ISOLATION AND MOLECULAR CHARACTERIZATION OF LOW DENSITY POLY-ETHENE DEGRADING BACTERIA AND FUNGI FROM DANDORA DUMPSITE-NAIROBI

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A Thesis Submitted to Pan African University, Institute of Science, Technology and Innovation in partial fulfilment of the requirement for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology.

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DECLARATION

This thesis is my original work and has not been presented in any other university or institution for the award of a degree.

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DEDICATION

This work is dedicated to my dear family; my husband Vinscent Andika, children; Gideon, Joy, Dorris and Kate. I appreciate the support you have accorded me during the course of my studies. Without your encouragement and understanding, this journey would have been longer and tougher. Lastly but not least, I also dedicate this piece of work to my dear mother Agnes Ndahebwa, whose enduring love and constant prayers was and still is my source of strength.

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MAY GOD BLESS YOU ALL!

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LIST OF ACRONYMS/ABBREVIATIONS

BLAST	Basic Local Alignment Tools
CH_4	Methane gas
CO_2	Carbon dioxide gas
DNA	De-oxy ribonucleic acids
FT-IR	Fourier Transform Infrared spectroscopy
GC-MS	Gas Chromatography-Mass Spectroscopy
GEM	Genetically Engineered Microorganism
GMM	Genetically Modified Microorganism
H_2O	Water
HCl	Hydrochloric acid
HDPE-	high density poly-ethene
HPLC-	High Performance Liquid Chromatography
LDPE	Low Density Poly-Ethene
LLDPE	linear low density poly-ethene
MDPE-	medium density
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology information
OD	Optical Density
PHA	Polyhydroxyalkanoates
PCR	Polymerase Chain Reaction
RNA	ribonucleic acids
rRNA	ribosomal RNA
SEM	Scanning Electron Microscopy
TLC	Thin Layer Chromatography

ABSTRACT

Dandora dumpsite is Nairobi's official main dumpsite and is close to the city's informal settlements. This study aimed at isolating and identifying bacteria and fungi with the capacity to degrade low density poly-ethene (LDPE) from the dumpsite. The level of biodegradation of LDPE sheets with bacterial and fungal inoculums from different sampling points was evaluated under laboratory conditions of 37˚C and 28˚C for bacteria and fungi respectively for sixteen weeks in a shaker incubator. Thirty bacterial isolates and 26 fungal isolates were isolated based on biodegradation outcomes. Twenty bacterial isolates and 10 fungal isolates were identified using 16S rDNA and 18S rDNA sequences for bacteria and fungi respectively. Bacteria of genus *Pseudomonas, Bacillus, Brevibacillus, Cellulosimicrobium, Lysinibacillus* and fungi of genus *Aspergillus* were implicated as poly-ethene degraders. The extent of biodegradation on the poly-ethene sheets was assessed by weight loss analysis, Fourier Transform Infrared Spectroscopy (FTIR) and GC-MS. The spectral analysis of the FTIR outcomes revealed appearance of aldehydes, ether and carboxyl functional groups on the poly-ethene sheets while GC-MS outcome indicated presence of a ketone which is an intermediary product in the culture media. An overall analysis confirmed that fungi are generally better degraders of poly-ethene than bacteria. The highest fungal degradation activity yielded a mean weight loss of 36.4±5.53 % attributed to *Aspergillus oryzae* strain A5, 1 (MG779508) while the highest degradation activity for bacteria had a mean of 35.72±4.01% and 20.28±2.30% attributed to *Bacillus cereus* strain A5,a (MG645264) and *Brevibacillus borstelensis* strain B2,2 (MG645267) respectively. The isolates were screened for their ability to produce extra cellular enzymes and the fungal isolates *Aspergillus fumigatus* strain B2,2 (MG779513) and *Aspergillus oryzae* strain A5,1-(MG779508) were confirmed to produce the highest laccase activity and esterase activity respectively. Investigation for the presence of genes that are responsible for the production of alkane degrading enzymes was done and the primer set Alkb1 was able to amplify the fragment of size 870 bp in 4 bacterial and 18 fungal samples. Presence of this gene in LDPE degrading bacteria and fungi is an indication of the inherent ability of these microorganisms to take

part in the bioremediation process since alkanes are major constituent of LDPEs. The optimum growth of the bacterial isolates at 600nm was found to be a temperature of between 30-40˚C, pH 6-8 and sodium chloride concentration of 0 while fungal optimum growth was at: temperature 30˚C, pH 8 and sodium chloride concentration of 0. From this study, it is notable that fungi and bacteria capable of degrading LDPE can be isolated from dumping sites of these materials and that *Aspergillus oryzae* strain A5, 1 and *Bacillus cereus* strain A5,a can be used in the bioremediation of poly-ethene from the environment. The possession of AlkB 1 gene for production of alkane degrading hydroxylase confirms the molecular basis for their LDPE degrading capacity which is further supported by their ability to produce enzymes implicated in this process. These, coupled with the right growth conditions may serve to better utilize microbes in the bioremediation of LDPE. We recommend further optimization of culture conditions for optimum activity of microbes that show potential of degradation especially where co-culturing is involved. Region wise bioprospecting for LDPE biodegrading microorganisms could lead to discovery of more and better degraders which may enable this kind of bioremediation to be done on a larger scale. Further, the knowledge of genes present in the identified microorganisms responsible for producing different polymer degrading enzymes will inform the use of the right microbial consortia in biodegradation processes. The use of recombinant DNA technology to insert genes that produce LDPE degrading enzymes into good degraders will serve to further improve their degrading potential.

CHAPTER ONE

INTRODUCTION

1.1: Background Information

Artificial polymers are very stable, and do not readily get assimilated into the natural degradation cycles of the earth (Shimao, 2001). Environmental pollution by artificial polymers like waste plastics and water-soluble artificial polymers in polluted waters has been identified as a large environmental problem due to unsustainable disposal strategies. Despite the presence of plastics in nature for a reasonable amount of time, evolution has not been able to design suitable enzyme structures capable of degrading them(Müller, 2005). Petroleum plastics fall in this category of non-biodegradable artificial polymers and they accumulate at the rate of 25 million tons each year, contaminating the soil and water as documented by Eubeler *et al*., (2009). Low Density Poly-ethene belongs to thermoplastics class (Pramilla and Ramesh, 2015) and is believed to have non-degradable nature due to hydrophobic backbone (Myint & Ravi, 2012). This has forced many governments to come up with measures to curb this menace. Bangladesh, for instance, imposed a ban on plastic bags in March 2002 following flooding caused by blockage of drains (EPHC, 2002) and most recently Kenya also imposed a ban on plastic carrier bags from September 2017 (Government of Kenya (GoK), 2017)

The artificial polymers are thus dumped into landfills, incinerated or recycled. Despite the fact that incineration burns off the plastic waste completely, it leads to heavy toxic

fume generation (Lettieri & Baeyens, 2009). Recycling is an environmentally-attractive solution, but the percentage of plastics that can be economically recycled is limited and the bulk ends up in land-fills (Lettieri & Baeyens, 2009; Bhatia *et al*., 2014). Thus, there is a need to develop an 'environment friendly' degradation solution. Microorganism–enhanced degradation of artificial plastics particularly fungi and bacteria has been reported to cause structural changes to the polymers (Myint & Ravi, 2012; Pramilla & Vijaya, 2015).

Biodegradation is defined as the process by which complex chemical compounds are biologically reduced or the process by which organic substances are broken down into smaller compounds by living micro-organisms (Marinescu & Dumitru, 2009). For bioremediation to take place, the microorganisms must be able to attack the pollutant through releasing suitable active enzymes (Müller, 2005). This is a complex process which involves several steps (Shah *et al*., 2008): bio-deterioration (the combined action of microbial communities and abiotic factors to fragment the materials into tiny fractions), depolymerization (Microorganisms secrete enzymes and free radicals able to cleave polymer into oligomers, dimers and monomers, assimilation (some molecules are identified by the cellular receptors of microbes and can go across the cell membrane) and mineralization (simple molecules as CO_2, N_2, CH_4, H_2O and different salts from intracellular metabolites that are completely oxidized are released). At least two groups of enzymes are actively involved in biological breakdown of polymers: extracellular and intracellular depolymerases (Gu, 2003).

