (p-chlorophenyl) ethane (DDD). None of the isolates was capable of transforming DDT into l, l-dichloro-2, 2-bis (p-chlorophenyl) ethylene (DDE). The ability of the six isolates to degrade DDT from highest to lowest over a period of 31 days was **102** (58.08%), **101** (44.31%), **103** (39.72%), **104** (30.33%), **105** (28.97%) and **110** (28.48%). The degradative ability of the six isolates combined over the same period was higher (82.63 %) than that of any individual isolate (range of DDT degraded by

the isolates was 28.48 %-58.08 %). The identity of the six isolates was determined through microscopic, biochemical and molecular techniques. Phylogenetic analysis of the 16S rRNA gene sequences of the isolates showed them to belong to genera *Bacillus* for isolate **101** with a 16S rRNA gene sequence similarity of 99 % to *Bacillus cereus.* Isolates **102** and **110** were members of the genera *Stenotrophomonas* with 16S rRNA gene sequence similarity of 98 % to *Staphylococcus sciuri.* Isolates **103**, **104** and **105** were members of the genera *Stenotrophomonas* with 16S rRNA gene sequences similarity of 95 %, 97 % and 94 % respectively to *Stenotrophomonas maltophilia*. Isolates **103**, **104** and **105** could be new species. There are DDT biodegraders in the tropical soils as evidenced by the isolates.

CHAPTER ONE

1.0 General Introduction

Organochlorine insecticides and their metabolites which mainly include Dichlorodiphenyltrichloroethane (DDT), Kelthane, chlorobenzilate, chloropropylate, methoxychlor, aldrin, dieldrin, heptachlor, lindane, endosulfan, isodrin, isobenzan, endrin, chlordane, toxaphene, mirex and Kepone are synthetic chemicals. They are highly efficient in controlling human and animal diseases carried by insects and mites and also against insect pests which cause great damage to agricultural crops (Newton and Bogan, 1978). These insecticides are more toxic to insects and less toxic to non target organisms on application. However they can be toxic even to non target organisms on prolonged exposure due to their persistence in the environment, bioaccumulation and biomagnifications along food chains (Albert *et al*., 1993). Therefore, these insecticides have important ecological effects in addition to those usually intended (Martin, 1966).

DDT has potent insecticidal properties and kills insects by opening sodium ion channels in insect neurons, causing the neuron to fire spontaneously (Kurihara *et al*., 1998). This leads to spasms and eventual death of the insects. Insects with certain mutations in their sodium channel gene may be resistant to DDT and other similar insecticides (Pfaender and Alexander, 1992).

DDT was initially used with great effect in Europe and North America, before and after World War II, to combat mosquitoes spreading malaria, typhus and other

insect-borne human diseases among both military and civilian populations (Mkoka, 2007). It was used extensively during World War II by allied troops and certain civilian populations around the world to control insect typhus and malaria vectors, nearly eliminating typhus as a result (Donald *et al.,* 1997). Civilian population used to spray the interior walls of their buildings. That killed mosquitoes that rest on the walls after feeding to digest their meal while the resistant strains are repelled from the area. In 1943, entire cities in Italy were dusted with DDT to control the typhus carried by lice (Briet *et al.,* 2005). DDT also sharply reduced the incidence of biting midges in Great Britain, and was used extensively as an agricultural insecticide after 1945. Malaria was eradicated from Brazil and Egypt, largely due to extensive DDT spraying (Walter, 1992). In May 1955 the Eighth World Health Assembly adopted a Global Malaria Eradication Campaign based on the widespread use of DDT against mosquitoes and of antimalarial drugs to treat malaria and to eliminate the parasite in humans (Gordon and Stephen, 1998). As a result of the Campaign, malaria was finally eradicated by 1967 from Europe and North America where the disease was endemic. Large areas of tropical Asia and Latin America were also freed from the risk of infection. The Malaria Eradication Campaign was only launched in three countries of tropical Africa since it was not considered feasible in the others. DDT was often applied directly to clothes or used in soap formulations. Indeed, DDT has on rare occasions been administered orally as a treatment for barbiturate poisoning (Rappolt, 1993).

Malaria afflicts between 300 million and 500 million people every year (WHO, 2000). The World Health Organization estimates that around one million people die

of malaria and malaria-related illness every year. About 90 % of these deaths occur in Africa, mostly to children under the age of five (WHO, 2000). The economic ramifications caused by malaria include costs of health care, working days lost due to sickness, days lost in education, decreased productivity due to brain damage from cerebral malaria, and loss of investment and tourism (Bate, 2001). In some countries with a heavy malaria burden, the disease may account for as much as 40 % of public health expenditure, 30-50 % of inpatient admissions, and up to 50 % of outpatient visits (Curtis *et al.,* 2004). Malaria halts economic development, places huge burdens on a country's health resources and causes massive productivity losses (Gallup and Sachs, 2001). It is also a barrier to foreign investors from developing countries, who are not keen on having their workforce suddenly become ill and unable to work (Bate, 2001). Economists believe that malaria is responsible for a "growth penalty" of up to 1.3 % per year in some African countries, which when compounded over time, leads to large differences in poverty between different nations (Biscoe *et al*., 1995). Malaria has been found to cause cognitive impairments, especially in children (Mills and Shillcutt, 2004). It causes widespread anaemia during a period of rapid brain development and also direct brain damage from cerebral malaria to which children are more vulnerable (Driessen, 2006).

DDT remains a major component of malaria control programs in southern African states, though many countries have abandoned or curtailed their spraying activities. Swaziland, Mozambique and Ecuador are examples of countries that have very successfully reduced malaria infestations by using DDT (African American Environmental Association, 2008). In Sri Lanka in the period from 1934-1955 there

were 1.5 million cases of malaria resulting in 80,000 deaths (Roberts and Andre, 1994). After the country invested in an extensive anti-mosquito program using DDT, there was a drastic change with only 17 cases being reported in 1963. Thereafter the program was halted hence malaria in Sri Lanka rebounded to 600,000 cases in 1968 and the first quarter of 1969 (Briet *et al.,* 2005). After South Africa stopped using DDT in 1996, the number of malaria cases in Kwa Zulu Natal province rose from 8,000 to 42,000 cases (Bate, 2004). By the year 2000, there had been an approximate 400 % increase in malaria deaths. That led to the reintroduction of DDT in the fight against malaria and by 2006, the number of deaths from malaria in the region was less than 50 per year (Hargreaves *et al.,* 2002). South Africa could afford and did try newer alternatives to DDT, but they proved less effective (Trane and Bate, 2004).

Many African nations like Kenya and Uganda would like to use DDT to control malaria and save lives but their agricultural exports may not be accepted by the Western countries if DDT spraying was widespread (Mkoka, 2007). These countries have been coming under pressure from international health and environment agencies like United Nations Environmental Programs (UNEP) to give up DDT or face losing aid grants (Bate, 2001). Uganda has begun permitting the use of DDT in anti-malarial efforts, despite a threat that its agricultural exports to Europe could be banned if they were contaminated with DDT (European Union, 2005). The Ugandan government has stated that it cannot achieve its development goals without first eliminating malaria (Edmund, 2006). The GDP in Uganda shows a striking correlation between malaria and poverty, where malaria is estimated to reduce per capita growth by 1.3 percent per annum (Lirri and Ntabadde, 2005).

In Kenya, approximately 70 percent of the land is prone to malaria epidemics (Neondo**,** 2003). The regular epidemics occur in the western highlands, the semi-arid regions in the north-eastern and eastern parts of the country (Ministry of Health, 2001a). Endemic areas are limited to areas surrounding Lake Victoria, the floor of the Rift Valley, the central parts of the Eastern and Central provinces and the coastal regions. Out of approximately 31 million Kenyans, over 20 million are at constant risk of malaria (Ministry of Health, 2001a). Malaria kills approximately 26000 children per year in Kenya and about 170 million working days are lost per year. Malaria accounts for 30 % of all outpatient attendance and 19 percent of all admissions to Kenyan health facilities (Ministry of Health 2001b, c). The Ministry of Health in Kenya had not initially chosen to use DDT for malaria control since DDT was banned as an agricultural product in 1986 (Bosire, 2004). The Kenyan National Environmental Management Authority (NEMA) is vehemently opposed to DDT reintroduction, as is the Pest Control Products Board (PCPB). PCPB had expressed concerns that DDT reintroduction would compromise Kenya's $300 million horticultural industry. Stakeholders such as the Fresh Produce Exporters' Association of Kenya (FPEAK) and the Kenya Flower Council (KFC) feel threatened by the prospect of losing their export markets if DDT is reintroduced (Bosire, 2004). The European Union constitutes approximately 90 percent of Kenya's horticultural export market (Kenya High Commission, 2004). Additionally, Kenya is the world's leading producer of natural pyrethrum, producing 80 percent of the global supply. The Pyrethrum Board of Kenya produces three vector control chemicals: Pylarvec (larviciding), Pymos for Indoor Residual Spraying (IRS) and Pynet for Insecticide Treated Nets (ITNs). Some government officials believe that national malaria control

strategies should support Kenya's own pyrethrum industry instead of DDT imports (Songa, 2004).

There are some insecticide alternatives to DDT like carbamates that work against mosquitoes, but they are at least twice and sometimes up to 20 times more expensive, and they are less effective as repellents (Corin and Weaver, 2005). Others like organophosphates are extremely dangerous and thus not appropriate to use at homes. Mosquitoes have built up a huge resistance to synthetic Pyrethroids, because they are used so extensively in agriculture. These substitutes are less persistent than DDT and this is what makes them so attractive to the environmentalists (African American Environmental Association, 2008). But when pesticides are used indoors, a persistent material is better because you might have to spray only once a year, and to a poor country, this kind of cost consideration could make all the difference (Bate, 2001).

Although DDT is very effective in killing or repelling mosquitoes its use has been severely reduced and restricted to indoor residual spraying, due to its persistence in the environment and ability to bioconcentrate in the food chain (Cousins *et al.,* 1998; Hickey, 1999). One of the removal processes with significant impact on the fate of DDT in the environment is biodegradation. Biodegradation and bioremediation are matching processes to an extent that both of these are based on the conversion or metabolism of pesticides by microorganisms (Hong *et al.,* 2007). The difference between these two is that, biodegradation is a natural process whereas bioremediation is a technology. In bioremediation microbes are used to degrade the pesticides *in situ*. A successful bioremediation technique requires an efficient bacterial strain that can degrade the largest pollutant to minimum level (Kumar and

Philip, 2006). Adequate rate of biodegradation is required to attain the acceptable level of pesticide residues or its metabolites at contaminated site in a limited time frame. The rate of biodegradation in soil depends on four variables: (i) Availability of pesticide or metabolite to the microorganisms, (ii) Physiological status of the microorganisms, (iii) Survival and / or proliferation of pesticide degrading microorganisms at contaminated site and (iv) Sustainable population of these microorganisms (Dileep, 2008). Therefore, to attain an achievable bioremediation, it requires the creation of unique niche or microhabitats for desired microbes, so they can be successfully exploited. The only difficulty may be the limited knowledge about the population dynamics of pesticide degrading microorganisms in relation to other microbes present in the same habitat. Temperature, pH, water potential, nutrients and the amount of pesticide or metabolite in soil may also act as limiting factor for pesticide degrading microorganisms, which requires further exploration in relation to total microbial population and their biochemical activities (Kearney and Roberts 1998). In consideration of suitable bioremediation technique, the understanding of pesticide dynamics in soil environment is also required because the soil has unique binding potential for a variety of pesticides or metabolites. It is also necessary to understand the physiology and genetics of degradation of a particular pollutant before using any bacterium for its biodegradation (Topp *et al*., 1993). There are three possible regions where pesticide contamination can occur in terrestrial ecosystem (i) surface soil, (ii) vadose zone and (iii) ground water or saturated zone. The biodegradation in surface is primarily aerobic and rapid because the surface soils have large numbers of aerobic microorganisms and their number usually decreases with the depth. Biodegradation is slow in other two zones i.e. vadose and ground

water. Therefore, different bioremediation technologies are required to deal with different regions of terrestrial ecosystem (Dileep, 2008).

1.1 Justification

DDT is still one of the first and most commonly used insecticide in indoor residual spraying because of its low cost, high effectiveness, persistence and relative safety to humans (Hecht, 2004). It is therefore a viable insecticide in indoor residual spraying owing to its effectiveness in well supervised spray operation and high excito-repellency factor. However, various toxic effects that would be difficult to detect without specific study might exist and could result in various risks. Responsible use of DDT should include research programs that would detect the most plausible forms of toxic effects as well as the documentation of benefits attributable to DDT. Environmental impact assessment of DDT versus its benefits in the control of malaria in Kenya has not been done hence the need to carry out this study as a starting point. A manageable way of using DDT in control of malaria in tropical countries should be established. This can be achieved by use of DDT in indoor residual spraying only, isolation and production of microorganism that can remediate soil containing DDT in case it finds its way there and initiation of an assessment on impact of DDT on the tropical soil environment. The great versatility of microorganisms offers an inexpensive, simpler and more environmentally friendly strategy to reduce environmental pollution than non biological options. Biodegradation is a natural process, where the degradation of a xenobiotic chemical or pesticide by an organism is primarily a strategy for their own survival. Most of these microbes work in natural environment but some modifications can be brought

about to encourage the organisms to degrade the pesticide at a faster rate in a limited time frame. This capability of the microbe is utilized as a technology for removal of contaminant from actual site. The aim of this research is to isolate and characterize microorganisms that can biodegrade DDT in the tropical soil. Knowledge of the genetics, physiology and biochemistry of these microbes may further enhance the microbial process to achieve bioremediation of DDT with precision and in a short time. The standard method for isolating microorganisms with the ability to degrade environmental pollutants is to enrich them from contaminated soils. This process has not been very successful for the isolation of micro-organisms that can mineralize DDT. A novel approach for isolating DDT-degrading microorganisms is to screen alternative sources like uncontaminated soil and other materials.

1.2 Hypothesis

Tropical soils that are uncontaminated with DDT contain microorganisms capable of biodegrading DDT

1.3 Objectives of the study

1.3.1 General objective

Enrichment, isolation and characterization of DDT microbial degraders from uncontaminated tropical soils

1.3.2 Specific objectives

i. To assess the growth of enrichment cultures at different DDT concentrations
ii. To isolate and characterize microorganisms that biodegrade DDT in tropical soils
iii. To assess the extent of DDT biodegradation by the isolates
iv. To compare growth and rate of DDT biodegradation of the isolates, mixed culture, and enrichment cultures

CHAPTER TWO

2.0 Literature Review

2.1 Introduction on Pesticides

A pesticide is a substance or mixture of substances used to kill or repel insects, plant pathogens, weeds, mollusks, birds, mammals, fish, nematodes and microbes that compete with humans for food, destroy property, spread or are a vector for disease or cause a nuisance (Kuhard, *et al.,* 2004). A pesticide may be a chemical substance, biological agent such as a virus or bacteria, antimicrobial, disinfectant or device used against any pest (Environmental Health Investigation Branch, 2008). Although there are benefits to the use of pesticides, there are also drawbacks, such as potential toxicity to humans and other animals.

2.2 Classification of pesticides

Pesticides can be classified on basis of their targets: Algaecides control algae, Antimicrobials kill microorganisms that cause diseases, Attractants attract specific insects, Avicides control birds, Bio pesticides which are naturally occurring substances that kill pests, Defoliants cause foliage to drop from plants, Desicants aid in drying of plants and insects, Fumigants produce vapour that control insects, Fungicides destroy fungi, Herbicides control weeds, Insect growth regulators accelerate or retard insect growth, Insecticides control insects, Acaricides kill mites, Molluscicides kill snails and slugs, Nematicide kill nematodes, Ovicides control insect eggs, Piscicides control fish, Plant growth regulator accelerate or retard the rate of plant growth, Predacides control vertebrate pests, Repellents repel pests such

as mosquitoes, flies, ticks and fleas and Rodenticides control mice, rats and other rodents (Gavrilescu, 2005). The above types of pesticides are further subdivided into different chemical classes (**Table 1**). They can also be classified as either ionic or non ionic on the basis of their physicochemical properties and behaviours in water and soil.

Table 1: Classes of pesticides (Gavrilescu, 2005**)**

Class	Group
Insecticides	Organochlorines
	Organophophosphates
	Carbamate esters
	Pyrethroids
	Botanical insecticides
Fungicides	Hexachlorobenzene
	Organomercurials
	Pentachlorophenol
	Phthalimides
	Dithiocarbamates
Herbicides	Chlorophenoxy compounds
	Bipyridyl derivatives
Rodenticides	Zinc phosphide
	Fluoroacetic acid and derivatives
	Napthyl thiourea (ANTU) anticoagulants
Fumigants	Phosphine
	Ethylene dibromide
	Dibromochloropropane

2.3 Pesticides as persistent organic pollutants

Persistent organic pollutants (POPs) are a set of chemicals that are toxic, persist in the environment for long periods of time, and biomagnify as they move up through the food chain (Gavrilescu, 2005). POPs have been linked to adverse effects on human and animals health such as cancer, damage to the nervous system, reproductive disorders, and disruption of the immune system (US EPA, 2005). In May 2001, a global convention intended to eliminate or restrict the production, use,

and release of POPs was signed in Stockholm (The Stockholm Convention on Persistent Organic Pollutants). The 12 initial POPs list, known as "the dirty dozen," includes aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, mirex, toxaphene, polychlorinated biphenyls (PCBs), hexachlorobenzene, dioxins, and furans (Chekol, 2005). Soil contamination with POPs is a major global problem because POPs released in one part of the world can travel to regions far from their source of origin through the atmosphere, water and other pathways. Further, its effect on human health can be felt directly via the food web (Chaney *et al.,* 1996). There is a hypothesis that suggests that soil can also be a source of atmospheric pollution through volatilization for some of the banned organic pollutants, such as PCBs (Cousins *et al.,* 1998). Understanding the health risks associated with pollutants, the nature of the contaminant and properties of the matrix is of paramount importance to the selection of an appropriate remediation technology and its ultimate success rate (Chaney *et al.,* 1996).