Since poly-ethene (PE) is widely used as packaging material, considerable investigations not only on production of biodegradable poly-ethene version but also on biodegradation of poly-ethene has been done (Bonhomme *et al*., 2003; Zhang *et al*, 2004). Biodegradation of poly-ethene has been known to occur by two mechanisms: hydro-biodegradation and oxo-biodegradation (Bonhomme *et al*., 2003). These two mechanisms rely on the modifications due to the two additives, starch and pro-oxidant, used in the synthesis of biodegradable poly-ethene. Starch blend poly-ethene which has a continuous starch phase that makes the starch hydrophilic and therefore, can be attacked by amylase enzymes. Microbes can easily access, attack and remove this part. Hence the past attempts to achieve biodegradation of poly-ethene have depended largely on pre-treatment of the polymer.

Researchers in an attempt to bring about biodegradation of non-pre-treated poly-ethene have isolated and used some bacterial genera from different sources that indicated potential for poly-ethene degradation. These included genera, *Pseudomonas, Acinetobacter, Brevibacillus, Rhodococcus and Micr*ococcus (Talkad *et al*., 2014; Hadad *et al*, 2005; Pramilla and Ramesh, 2015; Nanda & Sahu, 2010; Nowak *et al*., 2011 respectively). Fungal genera, *Gliocladium, Cunninghamella, Penicillium*, *Aspergillus*, *Fusarium, Mucor* and *Mortierella* from soil were also subjected to poly-ethene and showed degradation potential (Nowak *et al*., 2011) From these studies, the most commonly implicated genera in the biodegradation of poly-ethene are *Pseudomonas, Aspergillus* and *Penicillium* (Myint & Ravi, 2012). The biodiversity of Low Density Poly-Ethene (LDPE) degrading microorganisms varies in different

geographical regions as a result of varying environmental conditions hence the need to determine the available LDPE degraders in different environments.

Numerous microorganisms such as bacteria and fungi, are capable of degrading different petroleum products under different environmental conditions (e.g., aerobic and anaerobic conditions, at varied salinities and pH). The degradation occurs gradually by sequential metabolism of its compounds dictated by the enzymatic capabilities of the microorganisms. The genes involved in degrading petroleum enzyme production may be located on chromosomal or plasmid DNA (Broderick, 1999). Under aerobic conditions, oxygenase enzymes introduce oxygen atoms into hydrocarbons (monooxygenases introduce one oxygen atom to a substrate while dioxygenases introduce two (Cao *et al*., 2009). Alkane hydroxylases are alkane-degrading enzymes that are distributed among many different species of bacteria, yeast, fungi, and algae (Jan & Funhoff, 2007). Furthermore, Jan & Funhoff, (2007) proposed three categories of alkane-degrading enzyme systems: C1–C4 (methane to butane, oxidised by methane-monooxygenase-like enzymes), C5– C16 (pentane to hexadecane, oxidised by integral membrane nonheme iron or cytochrome P450 enzymes), and C17+ (longer alkanes, oxidised by essentially unknown enzyme systems). These authors also noted that microorganisms that are able to degrade alkanes can contain multiple alkane hydroxylases and can thus consume different substrate ranges. As cited by Van Hamme *et al*., (2003), to date, one of the most studied alkane degradation pathways is that described for Pseudomonas putida Gpo1, encoded by the OCT plasmid (van Beilen *et al*., 2001). In this case, the conversion of an alkane into an alcohol is first mediated by a membrane monooxygenase

(Van Hamme *et al*., 2003). Despite the fact that petroleum degradation under aerobic conditions occurs faster than under anaerobic conditions, it is notable that anaerobic degradation is also essential to the bioremediation process because in several cases the environmental conditions can include limitations of the oxygen availability, such as in mangroves, aquifers, and sludge digesters (Peixoto *et al*., 2011).

In Kenya for instance there is need to map out the different effective biodegraders in the different climatic zones as this will inform which microorganisms can be applied successfully in different localities. Kenya has embraced the 3R, Reduce, Recover and Recycle concept of solid waste management. Sustainable plastic/ poly-ethene waste management would be the lasting solution to this menace that is not only for Kenya but Africa and the whole world.

1.2: Problem Statement

Currently, incineration, burying in landfills and recycling are applied as disposal strategies of the plastic wastes produced in Kenya and around the world. When we resort to these methods of poly-ethene disposal as it has been the case, each of them comes with its own limitations and hence the problem persists. Incineration for instance burns off the plastic waste, but at the same time causes heavy toxic fume production (Lettieri & Baeyens, 2009; Crowley, 2003). Recycling is a very environmentally-attractive solution, but a very small fraction of the plastics is recycled while the remaining goes to the landfills (Bhatia *et al*., 2014). Dioxins produced from plastic incineration settle on the crops and in our waterways where they eventually enter into our food and hence the

body system. Its worst component, 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD), causes cancer and neurological damage (Verma *et al*., 2016). Plastics take 20 to 1000 years to break down hence when they are buried underground, and this occurs very often, then it means that the soil will be poorly aerated and therefore lead to death of microorganisms in soil. Thus, there is a need to develop an 'environmentally friendly' degradation solution.

Littering of plastic bags is associated with many environmental problems"(Baud, Post, & Furedy, 2016): Firstly, it causes visual pollution that affects sectors like tourism. Secondly, plastic wastes block gutters and drainage systems creating serious flowing water problems such as city flooding. Third, consumption of plastic bags by livestock causes death or when plastic wastes find their way into the sea and other water bodies, they kill aquatic wildlife upon ingestion by the animals which mistake them for food. Finally, the persistence in soil leads to poor aeration hence a threat to the soil microorganisms. According to Aurah, (2013), even though supermarkets and other market outlets in Kenya give "free" plastic bags to customers, in reality they are not free as the cost of the effects of poly-ethene in the environment are much higher than the poly-ethene production costs. In an effort to develop environmentally friendly poly-ethene disposal methods, researchers have resorted to the strategy of bioremediation where microorganisms are used to deplete these artificial polymers. The setback here has always been the rate at which the process takes place that is too slow for sustainable large scale use (Hadad *et al*., 2005). There is therefore need to increase bio-prospecting efforts to get more suitable microorganisms from our local environments that are better

adapted to carry out biodegradation processes. The use of a combination of the local LDPE degrading isolates could also improve the biodegradation potential.

1.3: Justification

According to the Kenya Vision 2030 strategy, the solid waste management systems in 5 leading municipalities and in the economic zones will require tightening regulations in order to limit production and usage of environmentally-detrimental plastic bags. Despite this vision, the importance of poly-ethene bags cannot be overemphasized and hence there usage and production continues. To reduce the problem of environmental pollution by LDPEs in a sustainable way, more environmental friendly approaches have to be applied. The use of microorganisms through the process of bioremediation is a preferred approach since it poses no threat to other life forms in the environment. In the natural environment, mixed cultures of microorganisms exist in addition to a mixture of carbon sources for the microbes. Under experimental conditions, the use of mixed pure cultures of microorganisms and the LDPE as the carbon source is expected to increase the rate of biodegradation of the substrate. A combination of LDPE degrading microorganisms from different sampling points under the right conditions could serve as a source of better degradation effectiveness.

There is need to understand the local, available genera of bacteria and fungi from the dumpsite, their optimal growth conditions and their contribution to the process of biodegradation of LDPEs since this data is currently scanty.

As recommended by Sangale *et al*., (2012), the status of poly-ethene pollution should be updated area-wise and the awareness campaign of the poly-ethene pollution should be promoted at mass level to the public. The microbes responsible for the degradation of poly-ethene should be isolated from all the sources and screened to know the efficient isolates. A combination of various genes that code for LDPE degrading enzymes through genetic manipulation into one host is expected to give rise to a candidate that has a greater biodegrading potential than the native isolates. Other than striving to degrade LDPEs in an environmentally friendly way, this study will also aim at assessing the environmental impact of the products of this whole process on living organisms and this will ensure that the approach is safe and sustainable.

The identification of isolates which are promising for application in LDPE degradation is a good step in the waste management sector as this will enable the use of environmentally friendly strategies of waste disposal. The ability of the microorganisms to produce enzymes involved in polymer degradation such as laccase and esterase can also be applied further through crude enzyme extraction which can also be incubated with the polymers under suitable conditions to bring about biodegradation. Optimum conditions of temperature, pH and salt concentration for incubation of the individual isolates are an important finding which when factored during the application of these microbes in bioremediation will ensure maximum activity. Application of known pure cultures of microorganisms under controlled experimental conditions subjected to use LDPE as their sole carbon source will make bioaugmentation with local microbes a success that can be considered for industrial use.

1.4: Objectives

1.4.1: General objective

To isolate, identify and carry out molecular characterization of LDPE degrading aerobic bacteria and fungi from Dandora dumpsite.

1.4.2: Specific objectives

1. To isolate LDPE degrading fungi and bacteria and determine their degrading effectiveness using physicochemical properties.
2. To identify the isolates at molecular level and determine their phylogenetic positions in relation to their taxonomically close relatives from the nucleotide sequence databases.
3. To screen for the presence of selected biodegrading enzymes in the effective bio-degraders
4. To determine suitable culturing conditions for effective biodegraders.

CHAPTER TWO

LITERATURE REVIEW

2.1: Bioremediation

Bioremediation is generally defined as the process by which living organisms, primarily microorganisms are used to degrade environmental contaminants hence reducing their concentration in the environment or convert toxic environmental contaminants into less toxic forms (Tehri *et al*, 2011). It uses naturally occurring bacteria or fungi to degrade or detoxify substances hazardous to human health or/and the environment. For bioremediation to take place, the microorganisms must be able to attack the pollutant through releasing suitable active enzymes. Most polymers are too large to pass through cellular membranes, so they must first be broken into smaller monomers before they can be assimilated and biodegraded within microbial cells (Mueller, 2006). At least two groups of enzymes are actively involved in biological breakdown of polymers: extracellular and intracellular depolymerases (Gu, 2003; Bamforth & Singleton, 2005). During degradation, exoenzymes from microorganisms released into the surrounding break down complex polymers yielding smaller molecules of short chains e.g., oligomers, dimers and monomers that are small enough to pass the semi-permeable outer bacterial membranes. The process is called depolymerization (Müller, 2005; Mueller, 2006; Mudasir & Uqab, 2016). These intermediary products are then utilized as carbon and energy sources (Sahadevan *et al*, 2013) through a process called process is called mineralization. The end products of mineralization are CO_2, H_2O, or CH_4 (Hamilton, Reinert, Hagan, & Lord, 2014) as shown (**Fig 2.1).**

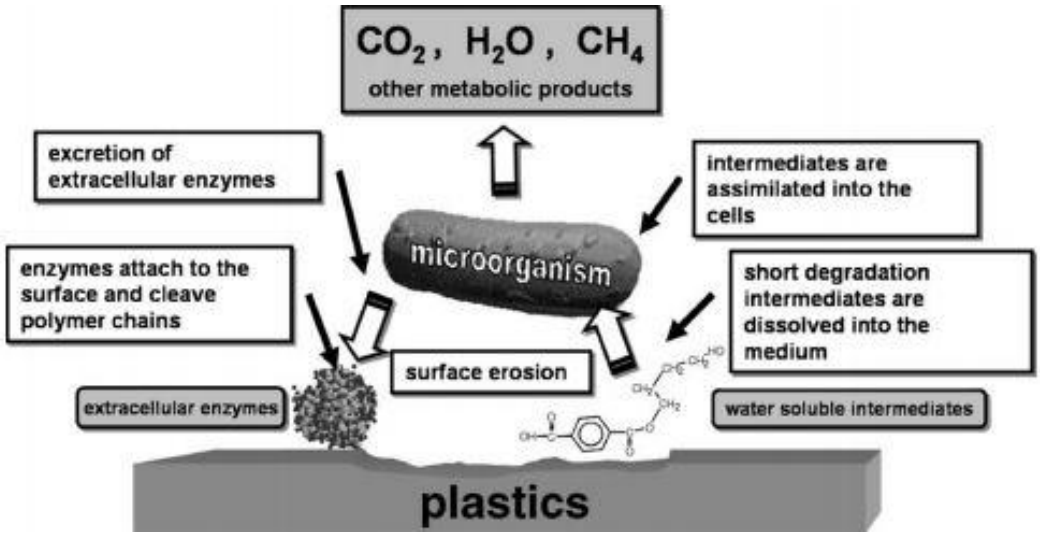


Figure 2.1: A flow chart of biodegradation of polymers in the environment (Mueller, 2006).

Bioremediation strategies include natural attenuation, biostimulation and bioaugmentation (Abdulsalam & Omale, 2009; Camargo & Okeke, 2005). Biostimulation involves identifying and adjusting physical/chemical factors such as soil temperature, pH, moisture content, nutrient content etc. that may slow down the rate of biodegradation of the pollutant by the indigenous microorganism in the affected site (Singh *et al*., 2017; Adams *et al*., 2015; Hassanshahian *et al*., 2014). There are many limiting factors to hydrocarbon biodegradation in soil including nutrients, pH, temperature, moisture, oxygen, soil properties and contaminant presence. The environment can be modified to stimulate existing bacteria capable of bioremediation through addition of various forms of limiting nutrients and electron acceptors, such as phosphorus, nitrogen, oxygen, or carbon, which otherwise are present in quantities low enough to constrain microbial activity (Adams *et al*., 2015). Bioaugmentation is the inoculation of an already enriched microbial consortium into the polluted site (Adams *et*

al., 2015; Hassanshahian *et al*., 2014). The rationale behind this method is that indigenous microbial consortia may not be capable of degrading the wide range of pollutants at present at the site or when the indigenous hydrocarbon-degrading population slow the speed of decontamination. Bioaugmentation with native microorganisms has been proved to be much more effective than with foreign microbial consortia (Camargo & Okeke, 2005). Natural attenuation on the other hand is soil's natural ability to degrade the contaminant (Hamilton *et al*., 2014; Abdulsalam & Omale, 2009). Optimization of biodegradation conditions can be achieved through knowledge of the characteristics of the contaminated site before beginning treatments. Basic information such as residual oil concentration, population density of the oil-degrading microorganisms and the biodegradation potential, are important factors to be considered for bio-remediation of hydrocarbon polluted sites.

2.2: Negative effects of poly-ethene accumulation in the environment

Worldwide, the harmful effects of poly-ethene have been on the rise prompting many governments to even ban the production of these material. Australia, Italy, United States of America, Tanzania, Ireland and some other countries have enacted laws to impose tax or to ban the production and use of poly-ethene bags for domestic and commercial purposes to try and reduce on the adverse environmental negative impacts the whole world is now facing (Ahmed & Gotoh, 2005; Jalil & Nannu 2013). Harmful environmental effects of plastics and poly-ethene include but are not limited to the following. Billions of poly-ethene bags are used by shoppers each year and far too many of these make their way into the streets and the countryside as unsightly litter (EU DG, 2011). This visual pollution affects sectors like tourism hence the economy is equally affected. Plastic bags can choke or poison fish, animals and birds when

ingested. When seabirds, sea mammals or fish ingest plastic particles, they are likely to block the gut and even kill the organism. .Birds can mistake the bags for fish or nesting materials (EU DG, 2011; Ahmed & Gotoh, 2005). They use up finite natural resource including oil, in their production. Up to 100 million barrels of oil are needed to make the world's plastic bags each year yet typical usage of a plastic bag is just 20 minutes (Oluwatosin *et al*., 2014).

In agriculture, the presence of poly-ethene bags which get into the soil either through land filling or careless disposal have immense impacts on agricultural activities. Their accumulation in soil cause difficulty to the development of plant roots and also reduce aeration of soil. Because poly-ethene takes long to degrade, those substances that are covered by poly-ethenes in the soil do not easily undergo composting(North & Halden, 2013; Grover *et al*., 2015) .The consumption of plastics in Kenya has increased to 4,000 tons per annum of poly-ethene bags which together with hard plastics end up scattered in the environment creating an eyesore commonly called "the plastics menace" (Baud *et al*., 2016). Littering of plastic bags is associated with many environmental problems in Nairobi city: Plastic wastes block the city's gutters and drainage systems creating serious flowing water problems leading to city (Aurah, 2013). Other adverse effects of poly-ethene waste occur as a result of the conventional methods of disposal that have been used for a long time and continue to be used even today (Verma *et al*., 2016).