2.4.1 Polychlorinated Biphenyls (PCBs)

Polychlorinated biphenyls, in which DDT belongs, are products of the chemical industry (Chekol, 2005). There are no known natural sources of PCBs. They have no distinct smell or taste and exist as either oily liquids or solids, which are colourless to light yellow in colour. PCBs are organic compounds in which one or more chlorine (Cl) atoms are attached to a biphenyl molecule. There are ten possible sites for chlorine (Cl) attachment on the biphenyl, giving rise to a group of 209 related compounds (congeners), which differ only in the number and position of chlorine atoms attached to the biphenyl molecule (Smith *et al.,* 1999). The International

Union of Pure and Applied Chemistry (IUPAC) nomenclature recognizes designations PCB-1 to PCB-209 for PCB congeners ranging from a biphenyl containing one Cl atom (2-chlorobiphenyl) to a ten Cl atoms congener (decachlorobiphenyl). PCBs mixtures differ in the number of congeners and average percent Cl content. The number and position of Cl atoms determine the physical, chemical, and toxicological properties of PCBs. The higher the number (more chlorinated), the more stable it becomes. Because of steric interference, only about half the 209 PCBs congeners are synthesized and less than 10 percent of them have been reported as having toxicological effects (Gan and Berthouex, 1994). Some of the reported health effects of PCBs include disruption of reproductive functions, neurobehavioral and developmental deficits especially in children, liver disease, diabetes, effects on the thyroid and immune system and increased cancer risks (Agency for Toxic Substances and Disease Registry, 2005; Johnson *et al.,* 1999). The manufacture and use of new products containing PCBs in the United States was stopped in 1977 because of the human and environmental health hazards of PCBs (Chekol, 2005).

2.4.2 Environmental Fate of PCBs

Although industrial use of PCBs has been severely reduced due to their extreme persistence in the environment and ability to bioconcentrate in the food chain, human health concerns are still warranted (Bedard, 1990). The soil and sediments are major sinks sequestering the greatest amount of PCBs in the terrestrial environment and represent environmental and human health risks that need remedial action (Cousins *et al.,* 1998; Hickey, 1999). In the soil PCBs, like any other organic contaminants,

are subject to retentive or removal processes. Sorption is a major retentive process of PCBs in soil and the soil organic matter is the primary sorbent for PCB partitioning (Chiou, 1989). The preferential strong adsorption of PCBs onto the soil organic matter is dictated by their physicochemical properties such as very high octanol-water partition coefficients (Hickey, 1999). The strong adsorption of PCBs to the soil organic matter can be instrumental in preventing the movement of these compounds into groundwater via leaching. Removal processes with significant impact on the fate of PCB in soils include volatilization and biodegradation. The loss of PCBs from soil through volatilization is highly dependent on the composition of the congeners. High molecular weight (highly chlorinated) congeners are less soluble in water and since volatilization represents a transfer across the air-water interface, losses due to volatilization are very limited. On the other hand, PCBs composed of mainly less chlorinated congeners are subject to volatile losses (Hickey, 1999; Smith *et al.,* 1999). Overall PCB losses from soil due to volatilization are variable and mainly depend on the soil organic matter content. In high organic matter soils, PCBs will preferentially sorb to the organic matter, and thus are less available for volatile losses (Chiou, 1989).

2.4.3 Biodegradation of PCBs

There are two distinct mechanisms of PCB biodegradation, aerobic and anaerobic dechlorination. Aerobic dechlorination is a stepwise oxidative degradation of the biphenyl molecule *via* a series of intermediate products. In contrast, anaerobic dechlorination takes place with the replacement of chlorine atoms by hydrogen atoms in the absence of oxygen (Smith *et al.,* 1999). Since the less chlorinated PCBs are

simple, more water-soluble and available, they are more amenable to aerobic biodegradation than the more chlorinated congeners (Bedard, 1990; Hickey, 1999; Higson, 1999). The principal dechlorination mechanism for highly chlorinated PCBs is anaerobic reductive dechlorination that requires a cometabolic substrate and usually takes place at the *meta* and *para* positions. This preferential dechlorination during anaerobic biodegradation invariably results in an increased percentage of the PCBs with *ortho* chlorines. Other removal processes with significant impact on the fate of PCB in soils include volatilization and biodegradation. Reports have also suggested that anaerobic reductive dechlorination of the more chlorinated PCBs results in the accumulation of mono-trichlorobiphenyls, which are easily degraded by aerobic microorganisms (Bedard, 1990). If the PCB-degrading bacteria are able to use cometabolic substrates of plant origin, then it will be feasible to select plant species that can provide these compounds and use them in the phytoremediation of PCB-contaminated soils (April and Sims, 1990).

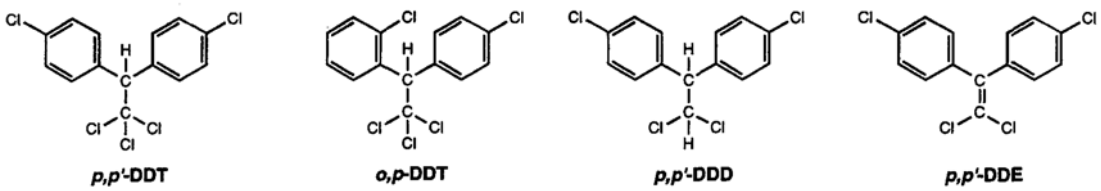
2.5.1 Dichlorodiphenyltrichloroethane (DDT)

Dichlorodiphenyltrichloroethane (DDT, $C_{14}H_9Cl_5$) was among the first modern organic pesticide (Foght *et al.,* 2001). DDT is also known under the chemical name 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane. It was first synthesized in 1874 by Othmar Zeidler (Gevao and Jones, 2002). However, its insecticidal properties were not discovered until 1939 by the Swiss scientist, Paul Hermann Muller, who was awarded the 1948 Nobel Prize in Physiology and Medicine for his discovery of the high efficiency of DDT as a contact poison against several arthropods (Carter, 2004). It is synthesized by the reaction of trichloroethanol with chlorobenzene (C_6H_5Cl)

(Brian, 2006). DDT is an organochlorine pesticide that has been used extensively since World War II to control hundreds of insect pests associated with agricultural practices, as a mosquito larvicide, as a residual spray for the eradication of malaria and as a delousing dust for typhus control (Albert *et al*., 1993). In 1972, DDT was banned from use in the United States. In developed countries, the use of DDT has been progressively restricted or phased out. However, in a number of tropical countries, DDT is still being used today (Bate, 2004).

2.5.2 Physical and chemical properties of DDT

DDT is an organochlorine insecticide. It is a white crystalline solid, tasteless, hydrophobic and colourless with a weak, chemical odour. It is nearly insolluble in water but has a good solubility in most organic solvents, fat and oils (Albert *et al*., 1993). DDT has two isomers, *p,p* isomer (**Figure 1a**) and *o,p* isomer (**Figure 1b**). These two compounds are abbreviated "*pp* DDT" and "*op* DDT", respectively. All isomers of the compound DDT have a relative molecular mass of 354.5. The melting range of *p p'* -DDT is 108.5 to 109 °C and its vapour pressure is 2.53 x 10^{-5} Pa (1.9 x 10^{-7} mmHg) at 20 °C. DDT is soluble in organic solvents as follows (g/100 ml): benzene, 110; cyclohexanone, 100; chloroform, 96; petroleum solvents, 4-10; ethanol, 1.5. It is highly insoluble in water (solubility approximately 1 µg/litre) but very soluble in animal fat (Wiktelius *et al.,* 1997).



(a) **(b)** **(c)** **(d)**

Figure 1: Chemical structure of *p,p*'DDT (a) *o,p*'-DDT (b) and its residues DDD (c) and DDE (d). DDT-1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane, DDD-1,1-dichloro-2,2-bis (p-chlorophenyl) ethane and DDE-1,1-dichloro-2,2-bis(p-dichlorodiphenyl) ethylene.

2.5.3 Uses of DDT in public health

Use of DDT in public health to control mosquitoes is primarily done inside buildings and through inclusion in household products and selective spraying (Mouchet *et al.,* 1998). This greatly reduces environmental damage compared to the earlier widespread use of DDT in agriculture (Brian, 2006). It also reduces the risk of resistance to DDT (Malaria International Foundation, 2006). This use only requires a small fraction of that previously used in agriculture (Roberts *et al.*,, 1997). Today, DDT continues to be used in tropical countries like Ethiopia, Tanzania and South Africa where mosquito-borne malaria and typhus are serious health problems (Mouchet *et al.,* 1998). According to the WHO paper (2000), Kenya began spraying DDT in the 1940s, with an immediate 98 % reduction in malaria incidences in some regions. This report has also credited DDT spraying for malaria not reoccurring in Nairobi after a flood in 1961. But the increase in malaria seems to be mirrored in the reduction of DDT use (Curtis, 2000). After the campaign to ban DDT use by Rachel Carson and other eco-activists, DDT use began to reduce and in 1990 Kenya

outlawed the insecticide's use. Now there is extensive debate in Kenya, as elsewhere, about bringing back DDT to control malaria.

2.5.4 Fate of DDT in the environment

DDT is a persistent organic pollutant with a reported half life of between two and 15 years and is immobile in most soils (Beunink *et al.,* 1988). Its half life is 56 days in lake water and approximately 28 days in river water (Kearney and Roberts, 1998). Routes of loss and degradation include runoff, volatilization, photolysis and biodegradation, both aerobic and anaerobic. These processes generally occur slowly. Breakdown products in the soil environment are DDD, 1,1-dichloro-2,2-bis (p-chlorophenyl) ethane (**Figure 1c**) and DDE, 1,1-dichloro-2,2-bis (p-dichlorodiphenyl) ethylene (**Figure 1d**). Both DDD and DDE are transformation products of DDT and are also highly persistent and have similar chemical and physical properties with DDT (Boul, 1995). These products together are known as total DDT. There is concern about the presence of DDT in the environment due to its toxic and teratogenic effects, and its potential to bioaccumulate in the food chain (Aislabie *et al.,* 1997). DDT has been detected at significant concentrations in the tissue of a number of animals (Hunnego and Harrison 1971; Gilbert *et al.,* 1990), and the organochlorine has also been reported to accumulate in grain, cotton and natural vegetation (Voerman and Besemer 2000). In addition, there have been concerns about chronic exposure of bird species to DDT and its effects on reproduction (Spynu, 1989). The persistence of DDT in the environment has been attributed to its low volatility, low water solubility and the presence of chlorine substituents on the molecule.

2.5.5 Biodegradation of DDT

Biodegradation of DDT largely involves cometabolism in which microbes growing at the expense of a growth substrate are able to transform DDTr without deriving any nutrient or energy for growth from the process (Bollag and Liu 1990). Diphenylmethane, a non-chlorinated analogue, is readily biodegradable. However, DDT is more resistant to microbial attack (Aislabie *et al.,* 1997). Although no micro-organisms have been isolated with the ability to degrade DDT as a sole carbon and energy source (Beunink and Rehm, 1988), organisms may degrade the organochlorine via co-metabolism under aerobic or anaerobic conditions. However, inherent problems with the microbial co-metabolism of DDT are the metabolic bottlenecks associated with both these pathways. The major transformation products produced, DDD and DDE are more toxic and recalcitrant than the parent compound. This is of concern as these compounds are metabolized slowly, if at all (Lal and Saxena 1999; Aislabie *et al.,* 1997).

Early investigations on the fate of DDT in rodents demonstrated that the indigenous microflora of animals were responsible for the conversion of DDT to DDD (Mendel and Walton, 1966). Since then a number of microbes have been shown to be capable of converting DDT to DDD in pure culture. These include the bacteria *Escherichia coli, Enterobacter aerogenes, Enterobacter cloacae, Klebsiella pneumoniae, Pseudomonas aeruginosa, Pseudomonas putida, Bacillus* species *"Hydrogenomonas",* and the fungi *Saccharomyces cerevisiae, Phanerochaete chrysosporium,* and *Trichoderma viridae* (Lai and Saxena, 1999; Alexander, 1985; Beunink and Rehm, 1988).

The chemical structure of DDT, including aromatic and alicyclic moieties, offers a variety of possibilities for biochemical attack. Most reports indicate that DDT is reductively dechlorinated to DDD under reducing conditions (Lai and Saxena, 1999; Rochkind *et al*., 1987). Bacteria and fungi are reported to metabolise DDT in this way and their pathways for biodegradation by this route have been determined. An alternative route for microbial attack under aerobic conditions has been described, involving strains of chlorobiphenyl degrading bacteria (Nadeau *et al*., 1994). Extensive biodegradation of DDT and DDT metabolites in some bacteria has been demonstrated (John and Steven, 1987). These studies have led to the elucidation of a pathway for DDT biodegradation (**Figure 2**). Although other reactions, most notably conversion of DDT to DDE may occur during its biodegradation, the major bacterial pathway appears to involve an initial reductive dechlorination of the trichloromethyl group to form DDD (**Figure 2**). DDD then undergoes further dechlorination to form l-chloro-2, 2-bis (pchlorophenyl) ethylene (DDMU) or directly to 1 -chloro-2, 2-bis (p-chlorophenyl) ethane (DDMS) (**Figure 2**). DDMS undergoes further dechlorination to form 2, 2-bis (p-chlorophenyl) ethylene (DDNU) (**Figure 2**). DDNU is oxidized to 2, 2-bis (p-hlorophenyl) ethanol (DDOH) and then to bis (p-chlorophenyl)-acetic acid (DDA) (**Figure 2**). DDA undergoes decarboxylation to form bis (4-chlorophenyl) methane (DDM) (**Figure 2**). DDM may then undergo cleavage of one of the aromatic rings to form *p*-chlorophenylacetic acid (PCPA), which may also undergo ring cleavage. The products of ring cleavage reactions are then further degraded to Krebs cycle intermediates and ultimately oxidized to carbon dioxide to complete the mineralization process. Alternatively, DDM may be converted to 4, 4'-dichlorobenzhydrol DBH or 4, 4'-dichlorobenzophenone DBP

(**Figure 2**). Both DBH and DBP may undergo reductive dechlorination of the aromatic ring to form the corresponding non chlorinated analogs which then undergo aromatic ring cleavage and further degradation.

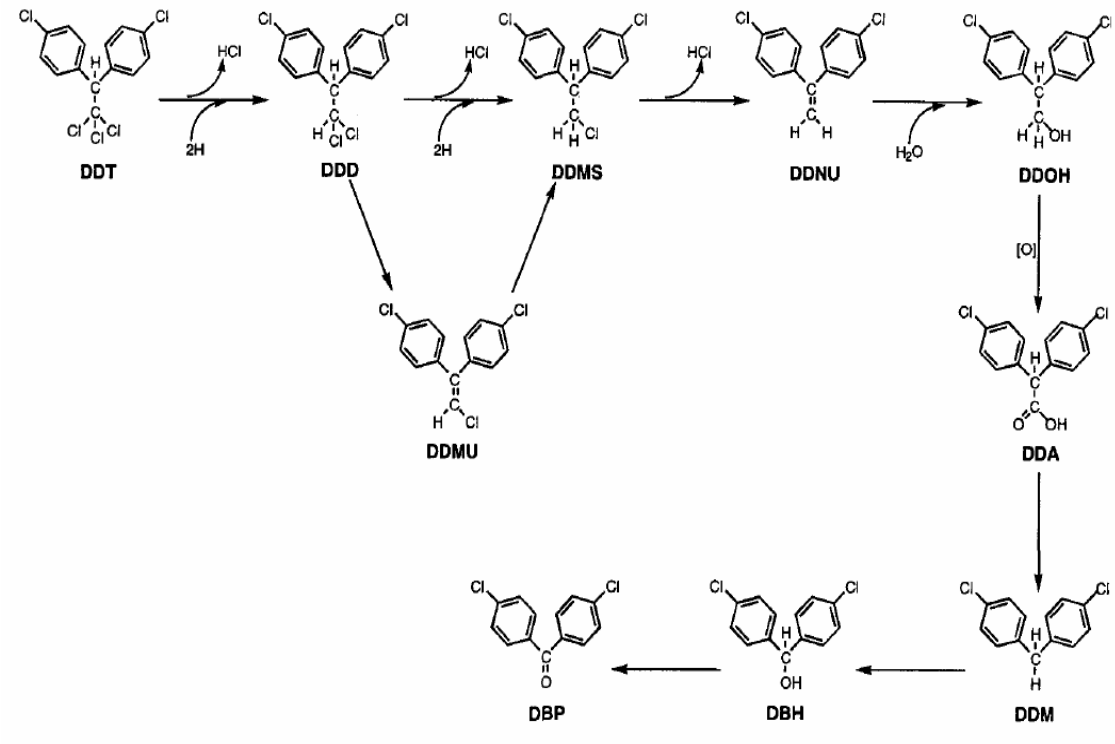


Figure 2: Proposed pathway for bacterial metabolism of DDT via reductive dechlorination (Wedemeyer, 1967; Langlois *et al.,* 1970 and Pfander *et al.,* 1972).

Several aerobic pathways for DDT metabolism in different organisms have been suggested (Nadeau *et al.,* 1994; Quensen *et al.,* 1998). A detailed study of the pathway for DDT degradation by *Pseudomonas aeruginosa* 640x, which was isolated from DDT-polluted soils helped to elucidate the pathway (Golovleva and Skryabin, 1981). This bacterium was observed to degrade DDT either completely or to the nonchlorinated compounds: phenylacetic, phenylpropionic, and salicylic acids. In aerobic biodegradation, DDE is attacked by a dioxygenase at the *ortho* and *meta*

positions (Higson, 1999). Such an attack would give rise to a 2, 3-dihydrodiol-DDE intermediate which proceeds *via* decarboxylation to yield 1,1-dichloro-(4'-chlorophenyl)ethane. The latter will undergo oxidation of the aliphatic side chain to yield 1, 1-dichloro-(4'-chlorophenyl) ethanol which is further oxidized to yield 4-Chloroacetophenone. The terminal methyl group of 1,1-dichloro-(4'-chlorophenyl) ethane may also undergo oxidation to yield phenyl acetic acid. The transformation of 4-chloroacetophenone to 4-chlorobenzaldehyde may be *via* complete oxidation and subsequent decarboxylation of the terminal methyl group (Hussain *et al.,* 1994). The ring-cleavage product would be further degraded to either a C-6- or C-5-chlorinated acid, depending on where the hydrolytic cleavage takes place. A C-6-chlorinated acid is formed if the hydrolytic cleavage took place between C-1 and an adjunct carbon on the cleaved phenyl ring. A C-5-chlorinated acid is formed if the hydrolytic cleavage took place in a way similar to that in the biphenyl pathway between C-5 and C-6 on the cleaved phenyl ring (John and Steven, 1987).

In soils DDT is converted to DDD and DDE where conversion of DDT to DDD is enhanced under anaerobic condition (You, 1995). Degradation of DDT in soils is dependent on the presence and numbers of microbes in the contaminated soil with the required degradative ability (Guenzi *et al.,* 1998). These microbes may be resident in the soil or they may be isolated from elsewhere and introduced to the soil. Microbes potentially useful for the biodegradation of DDT in soil include the bacteria and fungi that metabolise DDT *via* reductive dechlorination, ligninolytic fungi, and the chlorobiphenyl-degrading bacteria which carry out ring cleavage of DDT under aerobic conditions (Boul, 1995).