2.3: Biodegradation of Poly-ethene

Biodegradation is controlled by a number of factors that include polymer properties, type of organism, and nature of pretreatment (Mahalakshmi *et al*., 2012). The polymer characteristics such as molecular weight, the type of functional groups and substituents

present in its structure play an important role in its degradation (Esmaeili *et al*., 2013). Since poly-ethene (PE) is widely used as packaging medium, considerable work not only on biodegradable poly-ethene but also on biodegradation of poly-ethene has been done (Bonhomme *et al*., 2003; Zhang *et al*, 2004). These studies have indicated that poly-ethene is biodegraded following pretreatment i.e. photodegradation and/or chemical degradation (Mahalakshmi *et al*., 2012; da Luz *et al*., 2013). The two mechanisms by which the process occurs are: hydro-biodegradation and oxo-biodegradation (Bonhomme *et al*., 2003; Kawai *et al*., 2004). These two mechanisms are based on modifications due to addition of starch or pro-oxidant, used in the synthesis of biodegradable poly-ethene. Starch blend creates a continuous starch phase that makes the material hydrophilic and therefore, catalyzable by amylase enzymes, a property which the non-pretreated poly-ethene lacks (Antony & Govt, 2015). Microorganisms can easily access, attack and remove this part. In case of pro-oxidant additive (**Fig 2.2**), biodegradation occur following photo-degradation and chemical degradation (da Luz *et al*., 2013; Koutny *et al*., 2007). If the pro-oxidant is a metal combination, after transition, metal catalyzed thermal peroxidation and biodegradation of low molecular weight oxidation products occurs sequentially (Yamada-Onodera *et al*., 2001). EI-Shafei *et al*., (1998) investigated the ability of fungi and specific bacterial strains to attack degradable poly-ethene consisting of disposed poly-ethene bags with 6% starch. They also isolated 8 different strains of Streptomyces and two fungi *Mucor rouxii* NRRL 1835 and *Aspergillus flavus*. The non-pretreated poly-ethene is by nature hydrophobic making microbial attachment difficult (Gu, 2003; Haizhen *et al*., 2009).

However researchers have found out that it is also possible for microbial degradation of poly-ethene to occur without pretreatment (Yamada-Onodera *et al*., 2001). In a study where low density poly-ethene pieces buried in soil mixed with sewage sludge were examined microscopically after 10 months incubation, fungal attachment was found on the surface of the plastic, an indication of possible utilization of plastic as a nutrient source (Shah *et al*., 2008). The isolated fungal strains were identified as *Fusarium sp*. AF4, *Aspergillus terreus* AF5 and *Penicillum sp*. AF6. The ability to form a biofilm on poly-ethene was attributed to the gradual decrease in hydrophobicity of the poly-ethene surface with time (Gilan *et al*., 2004). This is a clear indication that bacteria and fungi can utilize poly-ethene as a carbon source even in the midst of other carbon sources hence the need to investigate the efficiency of poly ethene utilization by these microorganisms in the absence of other carbon source. In a study by Bonhomme *et al*., (2003), evidence by scanning electron microscopy (SEM) indicated that microorganisms (fungi) build up on the surface of the non-pretreated polymer (poly-ethene) and after removal of the microorganisms, the surface became physically pitted and eroded.

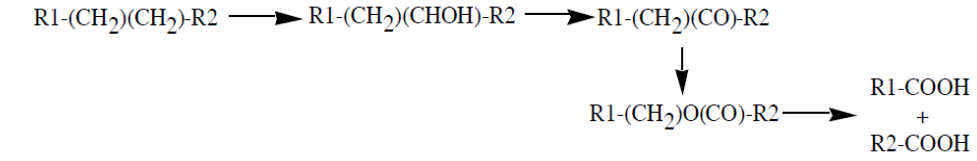
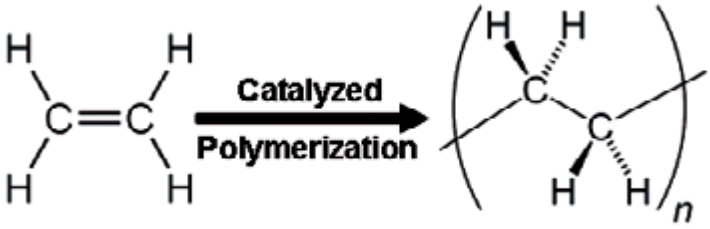


Figure 2.2: Flow chart of poly-ethene degradation with prior oxidation (Harayama, Kasai, & Shutsubo, 1999)

2.4: Structure and classification of poly-ethenes

Poly-ethene forms about 64% of all the artificial plastic waste produced worldwide (Shimao, 2001; Nowak *et al*., 2011). Its widespread use in almost all sectors of the economy is attributed to its strength, lightness, resistance to water and most water-borne microorganisms (Shah *et al*., 2008). Plastics have replaced paper and other cellulose-based products for packaging due to their properties. It has been reported to be the most commonly used non-degradable solid waste. Poly-ethene which is a linear hydrocarbon polymer consisting of long chains of the ethylene monomers (C_2H_4) has a general formula of C_nH_{2n}, where 'n' is the number of carbon atoms (Sangale *et al*., 2012). Poly-ethene is made from the cheap petrochemical stocks extracted from oil or gas through efficient catalytic combination of the monomers (Orhan & Bu, 2000) as shown below.



A variety of different poly-ethene types has been developed based mostly on density of the material and branching of the intrinsic molecular chains. The most widely used plastics used in packaging are low density poly-ethene (LDPE), medium density poly-ethene (MDPE), high density poly-ethene (HDPE), linear low density poly-ethene (LLDPE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polyurethane (PUR), poly-ethene terephthalate) (PET), polybutylene terephthalate (PBT)

and nylons (Khanam & Almaadeed, 2015; Ojha *et al*., 2017). Because of their durability and visibility in litter, poly-ethene has attracted more public and media attention than any other component of the solid waste stream. Poly-ethene materials are classified according to density as either LLDPE (0/9170-0.9200 g/cm^3), LDPE (0.9200-0.9250 g/cm^3), MDPE (0.9260-0.9400g/cm^3); or HDPE (>0.941g/cm^3) (Khanam & Almaadeed, 2015). The low density poly-ethene is the most widely used (Pramilla & Ramesh, 2015; Myint & Ravi, 2012). Poly-ethene waste is released during all stages of production and after consumption; hence every poly-ethene product is a waste.

2.5: Evolution of poly-ethene use and statistics

In 1993, the total world demand for plastics was over 107 million tones and it was estimated about 146 million tons in 2000. As the demand, production and utilization of plastics continues to rise, the disposal challenge continues to loom. With continuous growth for more than 50 years, global production in 2012 rose to 288 million tons (**Fig 2.3**), a 2.8% increase compared with 2011 (Antony & Govt, 2015). This global figure has been increasing by at a steady rate since 1950 to a peak of 245 million tons in 2008 (EU DG, 2011).