Fungi seem to play a significant role in the degradation of DDT. In this group of microorganisms DDD is the major metabolic product of DDT. It has been observed that DDT is extensively metabolized by different strains of *Trichoderma viride* isolated from soil (Matsumura and Boush, 1998). Out of 18 isolates tested for DDT metabolism, 8 produced DDD and dicofol, 3 produced DDD only and 1 produced DDE. Later, a "dicofol-like" compound was identified in place of dicofol which was subsequently confirmed to be DDNS (Matsumura *et al.,* 2000). Ligninolytic fungi have been shown to possess biodegradative capabilities for a broad spectrum of environmentally persistent compounds, including DDT. This capability has been attributed to their ability to degrade lignin (Shivaramaiah *et al.,* 2006). The majority of work on DDTr degradation by ligninolytic fungi has been carried out using a white rot fungus *Pseudomonas chrysosporium* (Bumpus *et al.*, 1985)

A bacterial strain of *Bacillus species* was shown to produce a number of phenolic metabolites from DDT, DDE, and DDD (Masse *et al*., 1989). No DDT ring cleavage products, however, were observed in these experiments. Nadeau *et al*. (1994) subsequently reported that *Alcaligenes eutrophus* A5 could metabolise both *o,p'*- and *p,p'-DDT* isomers when incubated at high cell density in resting cell cultures

The contribution of algae and protozoans is uncertain and has received relatively little attention. Algae, as compared with bacteria, are less efficient in the metabolism of DDT (Lai and Saxena 1999)

Most of these studies on biodegradation of DDT have been carried out with pure cultures under laboratory conditions, and results have been interpreted to indicate what may happen under natural conditions. However, in nature the metabolism of insecticides is influenced by environmental factors e.g., temperature, oxygen concentration, limited nutrients and competition for space. It is therefore possible that the same microorganisms might metabolize an insecticide differently according to the environmental conditions (Kuhard, *et al.*, 2004).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Chemicals

DDT and related metabolites were purchased from Sigma-Aldrich Chemical Company. All other chemicals, bacterial media and reagents were purchased from Oxoid limited- England, Scharlau Chemie- South Africa, Himedia laboratories and PVT limited- India. All the solvents and chemicals were high purity grade reagents.

3.2 Collection of soil samples

Six soil samples were collected from Jomo Kenyatta University of Agriculture and Technology (Juja campus, Kenya). The samples were collected from two different areas: cultivated and uncultivated areas. There had been no farming activities in the uncultivated areas for more than 20 years. DDT had not been used previously in the two areas hence the soil samples were uncontaminated with DDT. Three samples coded **A, B** and **C**, were collected from cultivated places and another three, **D**, **E** and **F** were collected from uncultivated places. Collection of the soil samples was through digging and scooping 10 cm^3 of the soil using a sterile spoon. The soil samples were carried to the laboratory in sterile plastic containers.

The sampling area (**Figure 3**) was largely covered by pyroclastic rocks (Muchena *et al.,* 1978). They contain crystals of orthoclase anorthoclase and rarely crystals of aegirine. The tuffs did not show any great variation in thickness or composition. The area had slopes ranging from 0-2 % and shows very little variation in physiography.

The soil was well drained with a gravely sandy clay to clay texture and a friable moist consistence. The soil structure was generally weak and fertility was low in the non cultivated places. The pH ranges from 5.6 in the topsoil to 8.4 in the subsoil. The soil had moderate to low organic matter from 1.0% to 1.7%. The nitrogen (N) status was low ranging from 0.11% to 0.14%. Phosphorus value was below 10 ppm. Calcium, magnesium and potash were adequate. The soils had high cation exchange capacity. The cultivated parts had higher nitrogen, phosphorus and organic matter.

(a) (b)

Figure 3: Map of a section of Jomo Kenyatta University of Agriculture and Technology (Juja, Thika district, Kenya) and the surroundings with the sampling sites at (a) uncultivated areas and (b) cultivated areas. From .http://earth.google.com/

3.3 Growth medium

Soil sample (1 g) was dissolved in 9 ml of sterile 0.85 % NaCl and diluted to dilution 10^{-6} in ten-fold dilution steps. Dilutions were inoculated in minimal media (MM), which was based on MM4 medium (Brune *et al.,* 1995), but without napthoquinone, yeast extracts, casamino acids, 7-vitamin solution, folic acid, riboflavin, and lipoic acid. MM4 medium contained NaCl [1.7 g], KCl [6.5 g], $MgCl_2.6H_2O$ [0.50 g], $CaCl_2.2H_2O$ [0.10 g], NH_4Cl [5.6 g], $NaS0_4$ [1.0 g] and KH_2PO_4 [1.0 g]. The following were also added from sterile stock solutions: 1 M Na-phosphate buffer [40 ml, pH 7.0], SL 11 [2 ml] (Eichler and Pfening, 1986) and Se/W solution [2 ml]. Cultures were incubated at 30 ^oC in the dark until growth became constant and began to decline. The incubation took 29 days.

3.4 Enrichment of soil samples in cultures with DDT

Dilutions of soil samples were enriched in cultures with the following concentrations of DDT: 0 ppm, 20 ppm, 50 ppm and 100 ppm. This was done to determine the highest concentration of DDT that can allow growth of microorganisms for subsequent tests. Then, an aliquot of 1.0 ml from dilution 10^{-6} from each of the six soil samples were inoculated in a 100 ml broth of MM4 with DDT (100 ppm). All experiments were prepared in duplicates. The controls were uninoculated medium with DDT and inoculated medium without DDT. Turbidity and amounts of DDT and its metabolites were measured after every two days until growth became constant and began to decline.

DDT whose purity was 100 % at a concentration of 5000 ug/ ml in methanol was used. A stock solution of 100 ug/ ml was made in methanol. Dilutions in media were

made so that a final concentration was 100 ppm DDT and was obtained by adding 100 µl to a flask containing 100 ml of sterile media just before inoculation.

3.5 Isolation of pure cultures

When growth became constant, aliquots of 0.1 ml from enrichment cultures of the soil samples were streaked on MM4 medium with DDT in 1.5 % agar. This was then incubated at 30^{o}C for six days. Individual colonies were then picked and inoculated in fresh medium and incubated for another six days.

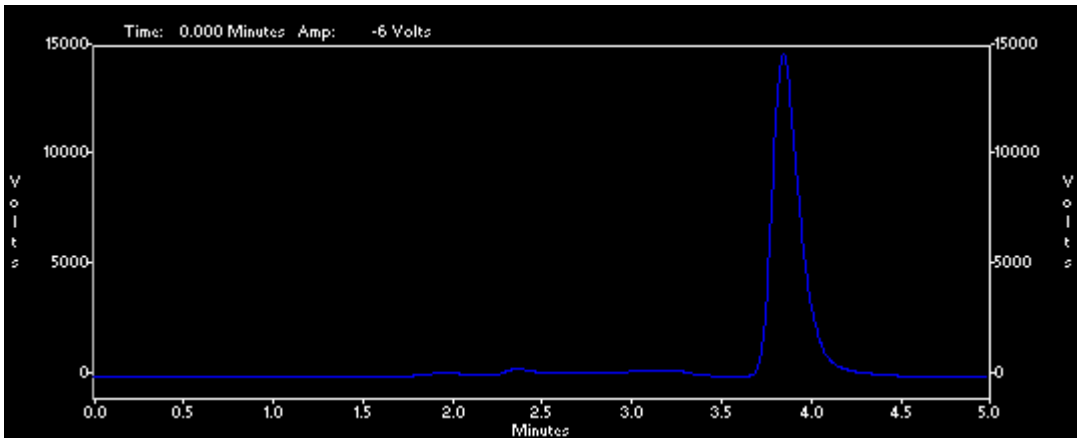
3.6 Testing isolates on their potential to biodegrade DDT

Isolates were inoculated in MM4 broth in which DDT [100 ppm] was added. The levels of DDT and turbidity were monitored using HPLC and spectrophotometer respectively. Isolates that grew and reduced levels of DDT producing DDD were taken to be biodegrading DDT.

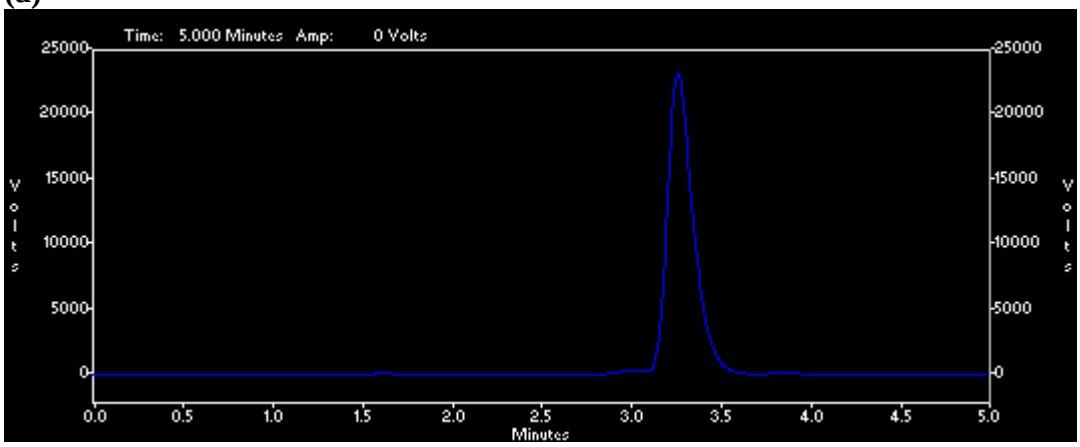
3.7 Analysis of DDT and its metabolites

The amount of DDT and its metabolites, DDD and DDE, were analyzed using High Performance Liquid Chromatography (HPLC, Shimadzu). This was done on a reverse phase C-18 Column 125 x 4 mm, 5 µM, equipped with UV-VIS detector (Ali and Aboul, 2002). Analysis was done at 25 ^{o}C and the mobile phase was 99.9 % methanol at a flow rate of 0.5 ml / min. Peak detection was at 240 nm. DDT was retained at a retention time of 3.78 minutes (**Figure 4a**) while DDD and DDE were retained at 3.2 and 4.3 minutes respectively (**Figures 4b and 4c**). Known concentrations of DDT, DDE and DDD were used to draw the standard curves

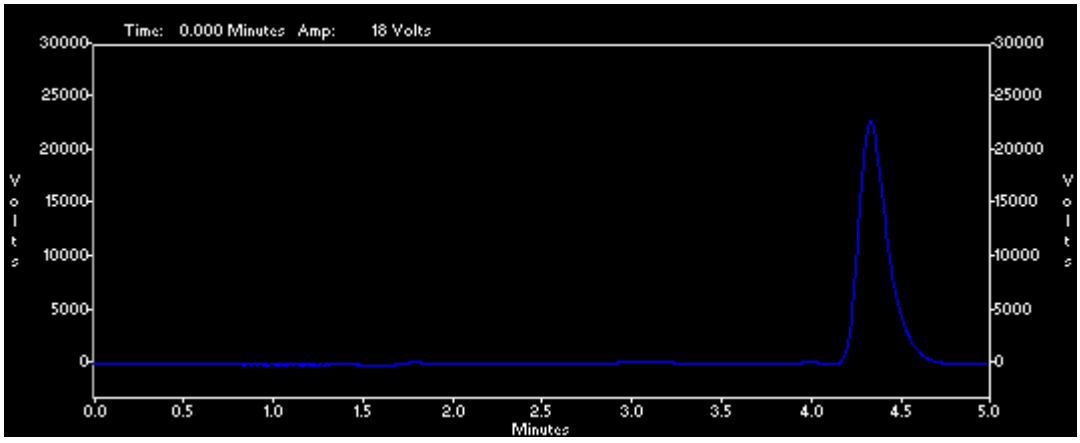
(**Figure 5a, 5b and 5c**) that were used to determine the amount of DDT, DDD and DDE in the enrichment cultures.



(a)

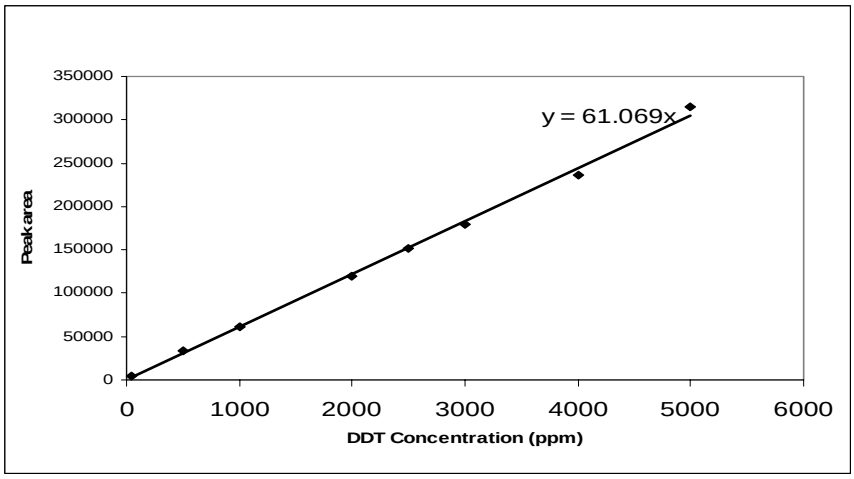


(b)

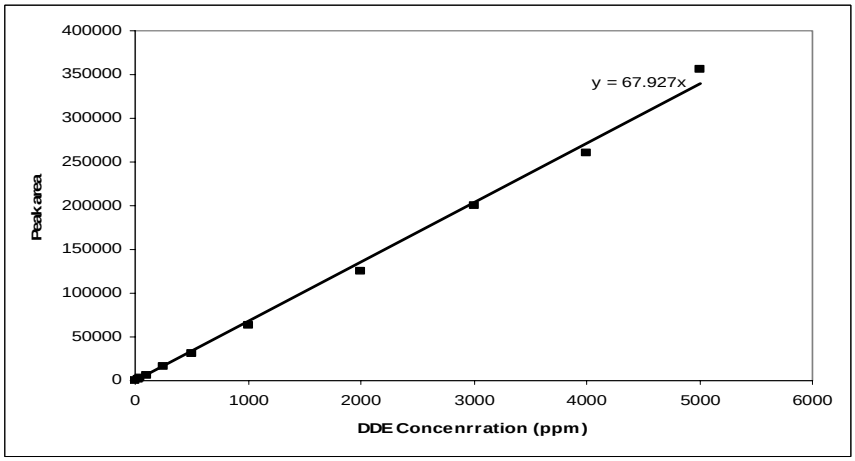


(c)

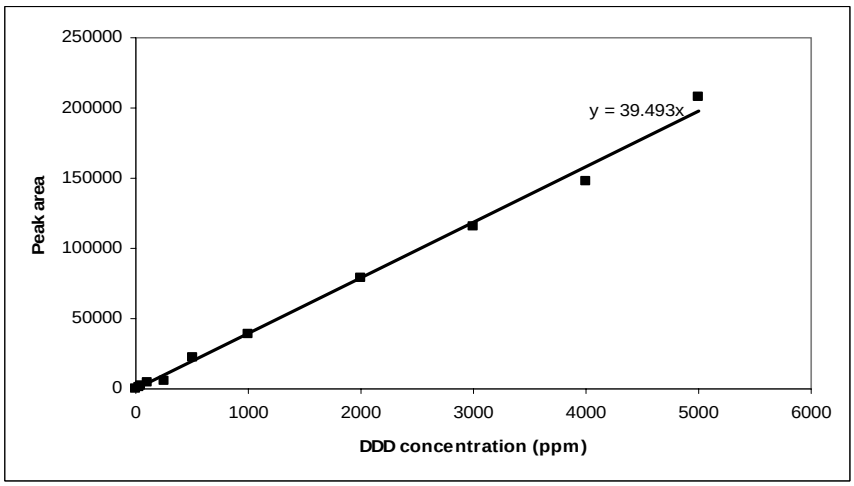
Figure 4: HPLC chromatograms of DDT (a), DDD (b) and DDE (c) detected at 240 nm at a retention time of 3.78, 3.2 and 4.3 minutes respectively.



(a)



(b)



(c)

Figure 5: Standard curves for DDT (a), DDE (b) and DDD (c)

3.8 Growth Curves and DDT Curves

The bacterial growth curve of enrichment cultures and of each pure isolate was determined after inoculation in conical flask with a 100 ml of MM4 medium with or without DDT. A 4 ml aliquot was aseptically removed from the conical flask after every two days and its turbidity at OD_{600} was determined using a spectrophotometer (Shimadzu UV240, Japan). The 4 ml culture was then centrifuged at 5000 rpm for five minutes and the amount of DDT, DDD and DDE monitored using the HPLC. MM4 medium with inoculum and MM4 Medium with DDT but without inoculum were used as the controls.

3.9 Characterization of isolates

3.9.1 Microscopic examination

Colony morphology was determined using a dissecting microscope (Leica zoom, USA) supplemented by a classical gram staining method and the 3 % (w/v) KOH test (Holt, 1994). Motility was assessed by inoculation in SIM agar and observing the ability of the strains to migrate from the point of inoculation through semisolid (0.3 %) agar plates (Ball *et al.,* 1996).

3.9.2 Biochemical characterization

3.9.2.1 Starch hydrolysis

Starch agar (1.5 %) containing 0.2 % soluble starch was used to demonstrate the hydrolytic activities of exoenzymes amylase and maltase. Starch in the presence of iodine imparted a blue-black colour indicating absence of starch splitting enzymes

thus a negative test. A clear zone of hydrolysis surrounding the growth of the organism represented a positive result (Cappuccino and Sherman, 2001).

3.9.2.2 Carbohydrate fermentation

Fermentative degradation was carried out in broth containing a durham tube. The fermentation media were lactose, dextrose and sucrose broths (Cappuccino and Sherman, 2001). Andrade indicator was also used with a variety of sugars like glucose, cellobiose, fructose, mannose, mannitose, sorbitol, rhamnose, arabinose, and melibiose. Following incubation, carbohydrates that were fermented and produced acid caused the phenol red to turn yellow and Andrade to turn pink, thereby indicating a positive reaction.

3.9.2.3 Methyl red – Voges –Proskauer test (MR-VP)

Methyl red –Voges–Proskauer (MR-VP) was used to determine the ability of the isolates to oxidise glucose with the production and stabilization of high concentrations of acids end products. MR-VP broth was inoculated with the isolates and incubated for 48 h. at 37 ^{o}C. Aliquots of each culture were added with either methyl red indicator or Barrits reagent. For positive cultures the methyl red indicator appeared red in colour and the absence of rose coloration was negative for VP (Holt, 1994).

3.9.2.4 Hydrogen sulphide (H_2S) production test

The blackening of lead acetate paper dipped in a culture incubated in 2 % peptone water and incubated at 37 ^{o}C for 24 hours indicates production of H_2S due to the activity of bacteria on sulphur containing amino acids ((Holt, 1994).

3.9.2.5 Citrate utilisation test

This tested the ability of the isolates to utilize citrate as the sole source of carbon and energy for growth. Isolates were inoculated in Koser's citrate medium and incubated at 37 ^{o}C for 24 hours. The change of bromothyl blue from green to blue indicated the utilization of citrate as a sole source of carbon and energy (Holt, 1994).

3.9.2.6 Urease test

The ability of the isolates to attack nitrogen and carbon bonds in amide compounds was determined using urea broth medium containing the pH indicator, phenol red. A pink colour indicated a positive reaction (Holt, 1994).

3.9.2.7 Litmus milk reactions

The enzymatic transformation of different milk substrates was carried out using litmus milk broth. For this test, a variety of different biochemical activities like gas formation, lactose fermentation, litmus reduction, curd formation, proteolysis (peptonisation) and alkaline reactions were tested (Cappuccino and Sherman, 2001).

3.9.2.8 Nitrate reduction test

This tested the presence of the enzyme nitrate reductase which reduces nitrate to nitrite. Isolates were inoculated in nitrate broth medium containing 1% potassium nitrate. Following incubation at 37 ^{o}C for 24 h., the addition of sulfalinic acid and alphanaphthylamine produced a cherry red colour indicating a positive test (Cappuccino and Sherman, 2001).

3.9.2.9 Gelatin liquefaction

The hydrolytic activity of gelatinase was determined by using a stab culture and incubating at 37 ^{o}C for 24 h. The cultures were then held at 4 ^{o}C for 20 min and those that remained liquefied were considered positive for gelatine hydrolysis (Cappuccino and Sherman, 2001).

3.9.2.10 Egg – yolk reaction

1.5 ml of egg yolk was aseptically added in a medium containing tryptone, disodium hydrogen phosphate, NaCl, $MgSO_4.7H_20$ and glucose. Isolates were inoculated and incubated at 30 ^{o}C and observed after five days with a white precipitate in or on the surface of the medium indicating a positive reaction (Holt, 1994).

3.9.2.11 Growth in 7% and 10% (w/v) NaCl

Isolates were inoculated in nutrient broth containing NaCl at indicated concentrations and incubating them at 30 ^{o}C for 3 days and observed for growth (Cappuccino and Sherman, 2001)

3.9.2.12 Deamination of phenylalanine

The action of deaminase enzyme to remove the amino group (-NH_2) from amino acids was determined by inoculation in phenylalanine agar and incubation at 37 ^{o}C for 24 hours. Formation of a green colour after addition of 10 % ferric chloride solution indicated a positive reaction (Cappuccino and Sherman, 2001).

3.9.2.13 Degradation of tyrosine

Isolates were inoculated with one streak in tyrosine agar containing L-Tyrosine and nutrient agar. The plates were incubated at 30 ^{o}C for 7 days and observed for clearing of tyrosine crystals around and below the growth (Cappuccino and Sherman, 2001).

3.9.2.14 Degradation of casein

Milk agar was used to demonstrate the hydrolytic activity of proteases. Following inoculation and incubation of the agar plate culture, isolates secreting proteases exhibited a clear area, zone of proteolysis, surrounding the bacterial growth (Cappuccino and Sherman, 2001).

3.9.2.15 Aerobic and anaerobic growth

Aerobic and anaerobic growth was determined by inoculation of the isolates into nutrient agar tubes (stab), nutrient broth tubes and thyoglycollate tubes and checking for growth after 72 hours at 30 ^{o}C (Holt, 1994).

3.9.2.16 Catalase test

Production of the enzyme catalase and degradation of hydrogen peroxide was tested by adding H_2O_2 to incubated trypticase soy agar slants with bubbling indicating a positive test (Cappuccino and Sherman, 2001).

3.9.2.17 Triple sugar iron agar test

Triple sugar iron agar slants were inoculated by stabbing the butt and streaking the slant and incubated for 24 hours at 37 ^{o}C. This was to test fermentation of glucose, sucrose and lactose shown by phenol and production of H_2S shown by ferrous sulphate which causes extensive blackening of the butt (Cappuccino and Sherman, 2001).

3.9.3 Metabolic versatility

The ability of the isolates to use various selected substrates was tested using MM4 medium with aromatic compounds at a concentration of 2 mM and non aromatic compounds at a concentration of 1 % (w/v). This was inoculated with 0.05 ml of 48 hours old culture grown in nutrient agar and incubated at 30 ^{o}C (Murray *et al.,* 1984) in an incubator shaker at 120 rpm for seven days. Turbidity was used to indicate growth. The aromatic compounds were resorcinol, isopropylamine salt of glyphosate (round up^{TM}), benzoic acid and diazinon while non aromatic compounds were mainly sugars.

3.9.4 Molecular characterization of isolates

Six bacterial isolates that biodegraded DDT were characterized using molecular techniques. DNA was extracted and the 16S rRNA gene amplified using PCR. The PCR products were purified and sequenced. 16S rRNA gene sequences of the isolates were compared to sequences in the public database with blast program of the NCBI.

3.9.4.1 DNA extraction

The isolates were grown in nutrient agar broth for two days at 30 ^{o}C and then centrifuged. The supernatant was discarded and the following added: 200 µl of Tris-EDTA-Sucrose (50 mM Tris at pH 8.5, 50 mM EDTA at pH 8.0, 25% sucrose solution), 10 ul of lysozyme (20 mg/ml), 10ul of RNAse A (20 mg/ml). Then it was mixed gently and incubated for one hour at 37 ^{o}C after which 600 ul of Tris-EDTA-SDS (10 mM Tris at pH 8.5, 5 mM EDTA at pH 8.0, 1% SDS) and 10ul of proteinase K (20 mg/m) was added, mixed gently and incubated at 50 ^{o}C for 30 minutes. DNA was precipitated by using equal volumes of phenol: chloroform and the precipitation process repeated. The mixture was then centrifuged for 15 minutes at 13000 rpm and the aqueous phase transferred into a new tube. An equal volume of chloroform: isoamylalcohol (24:1) was then added and centrifuged at 13000 rpm for 15 minutes. An equal volume of isopropanol and 0.1 volumes of 3M NaCl were then added and incubated overnight at -80 ^{o}C. After the overnight incubation, the samples were defrosted and centrifuged at 4 ^{o}C for 30 minutes to pellet the DNA. The pellets were washed twice with 70 % ethanol, centrifuged for five minutes at 13000 rpm. The ethanol supernatant was pippetted and discarded. The pellet was then air dried

on the bench at room temperature for 20 minutes, dissolved in 50 µl of water and stored at -20 ^{o}C. The DNA was verified by electrophoresis in 1.0 % agarose gel (Schmidt *et al.,* 1991).

3.9.4.2 PCR amplification of bacterial 16S rRNA gene

Purified total DNA from each isolate was used as a template for amplification of 16S rRNA gene. PCR amplification was performed with a model Gene Amp 9800 thermal cycler (Applied Biosystems) using universal primers 27F 5' -GAG TTT G(AC)T CCT GGC TCA G- 3' forward primer and 1492R 5'- TAC GG(CT) TAC CTT ACG ACT T-3' reverse primer (Lane, 2001). Each PCR reaction contained 3 µl of 10 X PCR buffer, 2.5 µl of 27 F forward primers (5 pmol), 2.5 µl of 1492R reverse primer (5 pmol), 4 µl of dNTPs (2.5 mM), 0.4 µl of Promega *Taq polymerase* (5U/µl), 1.5 µl of DNA template and 16.6 µl of PCR grade water in a final volume of 30 µl. A negative control contained all of the above except the DNA template. The initial activation of the enzyme was done at 94 ^{o}C for five minutes. Enzyme activation was followed by 35 cycles consisting of denaturation at 94 ^{o}C for 45 seconds, primer annealing at 55 ^{o}C for 55 seconds, extension at 72 ^{o}C for two minutes and final extension at 72 ^{o}C for 8 minutes. The amplification product was verified by electrophoresis in 1.0 % agarose gel.

3.9.4.3 Purification of the PCR Products

The PCR Products were purified using QuickClean 5M Gel Extraction Kit. The DNA bands from the agarose gel were excised using a clean, sharp scalpel and excess agarose removed. The gel slice was placed in a colorless, pre-weighed tube, weighed

and three volumes of binding solution II to one volume of gel slice (100 mg ≈ 100 µl) added. The above was incubated at 50 °C for ten minutes with occasional vortexing until the gel slice dissolved completely and the color of the mixture turned yellow. One volume of isopropanol (with respect to the original gel volume) was added, mixed, transferred to the QuickClean column and centrifuged at 12,000 rpm for 30 seconds. The flow-through from the tube was discarded and 500 µl of wash solution added to the column and centrifuged at 12,000 rpm for one minute. The column was then transferred to a clean 1.5 ml microcentrifuge tube, 50 µl of elution buffer added to the center of the column membrane and incubated at room temperature for one minute followed by centrifugation at 12,000 rpm for one minute to elute and collect the DNA. The purified PCR products were then sequenced.

3.9.4.4 Phylogenetic analysis

The forward and backward 16S rRNA gene sequences of the six bacteria isolates were viewed and edited using Chromas software package (www.technelysium.com.au). They were then aligned using Bioedit sequence alignment editor software package (Hall, 1999) to provide full sequences of about 1500 nucleotide bases. The sequences were compared to sequences in the public databases with the BLAST search program on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) to find closely related bacterial 16S rRNA gene sequences. The ARB database software package (Ludwig and Strunk, 1996) was also used to align and identify the closely related bacterial 16S rRNA gene sequences. The 16S rRNA gene sequences of the isolates and those of the closely related bacteria were then aligned and processed to produce

Phylogenetic trees using MEGA software package (www.megasoftware.net). The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test of 1000 replicates was used (Felsenstein, 1985). The trees were drawn to scale, with branch lengths being in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2007) and were in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset.

CHAPTER FOUR

4.0 RESULTS

4.1 Enrichment cultures

4.1.1 Growth of enrichment cultures at different DDT concentrations

The growth rate and maximum growth of cultures observed at DDT concentrations of 0 ppm, 20 ppm, 50 ppm and 100 ppm, after a period of 15 days (**Figure 6**), was different from each other. There was minimal growth of 0.05 (O.D $_{600}$) at DDT concentration of 0 ppm over the same period. At DDT concentration of 20 ppm there was growth of up to 0.25 (O.D $_{600}$) after which growth declined. The highest growth observed of 0.48 (O.D $_{600}$) was at DDT concentration of 50 ppm At DDT concentration of 100 ppm, growth was up to 0.39 (O.D $_{600}$), which is lower than the observed growth at 50 ppm.

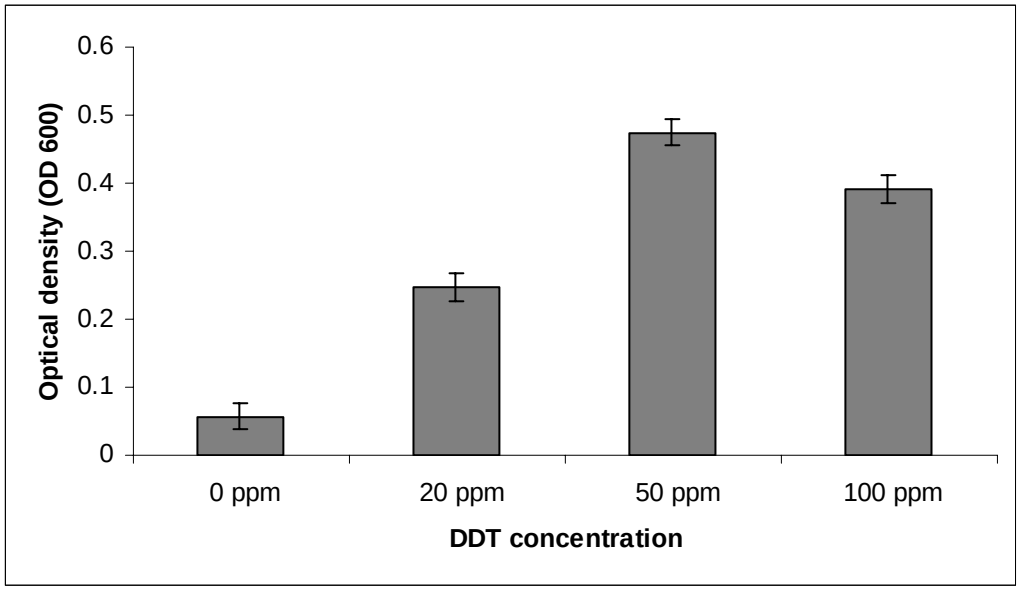
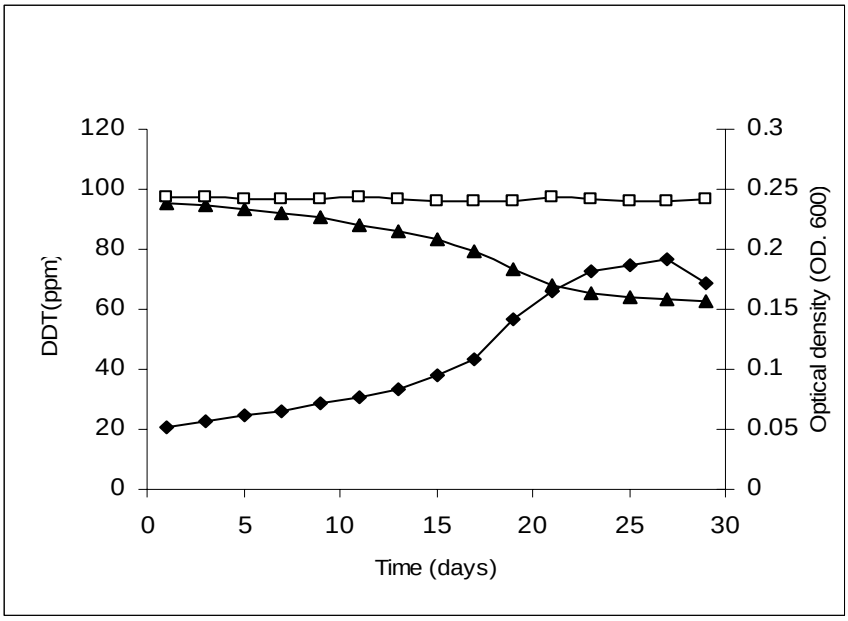


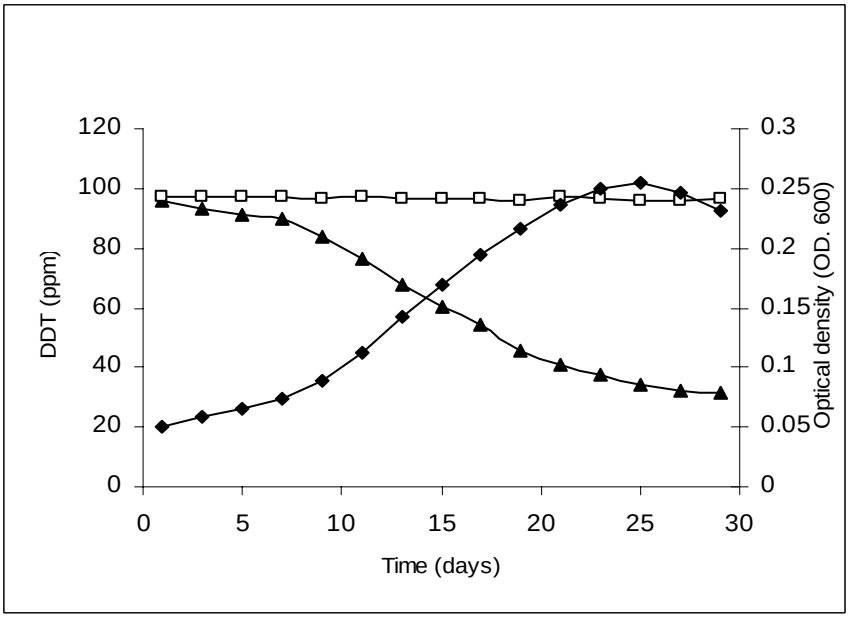
Figure 6: Growth of soil enrichment cultures at different concentrations of DDT. The values used were means ± SE (5%) of six replicates from both cultivated and uncultivated places.

4.1.2 Growth and rate of DDT degradation by the enrichment cultures

There was varying growth and DDT curves for the enrichment cultures from both cultivated and uncultivated places. In all the cultures, the more the growth, the more the DDT degradation hence DDT degradation was directly proportional to growth (**Figure 7**). The enrichment culture from cultivated places (**Figure 7a**) had a long lag phase and reached its highest cell mass at OD_{600} of 0.191 after 27 days which was lower than that of enrichment culture from uncultivated places (**Figure 7b**) that reached its highest cell mass of 0.247 after the same period of 27 days. In both, DDT degradation was noted after three days with the enrichment culture from the uncultivated places degrading 60.20 % after 29 days at a degradation rate of approximately 2.08 ppm DDT/ day while the one from cultivated places degraded 38.58 % of initial amount of DDT over the same period at a degradation rate of approximately 1.33 ppm DDT/ day. Out of the six isolates that were capable of degrading DDT, the two (isolates **102** and **101**) that degraded the highest amount of DDT (**Table 2**) were found in uncultivated places hence perhaps the higher degradation in enrichment culture from uncultivated places compared to the one from cultivated places.



(a)



(b)

Figure 7: Growth and DDT curves of soil enrichment cultures from (a) cultivated places and (b) uncultivated places. Results are means (n=6) where standard errors are <5% of means in all cases. The symbols represents: -□- Control, -▲- DDT concentration, -■- growth curve

4.2 DDT biodegrading isolates

4.2.1 Isolation of DDT biodegrading microorganisms

Through two independent enrichment steps, using DDT as the sole source of carbon and energy, ten bacterial isolates were initially isolated from both the cultivated and uncultivated places. From the ten isolates, only six isolates, designated as **101, 102, 103, 104, 105** and **110** (**Table 2**)**,** were found to individually biodegrade DDT into DDD. None of the isolates transformed DDT to DDE. The transformation of DDT to DDD was assessed using HPLC, which indicated a decrease in DDT concentration with an increase in DDD concentration (**Figures 8, 9 and 10**).

Table 2: Sources and amount of DDT degraded by isolates in pure and mixed culture

Isolates	% DDT degraded	Source	
		Cultivated	Uncultivated
101	44.31		√
102	58.08		√
103	39.72	√	√
104	30.33	√	
105	28.97	√	
110	28.48	√	√
Six (mixed)	82.63	√	√

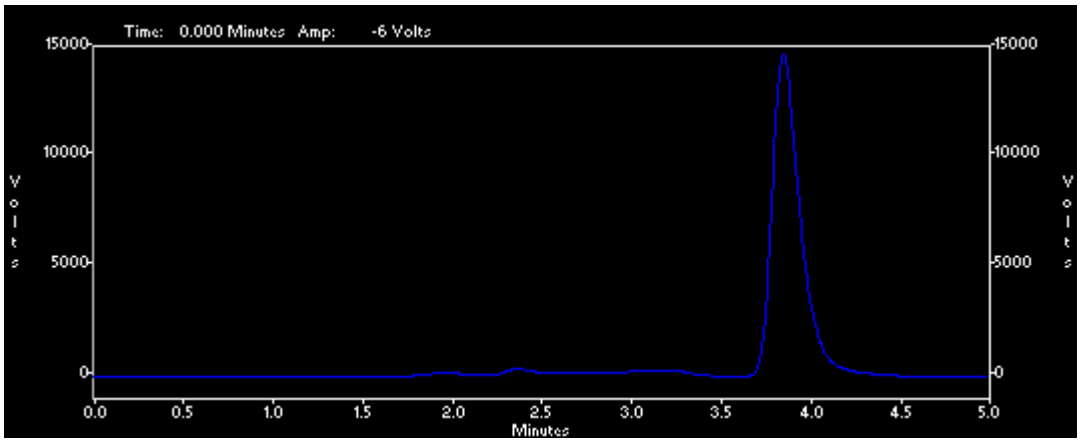
√ indicates the place from where the microbe was isolated.