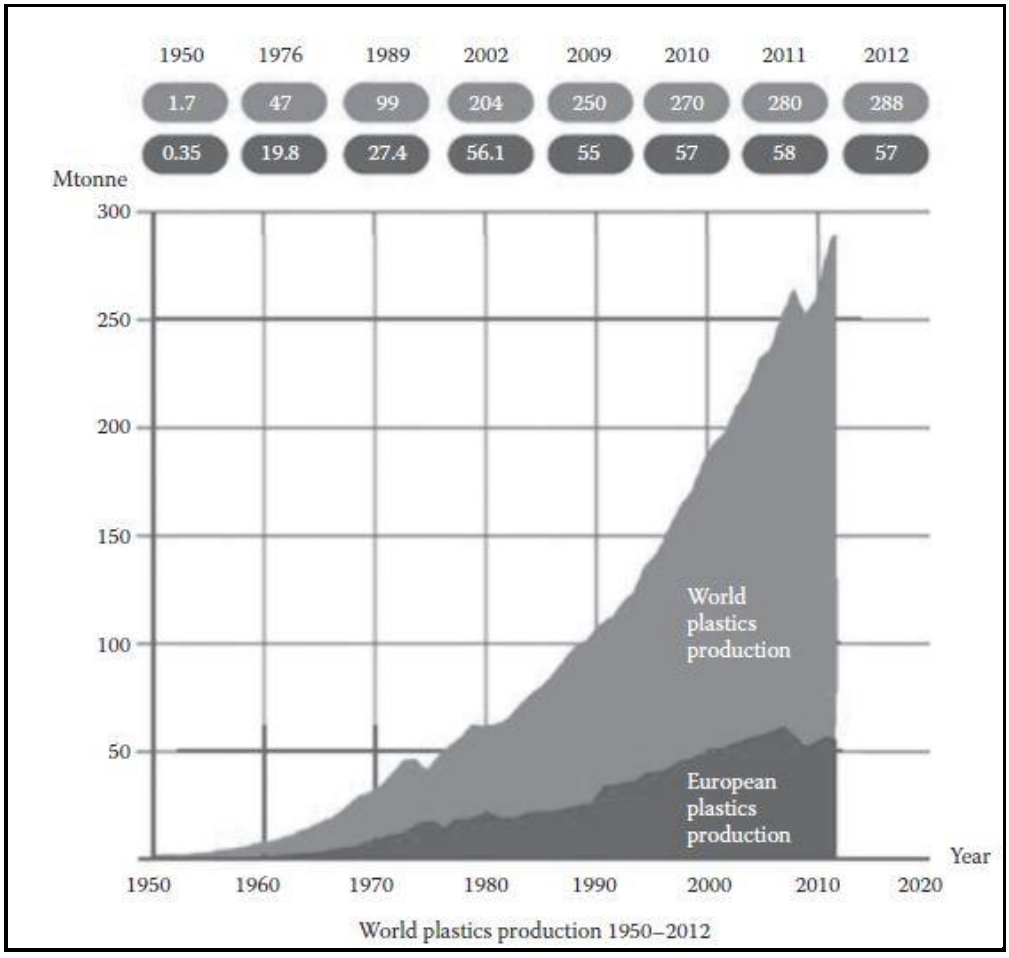


Fig 2.3: World plastics production Statistics (EU DG, 2011)

2.6: Conventional Methods of Poly-ethene disposal and Limitations

The increasing production and consumption of plastic products is consequently causing a proportional increase in plastic waste (UNEP, 2009). Mostly, plastic is thrown away after usage; and being durable they persist in the environment. Plastic waste is normally disposed off through landfilling, incineration and recycling (Aurah, 2013). 10-12% of the Municipal Solid Waste which is plastic is incinerated, releasing toxic gases into the environment which contains substances like Dioxins, Furans, Mercury and Polychlorinated Biphenyls (Verma *et al*., 2016; Crowley, 2003). Dioxins are the lethal

persistent organic pollutants (POPs) and some of its components are toxic compounds which cause cancer and neurological damage, disrupting reproductive thyroid and respiratory systems. Therefore, burning of plastic wastes increase the risk of heart disease, aggravates respiratory ailments and damages the nervous system (North & Halden, 2013). Landfilling has contributed to approximately 20% of Green House Gases. Landfilling as a method of disposal of poly-ethene is becoming unviable as land is fast becoming a scarce resource and the available landfills are overloaded with wastes posing health risks. The protective layers separating landfills from the soil and from underlying drinking water resources could rupture or leak over time. This poses a long term risk of contamination of soil and groundwater with plastics' components as well as with other contaminants contained in landfill leachate. The land utilized for landfills would otherwise be used for agricultural and other activities (Webb *et al*., 2013).Recycling may not be economically feasible due to the cost factor and loss of mechanical properties like tensile strength (Nanda & Sahu, 2010) and also due to the cost of sorting out. It has been noted that during recycling more toxic and larger amounts of hazardous volatile organic compounds are emitted from melted waste plastic pellets than during production of virgin plastics (Tsai *et al*, 2001). Recycled plastics have proved to be more hazardous than virgin plastic as during recycling it is mixed with a number of harmful colors, additives, stabilizers etc. In addition, volatile organic compounds produced during recycling have adverse health effects as they may contain massive hazardous compounds which are either non-cancer or cancer risks (He *et al*., 2015). The non-cancer effects include damages to the liver, kidneys and central nervous

system, asthma and other respiratory effects while the cancer effects are lung, *blood* (leukemia and non-Hodgkin lymphoma), brain, liver and kidney cancers (Rumchev *et al*., 2007). Because of poly-ethene persistence in our environment, communities and governments are now more sensitive to the impact of discarded plastic on the environment.

2.7: Isolation of Poly-ethene degrading microorganisms

Microorganisms can be isolated from many different environmental conditions. They can adapt and grow at subzero temperatures, as well as extreme heat, desert conditions, in water, with an excess of oxygen and in anaerobic conditions, with the presence of hazardous compounds or on any waste stream. The main requirement being carbon source (Vidali, 2001). Local microorganisms are the main agents used for bioremediation (Wolicka *et al*., 2009) due their adaptability hence they can be used to degrade or remediate environmental hazards The microbial consortia or individual strains utilized for bioremediation vary, depending on the chemical nature of the pollutants and need to be selected carefully as they only survive under a limited range of chemical conditions (Wolicka *et al*., 2009; Refugio, 2016). Poly-ethene degrading microorganisms can be isolated from Poly-ethene buried in the soil, Rhizosphere soil of mangroves, Plastic and soil at the dumping sites and Marine water.

Isolation of LDPE degraders involves particularly plating the sample on a medium that has LDPE as a carbon source and in this case only microorganisms that can utilize LDPE as a carbon source will be able to grow but non-utilizers of LDPE will not grow

on this media due to lack of a carbon source. Various preliminary protocols of isolation have been developed but in all of them, morphological characterization is usually employed to isolate pure colonies and sub culture them for use in the subsequent steps. Hadad *et al*, (2005) screened for the ability of micro-organisms ability to grow on poly-ethene as the sole source of carbon and energy by first growing them on liquid waxes. Soil samples taken from a poly-ethene-waste disposal site were plated on artificial medium supplemented with 10 ml of a mixture containing intermediate-size poly-ethene oligomers in the form of liquid waxes. Wax-degrading bacteria were identified by the production of clear zones around the colonies growing in the opaque wax-containing medium. These colonies of wax-degrading bacteria were further tested for their ability to grow in SM medium containing poly-ethene powder as the sole carbon source.

Pramilla & Vijaya , (2015) isolated LDPE degrading bacteria by preparing an artificial media in which pretreated poly-ethene was used as the sole carbon source. The poly-ethene was pretreated by boiling it in xylene for fifteen minutes, crushing it and then washing in 70% ethanol. The artificial media was then prepared by mixing (g/l) 1 $(NH_4)_2$ SO_4, 1 K_2HPO_4, 0.2 KH_2PO_4, 0.002 $CaCl_2$. $2H_2O$, 1 NaCl, KCl, 0.5 $MgSO_4$, 0.001$CuSO_4.5H_2O$, 0.0001 $ZnSO_4$.$2H_2O$, 0.0001 $MnSO_4$.$2H_2O$ supplemented with 500mg of LDPE powder in 1000ml distilled water. 50 ml of this was put in conical flasks in which the inoculum from soil was added alongside LDPE sheets and incubated for 30 days before plating on solid media. According to Ibiene *et al*., (2013), soil samples collected from the upper 0-5 cm layer of the mangrove soil were stored in plastic crates and transported to the lab where they were kept at room temperature.

LDPE films were then buried in the soil upon liming with $CaCO_3$ and fertilizer [$(NH_4)_2HPO_4$]. After 3 months of burial, the LDPE pieces were removed and transferred onto nutrient agar plates and incubated at 30^oC for 24 hours, for bacterial isolation from the surface of LDPE. To screen for poly-ethene and polyurethane degrading fungi, hydrolytic activity of the microorganisms was examined. This is based on the visual inspection of plates containing the lipid substrate tributyrine for indication of a clearing zone around the colony edges. Micro-organisms capable of degrading this polymer will show a zone of clearance around the growing culture (Russell *et al*., 2011). This is then followed by incubation of the isolated microbes with LDPE as the sole carbon source on artificial media (Vinay *et al*., 2016). The polymer over layer method used here uses glass petri dish covered with an LDPE film of 20-micron thickness previously sterilized with benzene and alcohol. The poly-ethene covered plate is then autoclaved and enriched nutrient media made of 5% tryptone, 5% NaCl and 1% yeast extract with 2% agar is carefully poured by raising the LDPE film with a sterile glass rod and gently laying it back on the solidifying agar. The whole process is done in a laminar flow to maintain sterility. 50 ml of bacterial culture is then spread upon the LDPE films and incubated at 37 ^oC in an inverted position. Monitoring the viability of the bacterial cells in the poly-ethene-based liquid culture was done after 15 days of incubation at 37˚C. 50 ml of the culture was taken and spread on an enriched medium agar plate for overnight at 37˚C after which the plate was observed for colony formation. Scanning electron microscopy was also done on the LDPE overlayer and presence of pits was an indication of microbial activity in an attempt to access the rich nutrient media underneath (Chatterjee

et al, 2010). Usually during screening for microbial growth on the polymer, colony shape, color, texture and size are used to distinguish between different bacterial cultures (James, 1988; Krieg & Staley, 2005). Bacteria and fungi have been greatly implicated in the degradation of both natural and artificial plastics (Gu, 2003).

2.8: Bacterial Polymer Bioremediation

Researchers have been able to isolate LDPE degrading bacteria even though the rates of degradation have remained low. Hadad *et al*., (2005) were able to isolate a thermophilic bacterium *Brevibaccillus borstelensis* strain 707 from soil samples taken from a poly-ethene-waste disposal site at the poly-ethene production plant of Carmel Olefins. These samples were plated on Artificial Medium with LDPE as the only carbon source to isolate LDPE degraders. The isolate *Brevibaccillus borstelensis* strain 707 after 30 days at 50°C reduced the gravimetric and molecular weights of poly-ethene sheets by 11 and 30% respectively. Bhatia *et al*., (2014) identified a novel strain of Pseudomonas-*Pseudomonas citronellolis* EMBS027 (KF361478) from a municipal landfill in Indore, India that had a degrading potential of 17.8% in 4 days. Similar results have also been obtained by (Kapri *et al*., 2010; Skariyachan *et al*., 2016; Shah *et al*., 2013). *Pseudomonas putida* isolated from sludge in industrial waste utilized *o*chloronitrobenzene (*o*-CNB) as its only carbon source (Haizhen *et al*., 2009). The highest degradation of *o*-CNB (85%) by *P. putida* was at 32°C and a pH of 8. This is an indication that the microorganisms that are involved in polymer degradation can work optimally under a narrow range of conditions.

2.9: Fungi and bioremediation

Fungi are widely used in bioremediation due to their robust nature and for their great source of diverse enzymes (Mahalakshmi *et al*., 2012). *Phanerochaete chrysosporium*, commonly known as white-rot fungus is among the most reported fungi and is able to degrade wide range of persistent pollutants and xenobiotic compounds under nutrient limited conditions due to its complex enzyme system. A number of fungi have been shown to use plastics as the sole carbon source (Russell *et al*., 2011; Yamada-Onodera *et al*., 2001), including in solid matrices, such as soil (Bhardwaj *et al*., 2012) and compost (Zafar *et al*., 2013) hence the implication of these organisms in the bioremediation of plastics. In a study, Low density poly-ethene sheets buried in the soil mixed with sewage sludge were examined microscopically after 10 months of incubation, fungal attachment was observed on the surface of the plastic, indicating possible utilization of plastic as a carbon source (Shah *et al*., 2008).The isolated fungal strains were identified as *Fusarium sp*. AF4, *Aspergillus terreus* AF5 and *Penicillum sp*. AF6. The attachment of the fungal strains in form of a biofilm on LDPE surface was attributed to the gradual decrease in hydrophobicity of its surface (Hadar *et al*, 2004).

2.10: Determination of Poly-ethene Degradation

The level of poly-ethene degradation can be determined by various methods as well as analytical techniques. Some of these methods include weight change determination, Scanning Electron Microscopy (SEM), Fourier Transform Infrared spectroscopy (FT-IR), Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC) and Gas Chromatography-Mass Spectrometry (GC-MS) (Orhan & Bu, 2000;

Juan-Manuel Restrepo-Flórez *et al*., 2014). According to Sen & Raut, (2015), the most convenient method to determine the degradation is by measuring the weight loss as the microbial enzymes catalyze the depolymerization causing weight reduction of poly-ethene. Loss of polymer integrity leads to weight loss and is directly proportional to polymer size (Sudhakar *et al*., 2007). Upon completion of the incubation time, the polymer is cleaned appropriately and oven dried before its weight is taken and used to calculate the change in weight (Pramilla & Ramesh, 2015). A comparison of weight reduction for LDPE and HDPE indicated dry weight loss ranging from 10.4% and 23.15 % for LDPE and 8.41% - 17.72% for HDPE where *Bacillus subtilis* was seen to be a better biodegrader than *Bacillus mycoides* (Ibiene *et al*., 2013). At topographical level, the Scanning Electron Microscopy (SEM) is used to see the level of physical utilization and attachment of the microbes on the surface of the poly-ethene before and after the microbial attack (Esmaeili *et al*., 2016; Esmaeili *et al*., 2013). Samples are fixed with 2.5% glutaraldehyde in 0.05 M cacodylate buffer and then rinsed before staining with a solution of 0.05% acridine orange to enhance visibility during examination under the epiflourescent microscope. This will enable visualization of microbial cells and surface deformations that occur during degradation (Bonhomme *et al*., 2003).

Fourier Transform Infrared spectroscopy (FT-IR) is useful in detecting compounds on the surface of the sample and can be documented via collection of large number of FT-IR spectra. Greater peak intensity indicates presence of certain compounds in higher concentrations. Functional groups on the surface of the poly-ethene sheet after incubation were observed in case of treated poly-ethene which indicated the breakdown

of polymer chain in the presence of oxidation products of poly-ethene. Non-degraded poly-ethene exhibited almost zero absorbancy at those particular wave numbers. Absorbance at 1700–1780 cm^{-1} (corresponding to carbonyl compounds) (**Fig 2.4**), 1640 cm^{-1} and 830–880 cm^{-1} (corresponding to (–C=C-), 1650 cm^{-1} (corresponding to vinyls) which appeared after incubation of treated samples with microbial consortia (Fariha *et al*., 2007; Juan-Manuel Restrepo-Florez., 2014). Typical degradation of LDPE as a result of oxidation is exhibited by formation of bands at 1620–1640 and 840–880 cm^{-1}. **Table 2.1** shows the criteria for characterization of various peaks in the FTIR spectrum. In the analysis of the polymer's spectral information special emphasis by researchers has been placed on the following functional groups: carbonyls (1715 cm^{-1}), esters (1740 cm^{-1}), and double bonds (908 cm^{-1}) (Antony & Govt, 2015).