4.2.2 HPLC chromatograms of the Isolates

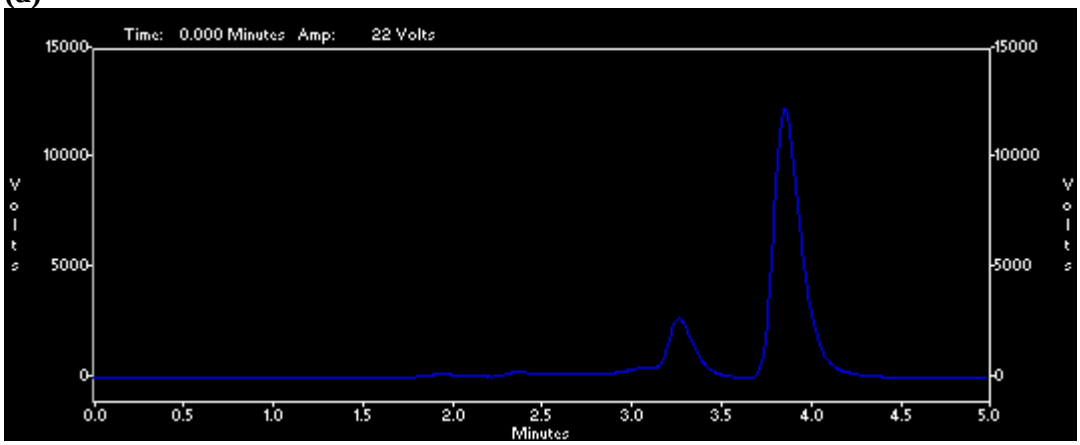
DDT was detected at a retention time of 3.78 minutes (**Figure 4a**) while DDD (**Figure 4b**) and DDE (**Figure 4c**) were detected at 3.2 and 4.3 minutes respectively. Degradation of DDT started after three days and the peak of DDD started to appear. From there on, the area and size of DDD peak continued to increase while that of DDT peak continued to decrease (**Figures 8, 9 and 10**). Had the DDT been transformed to DDE, a resultant peak at a retention time of 4.3 minutes (**Figure 4c**) could have appeared hence its absence indicated that DDT was degraded to DDD only. Biodegradation of DDT to DDD is enhanced in anaerobic conditions. In all the HPLC chromatograms, there was varying sizes and areas of both DDT and DDD peaks with time. A decrease in DDT peak with time indicated more degradation as was an increase in DDD peak and this was well pronounced after 25 days. The chromatogram of isolate **110**, which biodegraded the least amount of DDT (28.48 %) after 31 days, is shown (**Figure 8**). The chromatogram of isolate **102**, which biodegraded the highest amount (for individual isolates) of DDT (58.08 %) after 31 days, is also shown (**Figure 9**). For isolate **110**, the DDT peak as at 25th day is larger than DDD peak showing less degradation compared to isolate **102** whose DDT and DDD peaks are almost equal in area and size. The DDD peak of the mixed isolates as at 25th day (**Figure 10**) is larger in size and area than the DDT peak indicating the highest transformation of DDT to DDD.

In all the individual isolates and in the mixed isolates, DDD was the only product that resulted from the degradation of DDT. DDD was not degraded further. This was indicated by the increase in size of the DDD curve throughout the incubation period

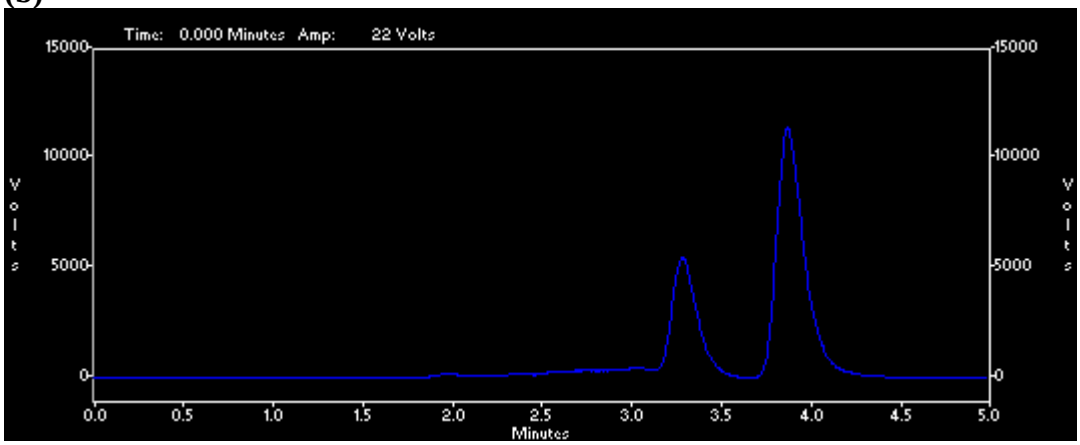
without any decrease at any time. Only the two peaks (for DDT and DDD) were observed.



(a)

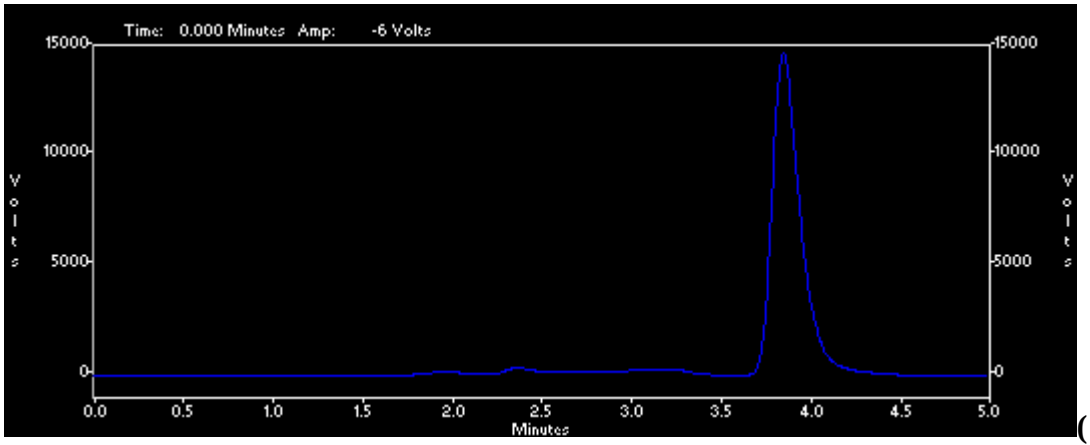


(b)

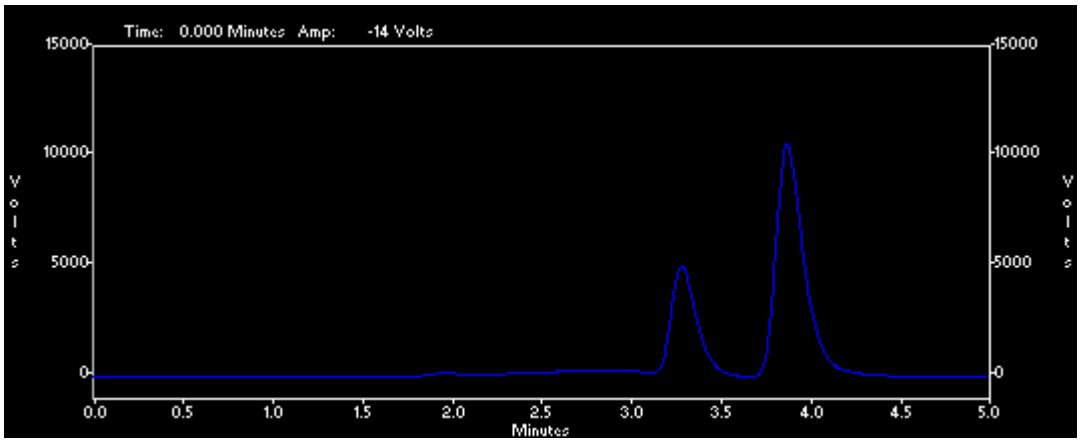


(c)

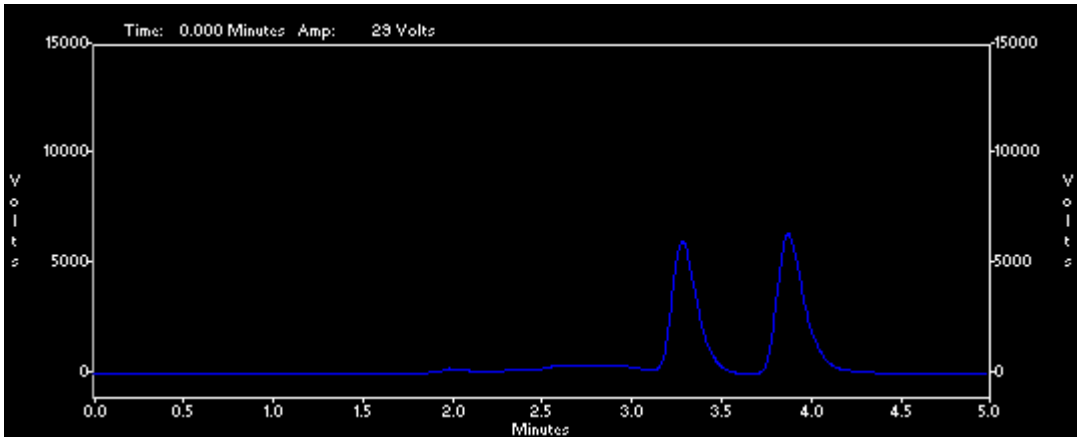
Figure 8: HPLC chromatograms of samples taken from growth culture of isolate 110 after 10 days (b), 25 days (c) and control (a). Detection was at 240 nm. Retention time for DDD and DDT was 3.2 and 3.78 minutes respectively.



(a)

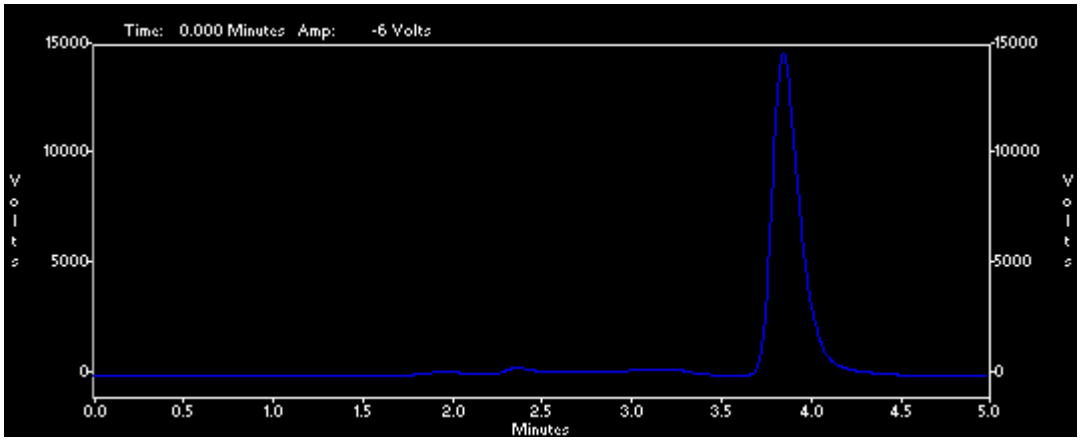


(b)

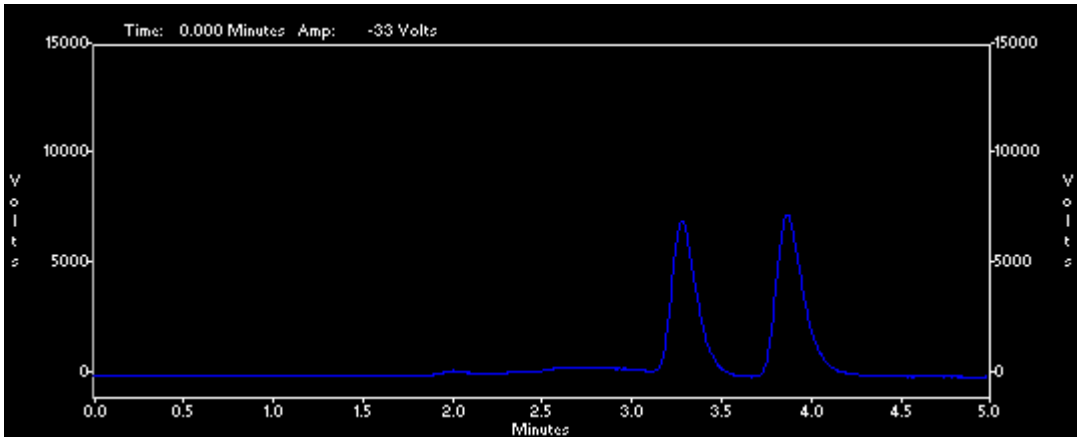


(c)

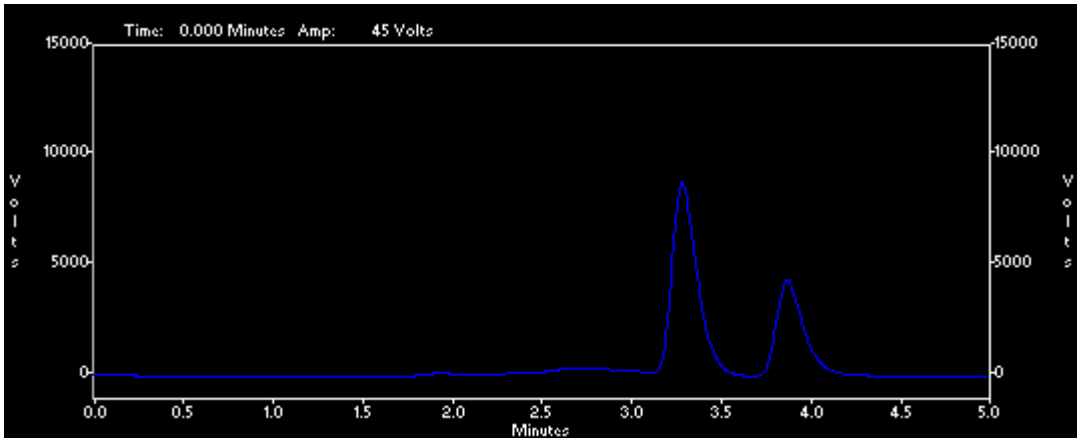
Figure 9: HPLC chromatograms of samples taken from growth culture of isolate 102 after 10 days (b), 25 days (c) and control (a). Detection was at 240 nm. Retention time for DDD and DDT was 3.2 and 3.78 minutes respectively.



(a)



(b)



(c)

Figure 10: HPLC chromatograms of samples taken from growth culture of six mixed isolates after 10 days (b), 25 days (c) and control (a). Detection was at 240 nm. Retention time for DDD and DDT was 3.2 and 3.78 minutes respectively.

4.2.3 Growth and rate of DDT degradation by the isolates

There was varying growth of the isolates and different DDT degradation rates hence the different DDT and growth curves for each individual isolate and the mixed isolates. It was observed that an increase in the observed growth rate corresponded to an increase in DDT degradation (**Figures 11** and **12**). Isolate **101** was isolated from uncultivated places and degraded 44.31 % of initial amount of DDT (**Table 2, Figure 11a**) over a period of 31days with a degradation rate of 1.43 ppm/ day. The isolate had a short lag phase and reached its highest cell mass (OD_{600}) of 0.157 after 27 days. Of the six isolates, isolate **102** (**Figure 11c**) had the highest cell mass of 0.168 after 27 days and also had the highest degradative ability after degrading 58.08 % of DDT (**Table 2**) in 31 days at a rate of 1.87 ppm/ day. Isolate **102** was isolated from uncultivated areas (**Table 2**). Compared to isolates **102** and **101**, isolate **103** (**Figure 11e**) which was isolated from both cultivated and uncultivated places (Table 2) had a lower growth and degradative ability. At day 25, isolate **103** had grown to its highest cell mass of 0.125 and had degraded 39.72 % of DDT in 31 days at a rate of 1.28 ppm/ day. Unlike the other isolates, isolate **104** which was obtained from cultivated places (**Table 2**) did not have a sharp decline in growth (**Figure 11b**) after it had reached its highest cell mass of 0.117 after 29 days. The DDT degradative rate of the isolate was 0.97 ppm DDT/day having degraded 30.33% of DDT in 31 days. The amounts of DDT degraded after 31 days by isolate **105** (28.97 %) at a rate of 0.93 ppm DDT/ day (**Table 2** and **Figure 11d**) and isolate **110** (28.48 %) at a rate of 0.91 ppm DDT/ day (**Table 2** and **Figure 11f**) was very close to each other but ranked the lowest compared to the other isolates. Isolate **105** was isolated from cultivated places and attained its highest cell mass of 0.097 after 27 days while

isolate **110** was from both cultivated and uncultivated areas and attained cell mass of 0.094 after 29 days.