To assess the mechanical properties of the poly-ethene after the microbial attack, various parameters are usually used i.e. determination of the percentage of elongation and change in tensile strength. Orhan & Bu, (2000) determined the percentage of elongation at room temperature using an Instron model 4502 Universal Tester (Instron, Canton, MA) operating at 500 mm/min with a 5-cm initial grip distance where percentage elongation value of LDPE/starch blend decreased in inoculated soil after 1 month whereas it remained constant for at least 3 months in un-inoculated soil. The products from poly-ethene degradation are also characterized using various techniques such as Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC) and Gas Chromatography-Mass Spectrometry (GC-MS) (Pramilla & Ramesh, 2015; Esmaeili *et al*., 2013; Shah *et al*., 2008)

Table 2.1: Characterization FTIR peaks (Vimala & Mathew, 2016)

No.	Wave number (cm^{-1})	Bond	Functional group
1	3000-2850	-C-H Stretch	Alkanes
2	2830-2695	H–C=O: C–H stretch	Aldehydes
3	1710-1665	-C=O Stretch	Ketones, Aldehydes
4	1470-1450	-C-H Bend	Alkanes, Akenes
5	1320-1000	-C-O Stretch	Acohols, Carboxylic acid, esters, ethers

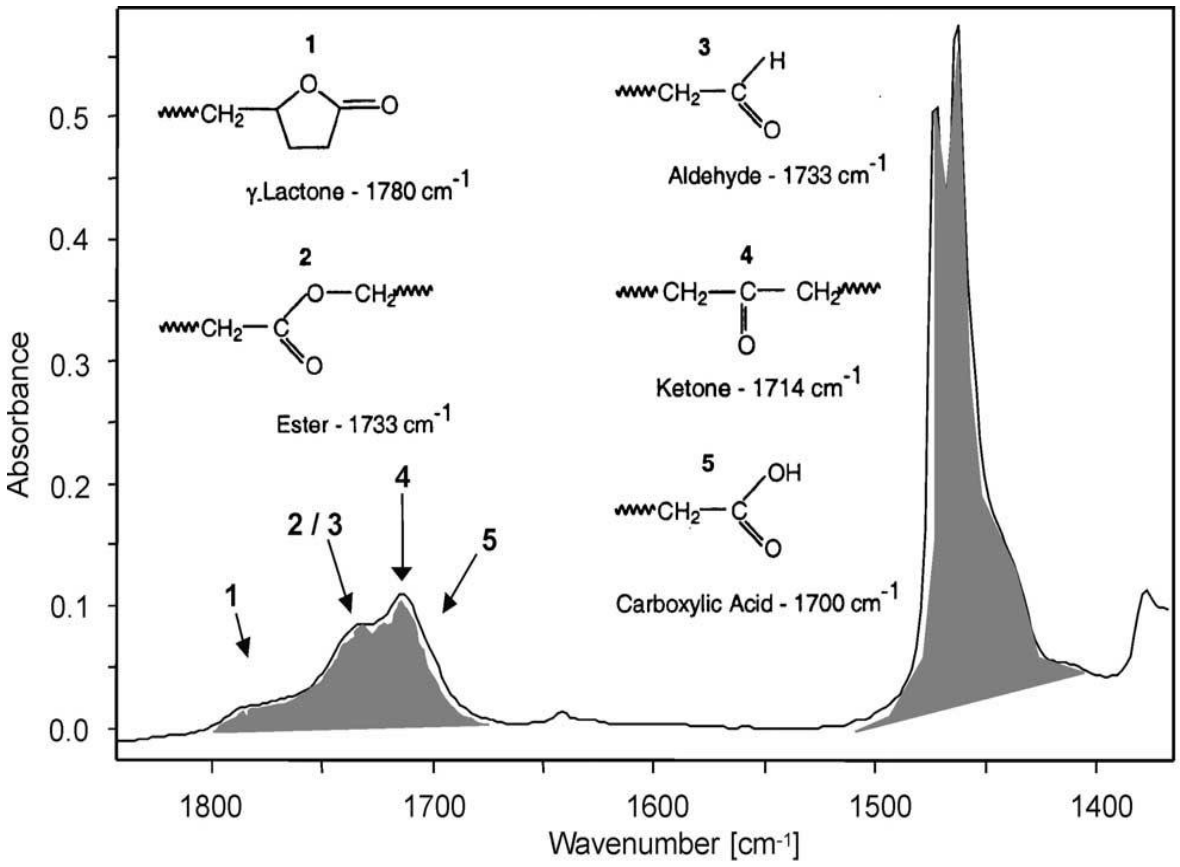


Figure 2.4: FTIR spectrum of LDPE showing peaks attributed to carbonyl groups (Gulmine *et al*., 2002)

2.11: Genetic modification of LDPE degrading Microorganisms

Other than environmental factors like temperature and presence of nitrogen and phosphorus sources, the genetic make-up of an organism influences its ability to utilize certain carbon sources. Molecular techniques can be applied to improve the level of a particular enzyme or series of enzymes in a microorganism leading to an increase in the reaction rate (Dua *et al*., 2002). The easiest way to come up with an appropriate genetically modified strain is to begin with an organism that already possesses much of the necessary degradative enzymatic machinery. Through the genetic modification of metabolic pathways, there is a possibility of extending the range of substrates that an organism can utilize. Genes useful in degradation of environmental pollutants like toluene, chlorobenzene and other halogenated pesticides have been confirmed. For every compound, one unique plasmid is needed but this is limited to just a specific compound. The plasmids occur four in four different categories namely OCT plasmid for degradation of octane, hexane and decane, XYL plasmid for degradation of xylene and toluene, CAM plasmid for camphor and NAH plasmid for naphthalene.

A multi-plasmid containing *Pseudomonas* strain- *Pseudomonas putida* containing the XYL and NAH plasmid together with a hybrid plasmid attained through recombination of parts of CAM and OCT via conjugation could biodegrade camphor, octane, salicylate, and naphthalene grew rapidly on crude oil because it capable of utelizing aliphatic, aromatic, terpenic and polyaromatic hydrocarbons. This product was called as superbug (oil eating bug). Genetic modification gives an opportunity to create new gene combinations that do not exist together naturally. The commonly used techniques

include engineering with single genes, physiological pathway construction and alterations of the sequences of resident genes (Cases & De Lorenzo, 2005). The first step is selection of suitable genes, insertion of the gene fragment into an appropriate vector and introducing it into the target host cells (Joutey *et al*., 2014). The genes selected are usually those coding for enzymes implicated for bio-degradation of desired specific substrates under investigation. Some of the important enzymes in artificial polymer degradation include depolymerases, manganese peroxidases, dehydrogenases, laccases, aldolases and oxygenases produced by bacteria for breakdown of Polyhydroxyalkanoates (PHAs) which have similar structure to petroleum polymers and are involved at different stages of polymer degradation. This is followed by production of many gene copies and selection of cells with the recombinant DNA (**Fig 2.5**)

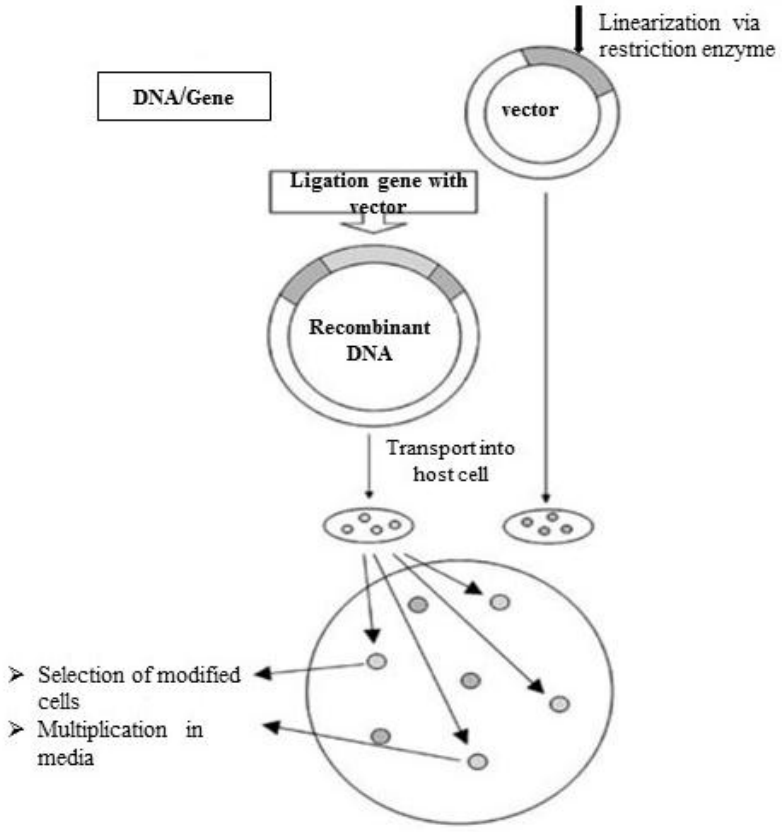


Figure 2.5: A recombination protocol for insertion of genes coding for LDPE degrading enzymes (Wasilkowski *et al*, 2012)

Nowadays, the artificial plasmid vectors in construction of GMMs are commonly used. They contain the best features obtained from a variety of natural plasmids such as ori-C (origin of replication), MCS (multi-cloning site) and marker genes. Currently, expression plasmids are mostly used because they enable the quick production of a large quantities of desired protein. Apart from the vectors, enzymes as a powerful genetic engineering tool in the cut-and-paste techniques are inevitable. They include restriction enzymes cutting DNA in a specific region and DNA ligases which close nicks in the

phosphodiester backbone of DNA. Among them, restriction endonuclease *Eco*RI, *Bam*HI and *Hind*III are commonly used in molecular biology (Wasilkowski *et al*., 2012).