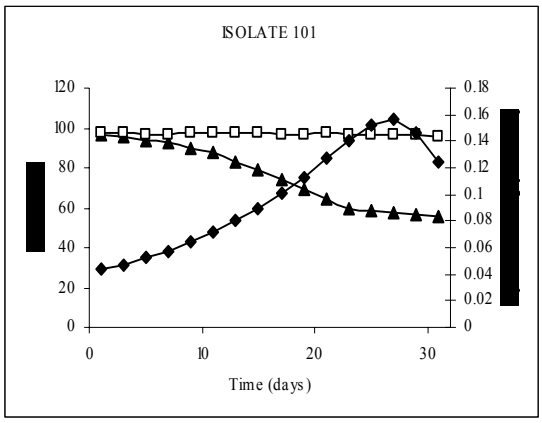


Figure 11a: Isolate 101

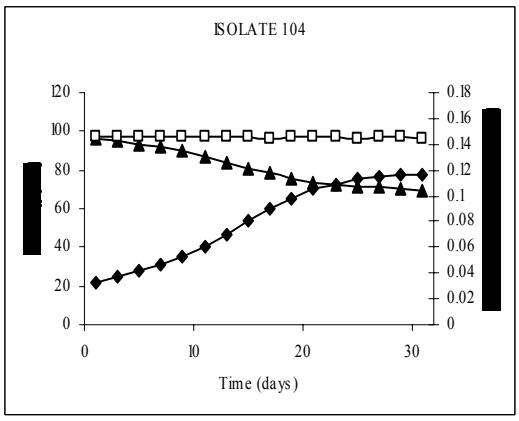


Figure 11b: Isolate104

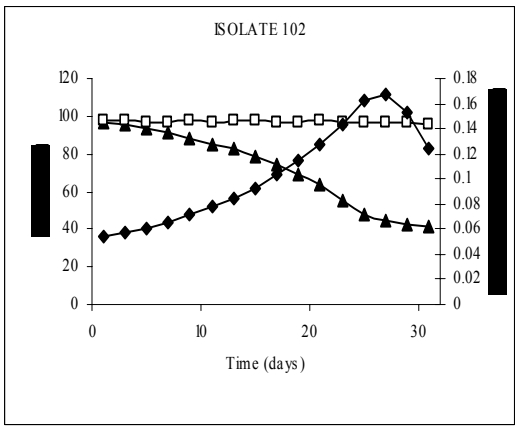


Figure 11c: Isolate 102

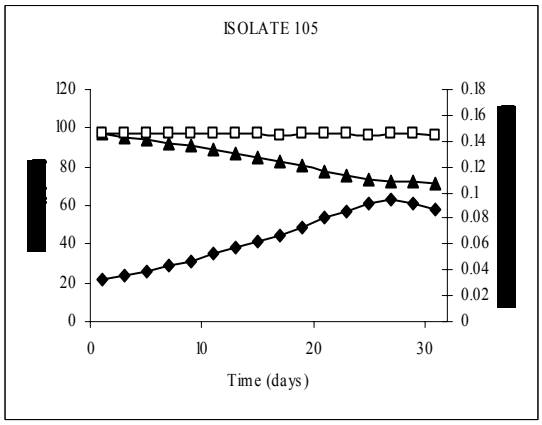


Figure 11d: Isolate 105

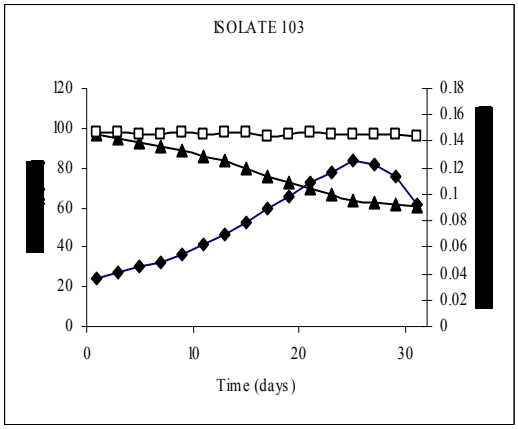
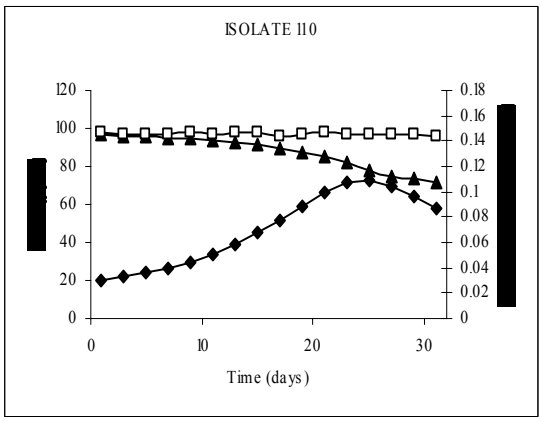


Figure 11e: Isolate 103



Figures 11f: Isolate 110

Figure 11: Mean growth and DDT curves of isolates 101 (a), 104 (b), 102 (c), 105 (d), 103 (e) and 110 (f), grown in MM4 Medium enriched with DDT (100ppm). n=2 where SE <5 % of means in all cases. The symbols represents: -□- Control, -▲- DDT concentration, -■- growth curve, n- replicates, SE- Standard errors

4.2.4 Growth and rate of DDT degradation by the mixed culture

The mixed culture had the highest cell mass at OD_{600} of 0.32 compared to individual isolates whose range was 0.12-0.17. DDT degradation was greatly enhanced when the six isolates were mixed (**Figure 12**). The mixed culture degraded 82.63 % of the initial amount of DDT after 31 days at the highest rate of 2.67 ppm DDT/ day. The amount of DDT degraded by the mixed culture was higher than that of individual isolates (the range of DDT degraded by the isolates was 28.48 %-58.08 %)

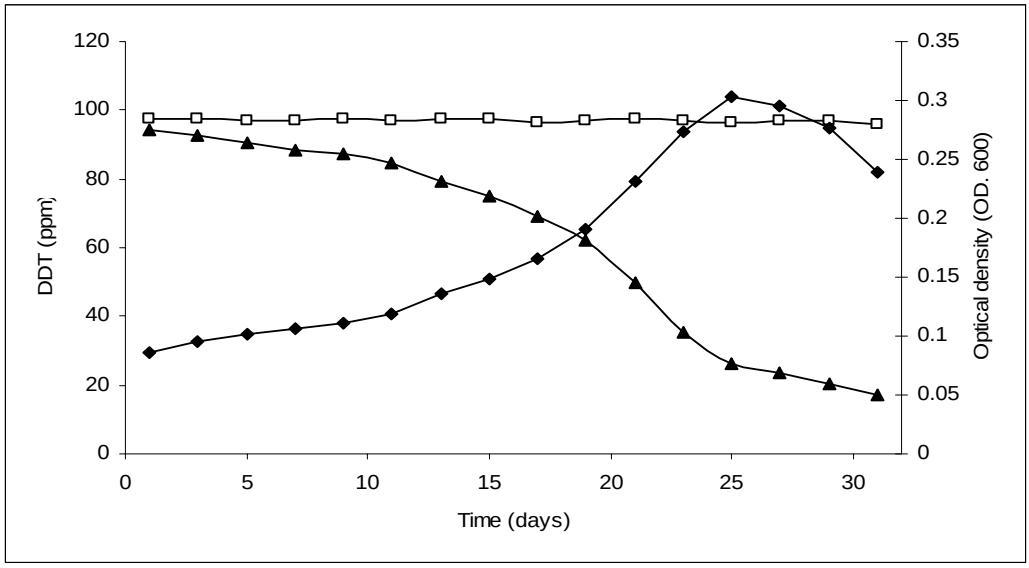


Figure 12: Mean growth and DDT curves of mixed isolates. n=2 where SE<5% of means in all cases. The symbols represents: -□- Control, -▲- DDT concentration, -■- growth curve, n- replicates, SE- Standard errors

4.3 Characterization of isolates

4.3.1 Morphological and cellular characteristics

The colonies of Isolate **101** were small in size, raised, round in shape with a smooth surface and had a cream pigmentation. Isolate **101** was gram positive with a rod shape (**Plate 1**). Isolate **102** and **110** were gram positive with Cocci shapes (**Plates 2 and 3** respectively). Their colonies were cream white and translucent in

pigmentation, moderate in size, raised, round in shape with smooth surfaces. Isolates **103**, **104** and **105** were gram negative with rod shapes (**Plates 3, 4 and 5** respectively). Isolates **103** and **105** had short thin rods while isolate **104** had thick rods. On nutrient agar plates, the colonies of isolates **103** and **104** were swamming and cream in pigmentation while isolate **105** was yellowish (**Plates 4, 5 and 6** respectively). The colonies of the three isolates were raised with smooth surfaces.

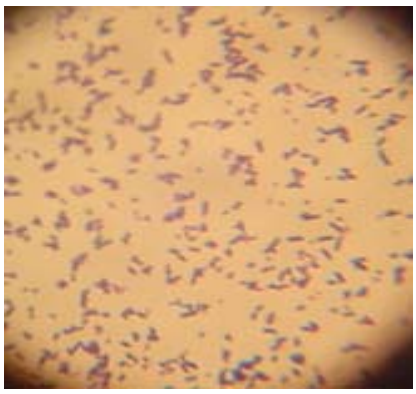
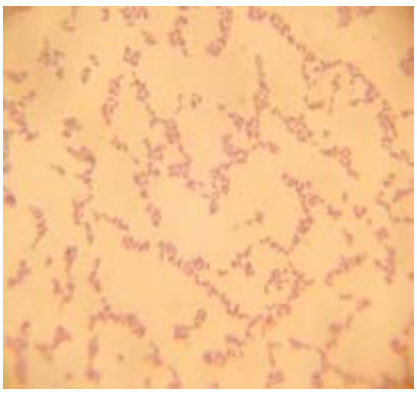


Plate 1: Isolate 101



Plates 2: Isolate 102

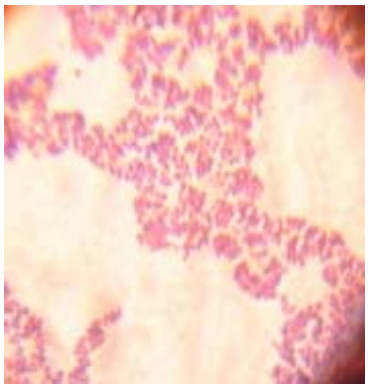
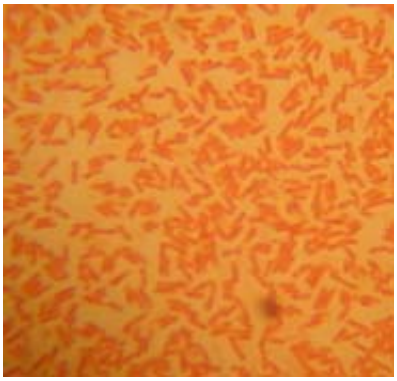


Plate 3: Isolate 110



Plates 4: Isolate 104

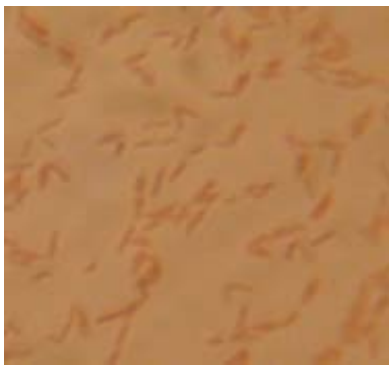
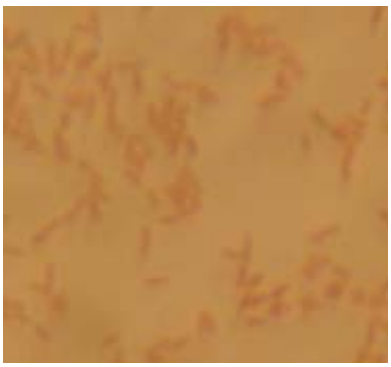


Plate 5: Isolate 105



Plates 6: Isolate 103

Plates 1-6: Photographs of gram stained isolates

4.3.2 Biochemical characterization of DDT degrading isolates

The ability of the isolates to excrete extracellular enzymes was tested through hydrolysis of starch, casein and gelatin. Only isolate **101** was positive for starch hydrolysis while isolates **102**, **103**, **104**, **105** and **110** were negative (**Table 3**). Starch is a high molecular weight – branching polymer composed of glucose molecules linked together by glycosidic bonds. Isolate **101** is the only one that produced the two starch hydrolytic enzymes amylase and maltase. Isolates **101**, **102**, **103**, **104**, **105** and **110** were positive for gelatin and casein hydrolysis (**Table 3**). Therefore, the isolates could excrete the extracellular enzymes proteases responsible for the step by step degradation of proteins into peptones, polypeptides, dipeptides and ultimately into amino acids in a process called proteolysis

The ability of the isolates to excrete intracellular enzymes was determined through tests on sugars fermentation, litmus milk reactions, hydrogen sulphide production, nitrate reduction, Catalase reactions, Urease, methyl red, voges-proskauer, citrate utilization and triple sugar- iron test. The isolates fermented glucose, dextrose, sucrose, mannose, fructose and maltose. Isolate **103** fermented mannitose while isolates **104** and **105** fermented lactose, cellobiose and mannitose (**Table 3**). Isolate **101** and **110** fermented cellobiose while only Isolate **102** and **110** fermented arabinose (**Table 3**). However, all the six isolates did not ferment rhamnose and melibiose (**Table 3**). Microorganisms use carbohydrates differently to obtain energy in a series of orderly and integrated enzymatic reactions, depending on their enzyme complement. Isolates **101**, **102** and **104** were positive for TSI agar test, both butt and slant (**Table 3**), confirming that they could ferment glucose and either sucrose or

lactose; Only the butt was positive for isolates **103**, **105** and **110** showing that they preferred glucose to either sucrose or lactose. All isolates were positive for methyl red test indicating that they fermented glucose and produced a lot of mixed acids as end products (**Table 3**). When barrits reagent was used to determine whether the isolates could produce nonacidic or neutral end products, such as acetylmethylcarbinol, from the organic acids that result from glucose metabolism, they were all negative, implying that the end products remained acidic (**Table 3**).

The isolates were negative for citrate utilization (**Table 3**). The ability to utilize citrate in the absence of glucose or lactose depends on the presence of the enzyme citrate permease that facilitates the transport of citrate into the cell so that it can be acted upon by the enzyme citrase which produces oxalacetic acid and acetate that are eventually converted enzymatically to pyruvic acid and carbon dioxide. Therefore, the isolates lacked the enzymes citrate permease and citrase. None of the isolates had the ability to produce enzyme cysteine desulfurase or thiosulfate reductase that are detected through production of hydrogen sulfide from substrates such as sulfur containing amino acids or inorganic sulfur compound, sodium thiosulfate (**Table 3**). The sim agar used to detect production of hydrogen sulfide was also used to check motility whereby only isolates **101** and **104** were able to migrate from the line of inoculation (**Table 3**). Motility is due to organisms being flagellated and could also be subject to growth conditions like temperature. Isolates **101**, **102**, **103**, **104** and **110**, were positive for Urease test (**Table 3**). Urease is a hydrolytic enzyme that attacks the nitrogen and carbon bond in amide compounds such as urea and forms the

alkaline end product ammonia that causes the phenol red in the urea broth medium to turn a deep pink. Isolate **105** could not produce enzyme Urease.

The isolates could reduce nitrate to nitrite liberating oxygen, a reaction catalyzed by the enzyme nitrate reductase (**Table 3**). All the isolates could produce the enzyme Catalase (**Table 3**) that degrades hydrogen peroxide and an extremely toxic superoxide into water and oxygen. Isolates **101**, **102**, **104** and **110** produced decarboxylase enzymes that are capable of removing the carboxyl group of the amino acid to yield end products consisting of an amine or diamine plus carbon dioxide (**Table 3**). None of the isolates contained deaminase enzymes that are capable of removing the amino group from amino acids and other NH_2 containing chemical compounds as all were negative for phenylalanine test (**Table 3**).

Table 3: **Biochemical characteristics of the isolates**

Biochemical Tests		**Isolates**					
		101	**102**	**103**	**104**	**105**	**110**
Gram stain	shape	Rods +	Cocci +	Rods -	Rods -	Rods -	Cocci +
Colony characteristics	color	Cream	Cream white	Cream white	Cream white	yellow	Cream white
	shape	round	round	round	round	round	round
	elevation	raised	raised	raised	raised	raised	raised
	surface	smooth	smooth	smooth	smooth	smooth	smooth
TSI agar test	Butt	+	+	+	+	+	+
	Slant	+	+	-	+	-	-
Citrate utilization		-	-	-	-	-	-
Gelatin liquefaction		+	+	+	+	+	+
MR test		+	+	+	+	+	+
VP test		-	-	-	-	-	-
Urease test		+	+	+	+	-	+
Nitrate reduction		+	+	+	+	+	+
Motility at 37^{o}C		+	-	-	+	-	-
Starch hydrolysis		+	-	-	-	-	-
Egg yolk reaction		+	+	+	+	-	+
Growth at 7% NaCl		+	+	-	+	-	-
Growth at 10% NaCl		-	+	-	+	-	-
Phenylalanine test		-	-	-	-	-	-
Tyrosine test		+	+	-	+	-	+
Aerobic growth		+	+	+	+	+	+
Anaerobic growth		+	+	+	+	+	+
H_2S Production		-	-	-	-	-	-
Casein hydrolysis		+	+	+	+	+	+
Catalase test		+	+	+	+	+	+
Fermentation							
Lactose		-	-	-	+	+	-
Dextrose		+	+	+	+	+	+
Sucrose		+	+	+	+	+	+
Glucose		+	+	+	+	+	+
Cellobiose		+	-	-	+	+	+
Fructose		+	+	±	+	+	+
Mannose		+	+	+	+	+	+
Arabinose		-	+	-	-	-	+
Rhamnose		-	-	-	-	-	-
Maltose		+	+	+	+	+	+
Mannitose		-	-	±	±	+	-
Melibiose		-	-	-	-	-	-

Symbols: + positive reaction, - negative reaction, ± variable reaction

4.4 Metabolic versatility of the isolates

The ability of the isolates to mineralize or transform a variety of aromatic and non aromatic compounds namely glucose, cellobiose, fructose, sodium acetate, benzoic acid, resorcinol, isopropylamine salt of glyphosate (Trade name, Round up^{TM}) and diazinon is summarized in Table 4. The isolates differ in their ability to use the above compounds. Isolates **101**, **102**, **103**, **104**, **105** and **110** could utilize Glucose, fructose, dextrose, sucrose and benzoic acid (**Table 4**). Isolates **104** and **105** could utilize lactose while isolates **101**, **104**, **105** and **110** could utilize cellobiose (**Table 4**). Isolates **101**, **102** and **110** could utilize sodium acetate (**Table 4**). Resorcinol could be utilized by isolates **103** and **104** while only isolate **102** could utilize sopropylamine salt of glyphosate (round up^{TM}) (**Table 4**). None of the isolates could utilize Diazinnon (**Table 4**).

Table4: Metabolic versatility of the isolates

Metabolic versatility tests	**Isolates**					
	101	**102**	**103**	**104**	**105**	**110**
Lactose	-	-	-	+	+	-
Dextrose	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+
Glucose	+	+	+	+	+	+
Cellobiose	+	-	-	+	+	+
Fructose	+	+	+	+	+	+
Sodium acetate	+	+	-	-	-	+
Benzoic acid	+	+	+	+	+	+
Resorcinal	-	-	+	+	-	-
Diazinnon	-	-	-	-	-	-
Round up^{TM}	-	+	-	-	-	-

Symbols: + positive for substrate utilization, - negative for substrate utilization.

4.5 Phylogenetic analysis

DNA was extracted from the isolates, amplified using universal primers 27F 5' - GAG TTT G(AC)T CCT GGC TCA G- 3' forward primer and 1492R 5'- TAC GG(CT) TAC CTT ACG ACT T-3' reverse primer (Lane, 2001). PCR products were first verified using gel electrophoresis in 1.0 % agarose gel (**Figure 13 a**). The PCR products were then purified and the purified product was verified by electrophoresis in 1.0 % agarose gel (**Figure 13b**). The purified PCR products were then sequenced using the above primers. The sequenced products were then blasted against samples at NCBI database at http://www.ncbi.nlm.nih.gov/.

The phylogenetic position of isolate **101** indicates that the isolate clusters with the genus *Bacillus*. This clustering pattern was supported by high bootstrap values of between 50-100 % (**Figure 14**). The isolate had sequence similarity of 99 % to *Bacillus cereus, Bacillus thuringiensis, Glacial ice bacterium* and *Bacillus anthracis* (**Table 5**).

Phylogenetic analysis of 16S rRNA gene sequences of isolate **102** and **110** showed that the two isolates clusters with the genus *Staphylococcu*s (**Table 6**). This clustering pattern was supported by high bootstrap values of between 50-100 % (**Figure 15**). The isolates had sequence similarity of 98 % to *Staphylococcus sciuri, Staphylococcus sp. XJU, Uncultured soil bacterium, Staphylococcus lentus, Uncultured rumen bacterium* among others (**Table 6**)

Phylogenetic analysis of 16S rRNA gene of isolate **103**, **104** and **105** showed that the three isolates clusters with the genus *Stenotrophomonas*. This clustering pattern was supported by high bootstrap values of between 50-100 % (**Figure 16**). The isolates had sequence similarity of 95 %, 97 % and 94 % respectively to *Stenotrophomonas maltophilia* (**Table 7**).

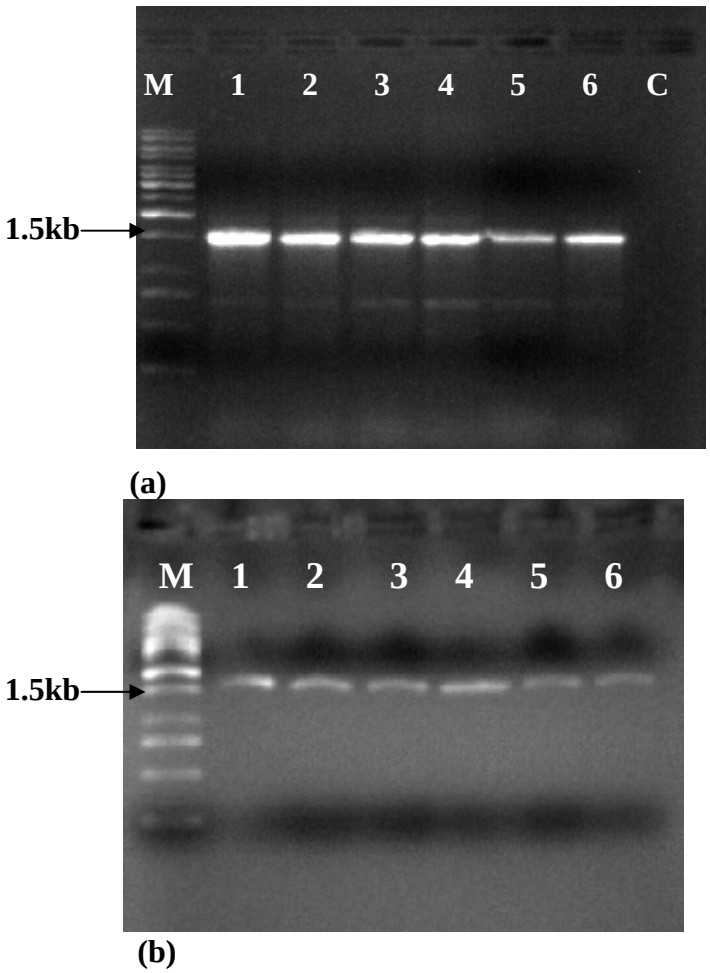


Figure 13: **1% agarose gel showing DNA bands visualized after ethidium bromide staining (a) after PCR amplification of 16S rRNA gene of the isolates (b) after Gel purification of PCR products**. Lanes: (M) 1kb DNA ladder used as a molecular marker; (1-6) PCR products.; (C) Control sample

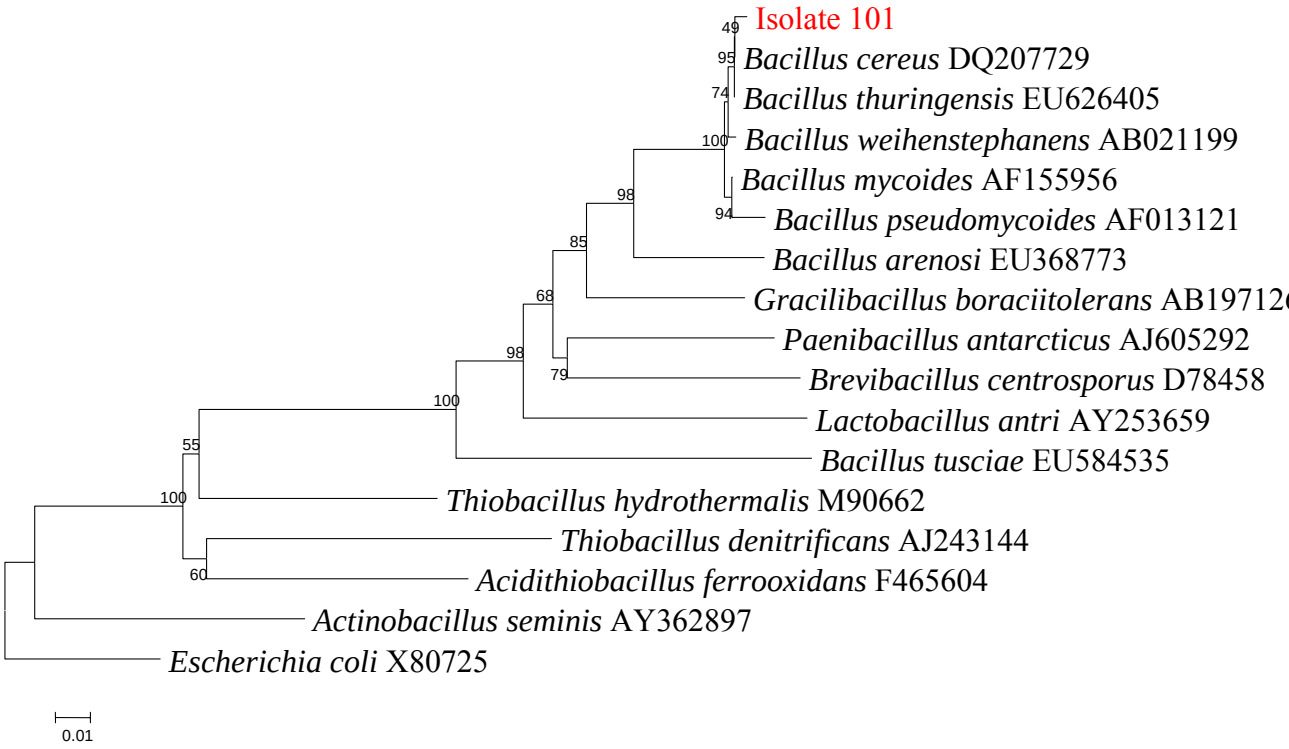


Figure 14: Phylogenetic tree showing position of isolate 101. The scale bar indicates approximately 1% sequence difference. Numbers at nodes indicate bootstrap values of each node out of 100 bootstrap resampling. The 16S rRNA gene sequence of *Escherichia coli* X80725 was used as an outgroup.

Table 5: 16S rRNA gene sequence similarity of isolate 101 to other close relatives

Strain	Accession number	% Relatedness
Bacillus sp. WPCB070	gi|198404121|FJ006883.1	99
Bacillus cereus strain CTSP45	gi|194399059|EU855219.1	99
Bacillus cereus strain	gi|122892480|EF178440.1	99
Bacillus thuringiensis strain	gi|158149007|AB363741.1	99
Glacial ice bacterium G500K-2	gi|19568759|AF479333.1	99
Bacillus thuringiensis serovar strain	gi|145578056|EF2**103**10.1	99
Bacillus anthracis strain	gi|33468788|AB116124.1	99

The accession numbers are from the NCBI database

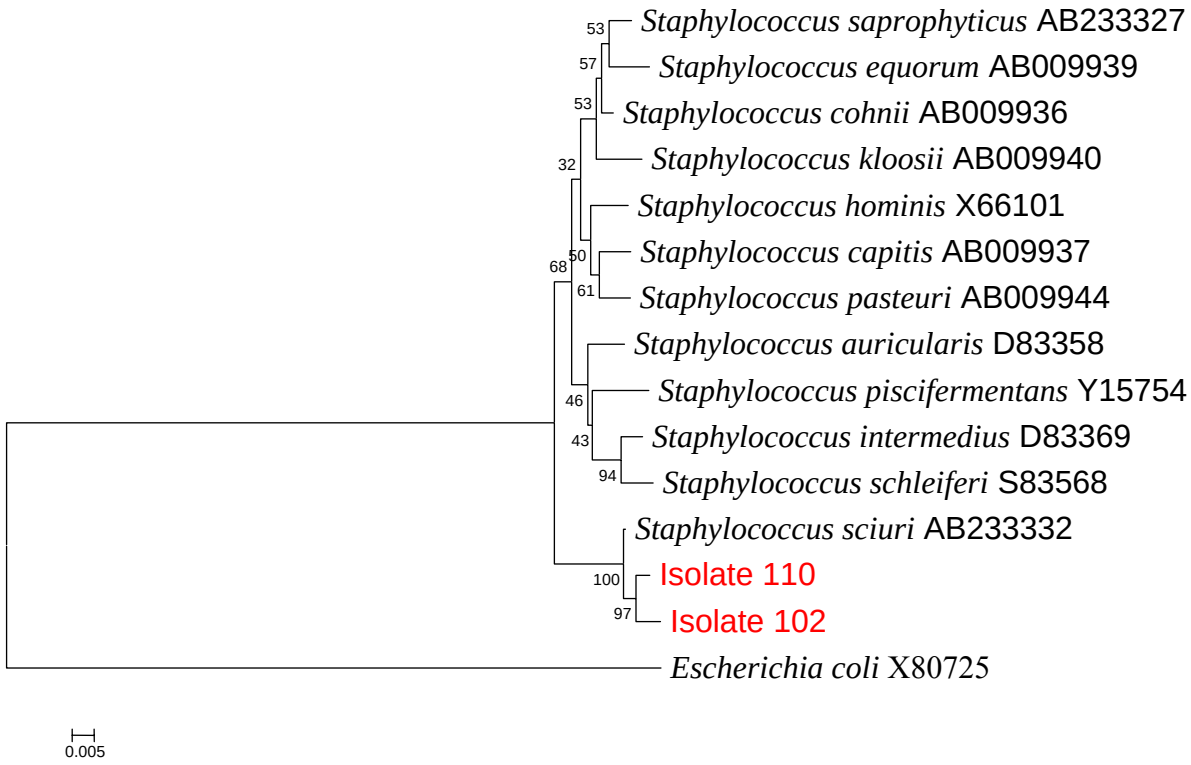


Figure 15: Phylogenetic tree showing position of isolates 102 and 110. The scale bar indicates approximately 0.5% sequence difference. Numbers at nodes indicate bootstrap values of each node out of 100 bootstrap resampling. The 16S rRNA gene sequence of *Escherichia coli*, X80725 was used as an outgroup

Table 6: 1**6S rRNA gene sequence similarity of isolate 102 and 110 to other close species**

Strain	**Accession number**	**% Relatedness**	
		102	**110**
Staphylococcus sciuri	gi|189034392|EU693523.1	98	98
Staphylococcus sciuri NT-7	gi|156567217|EU095646.1	98	97
Staphylococcus sciuri CTSP9	gi|194399031|EU855191.1	98	98
Staphylococcus sp. XJU	gi|111559207|DQ837544.1	97	98
Staphylococcus sp. TUT1203	gi|51491455|AB188210.1	98	98
Uncultured soil bacterium	gi|16517834|AF423242.1	98	98
Staphylococcus lentus strain	gi|146141367|EF528296.1	97	98
Uncultured rumen bacterium	gi|126653554|EF436333.1	98	98
Unidentified bacterium Z	gi|2209057|AB004765.1	98	98

The accession numbers are from the NCBI database.

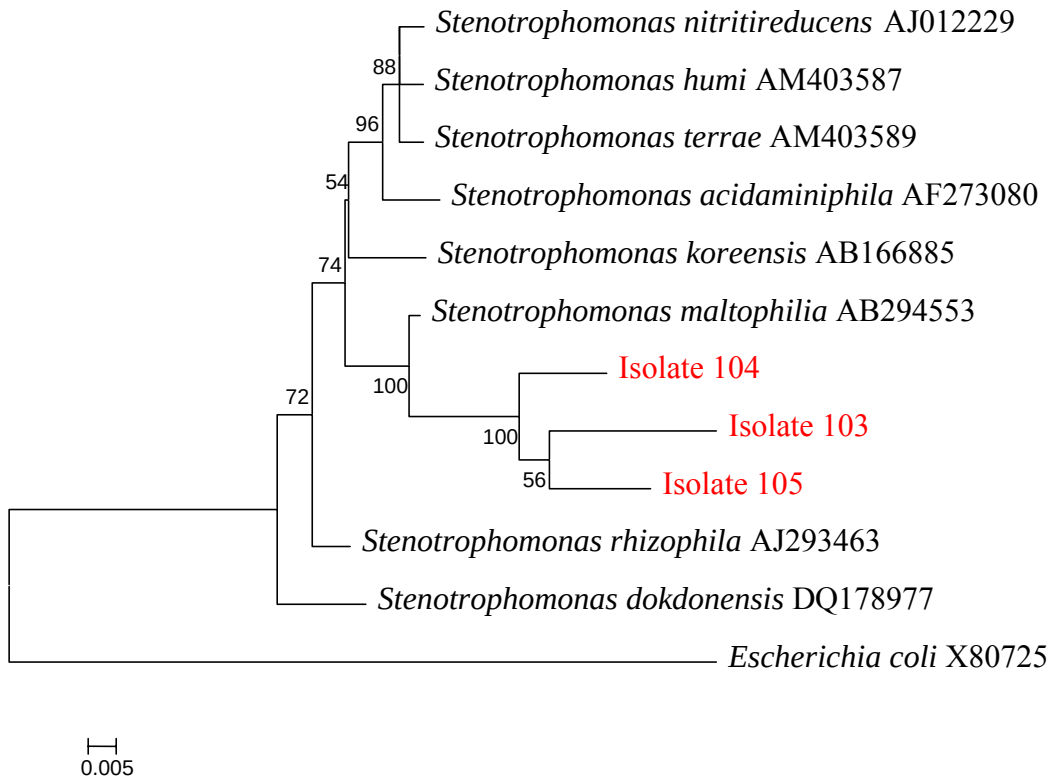


Figure 16: Phylogenetic tree showing position of isolate 103,104 and105. The scale bar indicates approximately 2% sequence difference. Numbers at nodes indicate bootstrap values of each node out of 100 bootstrap resampling. The 16S rRNA gene sequence of *Escherichia coli* X80725 was used as an outgroup.

Table 7: 16S rRNA gene sequence similarity of isolates 103, 104 and 105 to other close species

Strain	Accession number	% Relatedness		
		103	104	105
Stenotrophomonas maltophilia	gi|170673185|EU543577.1	95	97	94
Stenotrophomonas maltophilia strain 17A1	gi|147907867|EF580914.1	95	97	94
Stenotrophomonas maltophilia strain LMG 957	gi|4210842|AJ131114.1	95	97	94
Stenotrophomonas maltophilia strain SWCH-9	gi|189409748|EU708603.1	94	97	94
Stenotrophomonas sp. Ellin162	gi|33309404|AF409004.1	95	97	94
Stenotrophomonas sp. EP01	gi|115306407|AM402950.1	94	97	94
Stenotrophomonas maltophilia strain B25R	gi|91805183|DQ466574.1	95	97	94
Uncultured gamma proteobacterium clone	gi|22218228|AF529349.1	95	96	94
Uncultured bacterium clone P5D15-671	gi|138240652|EF511438.1	95	97	94
Pseudomonas geniculata strain XJUHX-18	gi|159906319|EU239476.1	95	97	94

The accession numbers are from the NCBI database.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Growth of enrichment culture at different DDT concentrations

From the results, it was found that the extent and rate of microbial growth is affected by the concentration of DDT in the media (**Figure 6, page 39**). The maximum growth observed was different at various DDT concentrations. The highest growth was observed at DDT concentration of 50 ppm (**Figure 6, page 39**). At 100 ppm, growth was reduced as was indicated by turbidity that was lower than at 50 ppm. Langlois and Collins, (2000) also found out that growth of *Staphylococcus species* in trypticase soy broth was affected by increase in DDT concentration in the media. This means that the presence of DDT in the environment reduces microbial growth and this effect will be more pronounced when its concentration increases.

5.2 Isolation of DDT biodegrading bacteria

In this study, six DDT biodegrading bacteria (Isolates **101**, **102**, **103**, **104**, **105** and **110**) were isolated from the tropical soil uncontaminated with DDT (**Table 2, Page 42**). DDT-metabolising microbes have been isolated from a range of habitats including animal faeces, soil, sewage, activated sludge, and marine and freshwater sediments (Lai and Saxena, 1999; Rochkind *et al*., 1987). Microorganisms could be classified into three categories with respect to their exposure to DDT or any other pesticide (Smith *et al,* 1999). First, there are those whose growth is totally inhibited by DDT and this includes the ones that could not grow in media containing DDT hence could not be isolated. The second group is the ones that can tolerate presence

of DDT and their growth is not inhibited, though they do not degrade it. However, to identify those two categories of microorganisms, further research should be done whereby different known concentrations of DDT are added to a media which has another source of carbon and compare their growth with a control without DDT. The third group includes the microorganisms that can degrade DDT. Langlois *et al.,* 2002 found out that not all species are capable of degrading DDT and amount of degradation is affected by growth medium especially availability of oxygen during incubation. The standard method for isolating microorganisms with the ability to degrade environmental pollutants is to enrich them from contaminated soils. A novel approach for isolating DDT-degrading microorganisms is to screen alternative sources like uncontaminated soil and other materials. So, tropical soils, uncontaminated with DDT, also contain some microorganisms that can degrade DDT and can be used as a source of the same.

5.3 Identification of DDT biodegrading isolates

5.3.1 Isolate 101.

Phylogenetic analysis of 16S rRNA gene sequence of isolate **101** showed that the isolate belongs to genera *Bacillus. Bacillus* represents a genus of Gram positive bacteria, rod-shaped, beta-haemolytic and a member of the division Firmicutes, Class Bacilli, Order Bacillales and Family Bacillaceae (Graumann *et al.,* 2007). *Bacillus* species are either obligate or facultative aerobes, and test positive for the enzyme catalase. They are ubiquitous in nature: soil, water, and airborne dust (Scheffers *et al.,* 2007). Some species are natural flora in the human intestines (Ryan *et al.,* 2004). A unique characteristic of these bacteria is their ability to produce endospores when

environmental conditions are stressful (Graumann *et al.,* 2007). The only other known spore producing bacterium is *Clostridium*. Isolate **101** adheres to all phenotypic traits of the genus *Bacillus* (**Plate 1, Page 50**) including the fact that they are gram positive, obligate or facultative, rod shaped and catalase positive (**Table 3, page 54**). These characteristics together with other biochemical properties distinguish isolate **101** as a member of the genus *Bacillus*. Although most species of *Bacillus* are harmless saprophytes, two species are considered medically significant: *B. anthracis* and *B. cereus.* Phylogenic analysis showed that Isolate **101** clustered with members of the genus *Bacillus.* This was supported by high bootstrap values of between 50-100 % (**Figure 14, page 58**). Other members of the genus *Bacillus* and other bacteria whose 16S rRNA gene sequences were 99 % similar to those of isolate **101** are unidentified *Bacillus sp*. FJ006883, *Bacillus thuringiensis strain* AB363741 and *Glacial ice bacterium* AF479333 (**Table 6, page 59**). In addition to other biochemical characteristics, isolate **101** was motile, hydrolyzed gel, fermented glucose, maltose, fructose, Lactose, dextrose, sucrose, cellobiose and mannose. This together with the phylogenetic position in the phylogenetic analysis and the high bootstrap values make it closer to *B. cereus*. Katayama *et al*. (1993) reported the isolation of two strains of bacteria, *Bacillus* sp. B75 and an unidentified Gram-variable rod Bl 16, which degraded DDT at the extremely low level of 10 pg /ml. Therefore, isolate **101** obtained from uncultivated areas (**Table 2, page 42**) degraded DDT and was a strain of *Bacillus cereus* in the genus *Bacillus*.

5.3.2 Isolates 102 and 110

Phylogenetic analysis of 16S rRNA gene sequence of isolates **102** and **110** showed that the isolates clustered with members of the genus *Staphylococcus. Staphylococcus* is a genus of Gram-positive bacteria, facultative anaerobes capable of growth both aerobically and anaerobically (Madigan *et al.,* 2005). *Staphylococcus species* can be differentiated from other aerobic and facultative anaerobic Gram positive cocci by several simple tests. All species grow in the presence of bile salts and are catalase positive (Madigan *et al.,* 2005). Growth also occurs in a 6.5 % NaCl solution. On Baird Parker Medium *Staphylococcus species* are fermentative, except for *S. saprophyticus* which is oxidative. Under the microscope, they appear round (cocci), and form in grape like clusters (Ryan *et al.,* 2004). *Staphylococcus species* are resistant to Bacitracin (0.04 µg resistance <10mm zone of inhibition) and susceptible to Furazolidone (100 µg resistance <15mm zone of inhibition). Most are harmless and reside normally on the skin and mucous membranes of humans and other organisms. Found worldwide, they are a small component of soil microbial flora (Madigan *et al.*, 2005). Most of the above biochemical and cellular characteristics of the genus *Staphylococcus* agree with those of isolates **102** and **110** (**Table 3, page 54; Plate 2 and 3, page 50**). Phylogenetically, 16S rRNA gene sequences analysis of isolates **102** and **110** shows that they cluster with members of the genus *Staphylococcus*. This was supported by high bootstrap values of between 50-100 % (**Figure 15, page 59**). Other members of the genus *Staphylococcus* and other bacteria whose 16S rRNA gene sequences were 98 % similar to those of isolate **102** and **110** are unidentified *Staphylococcus sp.* EU784844, *Uncultured rumen bacterium* EF436333, *Uncultured soil bacterium* AF423242 and *Staphylococcus*

lentus strain EF528296 (**Table 6, page 59**). However, the phylogenetic positions shown in the Phylogenetic analysis (**Figure 15, page 59**) indicate that isolates **102** and **110** cluster closely to *Staphylococcus sciuri*. Langlois and Collins (2000) also isolated *Staphylococcus species* in trypticase soy broth containing DDT. Therefore, isolates **102** and **110** obtained from both cultivated and uncultivated areas (**Table 2, page 42**) degraded DDT and are strains of *Staphylococcus sciuri* in the genus *Staphylococcus*.

5.3.3 Isolates 103, 104 and 105

Phylogenetic analysis of 16S rRNA gene sequence of isolates **103**, **104** and **105** showed that the isolates clustered with members of the genus *Stenotrophomonas.* Phylogenetic position of the three isolates shows that they cluster more closely with *Stenotrophomonas maltophilia* (**Figure 16, page 60**). This is supported by high bootstrap values of 50-100 %. *S. maltophilia* belongs to the Phylum Proteobacteria, Class Gamma Proteobacteria, Order Xanthomonadales, Family Xanthomonadaceae and Genus *Stenotrophomonas* (Vazquez *et al.,* 1995). *S. maltophilia* was initially classified as *Pseudomonas maltophilia* and grouped in the genus *Xanthomonas* before eventually becoming the type species of the genus *Stenotrophomonas* in 1993 (Palleroni *et al.,* 1993; Denton and Kerr *al.,* 2001). *S. maltophilia* is an aerobic, non fermentative, motile due to polar flagella, gram-negative, slightly smaller (0.7-1.8 x 0.4-0.7 μm) than other members of the genus (Gilligan *et al.,* 2003). They grow well on MacConkey agar producing pigmented colonies (Kerr *et al.,* 1998). *S. maltophilia* are catalase positive, oxidase negative which distinguishes them from most other members of the genus and have a positive reaction for extracellular DNase (Berg *et*

al., 1999; Al-Jasser, 2006). Most of the above cellular and biochemical characteristics of *S. maltophilia* agree with those of isolates **103, 104** and **105** (**Table 4, page 55**). 16S rRNA gene sequences of isolate **103**, **104** and **105** had a sequence similarity of 95 %, 97 % and 94 % respectively to 16S rRNA gene sequences of *Stenotrophomonas maltophilia, Stenotrophomonas maltophilia strain* 17A1, *Stenotrophomonas maltophilia strain* LMG 957, *Stenotrophomonas sp. Ellin*162, *Stenotrophomonas maltophilia strain* B25R, *Uncultured gamma proteobacterium clone Uncultured bacterium clone* P5D15-671, *Pseudomonas geniculata strain* XJUHX-18. However, the phylogenetic positions shown in the Phylogenetic trees (**Figure 16, page 60**) indicate that isolates **103, 104** and **105** cluster closely to *S. maltophilia.* The 16S rRNA gene sequence similarity of 95 %, 97 % and 94 % of isolates **103, 104** and **105** respectively to *S. maltophilia* (**Figure 16, page 60**) shows that the three isolates could be new species that cluster closely to *S. maltophilia.* Juhasz and Naidu (2000) in their Microbial analysis of contaminated soil and uncontaminated plant and faecal material resulted in the enrichment of a Strain AJR^{3}9, 504, isolated using DDT and peptone that closely resembled *S. maltophilia.* In addition, the degradation of DDT (100 mg 1^{-1}) by isolate AJR^{3}9, 504 resulted in a 35% decrease in DDT concentration after 28 days with a concomitant increase in DDD concentration. Peter *et al.* (1995) also found that *Stenotrophomonas maltophilia* obtained from a mixed microbial culture isolated from soil enrichments under aerobic and nitrogen-limiting conditions was capable of degrading DDT. Therefore, isolates **103, 104** and **105** obtained from both cultivated and uncultivated areas (**Table 2, page 42**) degraded DDT and are probably new species that cluster closely to *S. maltophilia* in the genus *Stenotrophomonas*.

5.4 Degradation of DDT by enrichment cultures and the isolates

5.4.1 Enrichment cultures from cultivated and uncultivated places

From the results, it was found that the enrichment culture from uncultivated places (**Figure 7b, Page 41**) degraded higher amount of DDT (60.20 %) than enrichment culture from cultivated areas (**Figure 7a, Page 41**) that degraded 38.58 %. The two isolates with the highest degradative rate, *Bacillus* isolate **101** and *Staphylococcus* isolate **102** (**Figure 11a** and **11c, Page 48**), were from uncultivated places and this explain the higher DDT degradation in uncultivated places. Of particular relevance to the degradation of DDT in soils are the presence and numbers of microbes with the ability to metabolise DDT, environmental factors which limit both growth and activity of the DDT metabolising microbes and access of the microbes to DDT (Dileep, 2008). Therefore, this study agreed with Dileep (2008) who found that degradation of DDT in soils is dependent on the presence and numbers of microbes in the soil with the required degradative ability. Although the six microbes isolated are evidence for the existence of DDT metabolising microbes in soils, their prevalence is unknown.

5.4.2 DDT degradation by the isolates

Figure 11, Page 48, shows that DDT was degraded by pure cultures of *Bacillus* isolate **101**, *Staphylococcus* isolates **102** and **110** and *Stenotrophomonas* isolates **103**, **104** and **105**. Degradation was measured by DDT disappearance using HPLC and appearance of metabolite, DDD. Degradation of DDT in pure culture from highest to lowest was *Staphylococcus* isolate **102** (58.08 %), *Bacillus* isolate **101** (44.31%), *Stenotrophomonas* isolate **103** (39.72%), *Stenotrophomonas* isolate **104** (30.33%),

Stenotrophomonas isolate **105** (28.97%) and *Staphylococcus* isolate **110** (28.48%). The mixed culture of the six isolates (**Figure 12, Page 49**) degraded 82.63 % of the initial amount of DDT, which was higher than the amount degraded by any individual isolate. Therefore, DDT degradation is greatly enhanced in a mixed culture perhaps due to their synergistic effect. These findings agreed with Aislabie *et al.* (1997) who found out that a mixed culture of two or more isolates increased the rate of DDT degradation. Degradation of DDT was a slow process as it took the isolates 31 days to degrade between 28.48 % to 58.08 % of the initial amount of DDT. This is in line with the fact that DDT persists in the environment. Juhasz and Naidu (2000) proposed that the tri Chlorine molecule is responsible for the resistance of DDT to degradation. DDT was degraded to DDD by the six isolates and none produced DDE (**Figures 8, Page 44; Figure 9, Page 45 and Figure 10, Page 46**). DDD is formed from DDT through reductive dechlorination, either microbially mediated (Wedemeyer, 1966) or as the result of chemical reactions (Wilson and Jones, 2000) some of which are mediated by biomolecules. DDE is formed from DDT through photochemical reactions in the presence of sunlight and through dehydrochlorination in bacteria (Pfaender and Alexander, 1991) and animals (Kurihara *et al.,* 1998). Under reducing conditions reductive dechlorination is the major mechanism for the microbial conversion of both the *o, p'*-DDT and *p, p*'-DDT isomers of DDT to DDD (Bushway and Hanks, 2006). A decrease in the concentration of DDT was observed when the isolates were grown in MM4 medium with DDT. The degradation of DDT by the isolates resulted in the concomitant increase in DDD concentration which accumulated in the medium. DDD has been identified as one of the major anaerobic transformation products of DDT (Aislabie *et*

al., 1997). Guenzi *et al.,* (1998) reported the accumulation of DDD in anaerobic soil systems. Transformation of DDT by sewage sludge micro-organisms resulted in the production of DDD (Pfaender and Alexander, 1991). DDD formation by the isolates probably resulted from the reduction dechlorination of the aliphatic part of the DDT molecule. Bogardt and Hemmingse (2006) proposed that the conversion of DDT to DDD by *A. aerogenes* occurred with the replacement of chlorine by hydrogen or hydride ion. In pure culture systems, dechlorination of DDT by *Escherichia coli*, *Enterobacter aerogenes*, *Eterobacter cloacae, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Aerobacter aerogenes* has been reported (Aislabie *et al.,* 1997). The reaction involves substitution of aliphatic chlorine for a hydrogen atom. Katayama *et al.,* (1993) propose that degradation of DDT involved two processes: The uptake of DDT into the cell and the transformation of DDT in the cell. The rate of uptake into the cells is unlikely to be important as DDT is extremely hydrophobic; rather, the rate at which the chemical is transformed in the cell would be the rate-limiting step.

This study shows that as the DDT peaks were reducing, the DDD peaks were increasing and at no particular point did the DDD peak start to decrease even with the mixed cultures (**Figures 8, Page 44; Figure 9, Page 45 and Figure 10, Page 46**). This shows that under the conditions, DDD was the only product hence this was not a complete break down of DDT to CO_2 or to non chlorinated compounds like phenylacetic, phenylpropionic, and salicylic acids. These findings agree with Alexander (1985) who found that in various ecosystems, microorganisms cause only modest changes in the DDT molecule. Pfaender and Alexander (1992) also reported that pure cultures of bacteria decompose DDT extensively only through a cometabolic process. Further, Bogardt and Hemmingse (2006) also found out that,

only the first step in the process, the reductive dechlorination of DDT to DDD can take place without an additional substrate, as was the case in this study. While using different growth medium like Skimmilk and trypticase soy broth, McAllister *et al.,* (2003) identified as many as eight products from the degradation of DDT depending on microbial species as well as availability of oxygen. The first products formed were DDD and smaller amounts of DDE. The major transformation products produced, DDD and DDE, are more toxic and recalcitrant than the parent compound. This is of concern as these compounds are metabolized slowly, if at all (Lai and Saxena 1999; Aislabie *et al.,* 1997).

5.5 Conclusion and Recommendation

5.5.1 Conclusion

- The first conclusion is that there are microorganisms in the soil that can degrade DDT. This study identified six DDT biodegrading bacteria.
- The second conclusion relates to the phylogenetic analysis which showed that isolate 101 was a member of the genus *Bacillus*. This was supported by the clustering pattern on the phylogenetic tree which indicated the isolate clustering with *Bacillus.* This clustering pattern was supported by high bootstrap values of between 50-100 % (**Figure 14, Page 58**). This result was further confirmed after blasting the results with accession numbers which showed isolate 101 relating to a member of the *Bacillus* genus with a sequence similarity of 99 % to *B. cereus*
- The third conclusion relates to the phylogenetic analysis which showed that isolate **102** and **110** were members of the genus *Staphylococcu*s. This was

supported by the clustering pattern on the phylogenetic tree which indicated the isolates clustering with *Staphylococcus*. This clustering pattern was supported by high bootstrap values of between 50-100 % (**Figure 15, Page 59**). These results were further confirmed after blasting the results with accession numbers which showed isolates **102** and **110** relating to members of the *Staphylococcus* genus with a sequence similarity of 98 % to *Staphylococcus sciuri*

- The fourth conclusion also relates to the phylogenetic analysis which showed that isolate **103, 104** and **105** were members of the genus *Stenotrophomonas*. This was supported by the clustering pattern on the phylogenetic tree which indicated the isolates clustering with *Stenotrophomonas.* This clustering pattern was supported by high bootstrap values of between 50-100 % (**Figure 16, Page 60**). These results was further confirmed after blasting the results with accession numbers which showed isolates **103, 104** and **105** relating to members of the *Stenotrophomonas* genus with a sequence similarity of 95 %, 97 % and 94 % respectively to *Stenotrophomonas maltophilia.* This suggests that the three isolates could be new species.
- The fifth conclusion is that the rate of degradation of DDT in soils is dependent on the presence and numbers of microbes in the soil with the required degradative ability and environmental factors which limit both growth and activity of the DDT metabolising microbes and access of the microbes to DDT. This was supported by the presence of isolates **101** and **102** in the enrichment cultures from uncultivated areas (**Table 2, Page 42**). Their presence resulted into a higher rate of DDT degradation in the

enrichment cultures from uncultivated areas compared to those from cultivated areas (**Figure 7, Page 41**).

- The sixth conclusion is that the rate of DDT degradation by the mixed culture is higher than that of the pure culture. This is supported by the enhanced DDT degradation in the mixed culture (**Figure 12, Page 49**) compared to pure cultures (**Figure 11, Page 48**)
- Complete degradation of DDT is a cometabolic activity and only the first step, the conversion of DDT to DDD can take place without an additional carbon source. This was shown by the degradation of DDT to DDD only, in a media where DDT was the only carbon source (**Figures 8, Page 44; Figure 9, Page 45 and Figure 10, Page 46**).

5.5.2 Recommendations

- It is important to establish the lowest induction levels for biodegradation of DDT by microorganisms. In this study, MM4 media with DDT at a concentration of 100 ppm was used where growth and DDT biodegradation was observed. DDT in the environment may be at a lower concentration hence the need to find the lowest induction levels.
- A selective media for the isolates should be used and test both their prevalence in the environment and rate of DDT degradation. In this study, a general approach was used to enrich and isolate DDT biodegraders. Since their identity has been established, a selective media and optimum growth conditions should be used.

- The ability of the isolates to completely biodegrade DDT to CO_2 or to non chlorinated compounds should be assessed using cometabolism where an additional carbon source should be explored. Their pathway for DDT degradation should be elucidated. In this study, DDT was the only carbon source hence degradation of DDT to DDD only.
- It is important to determine the effect of DDT on microbial diversity.

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APPENDIX

Appendix A: Biochemical and other characteristics of *S. maltophilia from* Clinical Microbiology Reviews

Test	Reaction
Indophenol oxidase	−
Catalase	+
Growth	
5°C	−
18°C	+
37°C	+
Motility	
18°C	+
37°C	v
Indole	−
Lysine decarboxylase	+
Ornithine decarboxylase	−
Methyl red	−
Voges-Proskauer	−
Hydrogen sulfide	−
Reduction of nitrate to nitrite	v
Citrate	v
Phenylalanine deaminase	−
β-Galactosidase (ONPG)	v
Hydrolysis	
Esculin	+
Gelatin	+
Tween 80	+
DNA	+
Starch	−
Urea	−
Carbon sources for growth	
Adonitol	−
Arabinose	−
β-Hydroxybutyrate	−
Cellobiose	v
Dulcitol	−
Glucose	+
Fructose	v
Galactose	v
Mannitol	−
Mannose	v
Rhamnose	−
Salicin	−
Sorbitol	−
Trehalose	−

+, >85% strains positive; v, 16 to 84% strains positive; −, ≤15% strains positive

Appendix B. SL11 (Trace element solution)

Salts	Mg/L
$FeC_{2.}4H_2O$	2000
$ZnCl_{2.}$	70
$MnCl_{2.}.4\ H_2O$	100
$CuCl._{2.}\ H_2O$	2
$NiCl.\ H_2O$	24
$Na_{2.}Mo0_4.2\ H_2O$	36
H_3BO_3	6
$CoCl_{2.}6\ H_2O$	190
$EDTA.Na_{2.}^{2}$	5.2g
H_2O	1000 ml

Eichler and Pfening (1986) Bergeys manual of systematic bacteriology, vol 3.