However, ecological and environmental concerns and regulatory constraints are major bottlenecks in the testing of GEM under field conditions (Menn *et al*., 2001). There are at least four principal approaches to GEM development for bioremediation application (Menn *et al*., 2001). These include: Modification of enzyme specificity and affinity; Pathway construction and regulation; Bioprocess development, monitoring and control and Bio-affinity applications for chemical detection, toxicity reduction and end point analysis.

2.12: Abiotic Factors Affecting Bioremediation

Effective bioremediation can only occur where environmental conditions support microbial growth and activity, its application mostly involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate. The right temperature, pH and salt concentration vary for different microorganisms and hence need to be adjusted accordingly to enhance microbial activity. Fungi of genus *Rhizopus* and *Penicillium* were incubated with LDPE for six months at 30˚C and they were able to degrade the polymer resulting in a maximum weight loss of 4% (Mahalakshmi *et al*., 2012). The bacterial optimum incubation temperature for LDPE *Enterobacter cloacae* KU923381 isolated from petroleum contaminated soil was at a temperature of 35˚C and a pH of 7 (Ramasamy *et al*., 2017). According to Skariyachan *et al*., (2015), *Pseudomonas spp* was able to utilize plastic as

a sole source of carbon and showed 20–50% weight reduction over 120 days and this rate was optimally achieved at 37 °C in pH 9.0. Microbial enzymes directly influence the rate of activity of the microbes and hence the optimum conditions at which certain enzymes work will equally dictate the maximum conditions for incubation of the microorganisms. Laccase production by fungi for instance is influenced by culture conditions such as type and concentration of carbon sources, pH and temperature (Khalil *et al*, 2014). The optimum temperature for activity of laccase enzyme by fungi was found to be between 25˚C and 30°C as laccase activity reduces with higher temperatures although this can vary highly with the fungal strains. There is a clear indication that the optimum pH conditions for biodegradation of LDPE vary depending on the microbial strain and source. In a study done by (Pawar *et al*, 2013), it was observed that soil pH of 7.5 was most suitable for the degradation of all the PHAs as 50% degradation was observed at pH 7.5 and *Aspergillus species* was also found to be more prevalent at this pH (7.5-8.0).

2.13: Role of Enzymes in bioremediation

Some of the enzymes that could be of interest are PHA depolymerases produced by bacteria for breakdown of PHAs which have similar structure to petroleum polymers. Manganese peroxidases, dehydrogenases, laccases, aldolases and oxygenases (Pramilla *et al*., 2015) are involved at different stages of polymer degradation. This mechanism is as a result of adaptation of the works presented by different authors (Juan-Manuel Restrepo-Flórez, 2014) (**Fig 2.6**). In the first ever reported enzyme that degrades polyethylene, it was postulated that copper affects laccase activity and ultimately the

biodegradation of polyethylene since an increase in the molarity of copper ions increased the degradation of polyethylene leading to molecular weight reduction and an increase in the keto-carbonyl index (Santo *et al*., 2013).

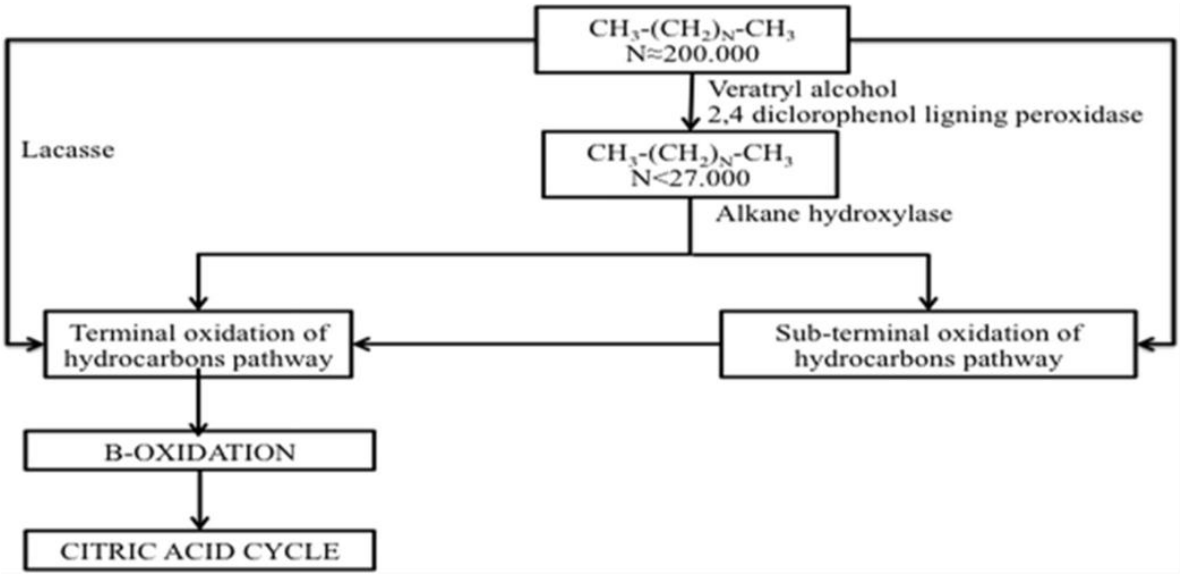


Figure 2.6: Role of laccases in poly-ethene degradation pathway

AlkB and alkB related genes, encoding the alkane hydroxylase which catalyzes the first step of the alkane degradation process is an important component of the LDPE degradation studies (Yoon *et al*., 2012). This group of enzymes are known as oxygenases and their role is to introduce oxygen atoms derived from molecular oxygen into the alkane substrate. Terminal oxidation of alkanes causes activation to the corresponding primary alcohol, which is further oxidized by alcohol and aldehyde dehydrogenases to the resulting fatty acids which enter the β-oxidation cycle (Van Beilen *et al*., 2003).

CHAPTER THREE

MATERIALS AND METHODS

3.1: Study Site

Dandora dumpsite is Nairobi's official main dumpsite and has been operational since the 1970s. It's about 8 km away from the city and is close to the heavily populated low income estates which include Dandora, Korogocho, Baba Dogo and Huruma. It is home to a 1\4 a million people and it stands on over 30 solid acres. The site was among the Blacksmith Institute list of the dirty 30 most polluted places in the world (Black Smith Institute, 2007). It contains industrial wastes that include expired goods, agricultural wastes such as pesticides, and hospital wastes such as packaging materials, sharps, pharmaceuticals and syringes, biological wastes, heavy metals such as lead and mercury and persistent organic pollutants such as DDTS. The sampling points were as follows: **A:** S01˚14.633' E036 ˚54.063' Elevation 1578m, 21.48Km SW **, B:** S01˚14.652' E036 ˚54.031 Elevation 1589m , 21.55Km SW **, C:** S01˚14.661' E036 ˚53.977' Elevation 1590m 21.63km SW **D:** S01˚14.688' E036 ˚53.986' Elevation 1594m 21.65km SW and **E:** S01˚14.710' E036 ˚53.977' Elevation 1596m 21.69km SW. From the photographs of the dumpsite **(Fig 3.1 and 3.2)**, it is evident that poly ethene is a major component of the garbage.

Figure 3.1: A photograph of Dandora dumpsite indicating that poly-ethene is the major waste at the site.

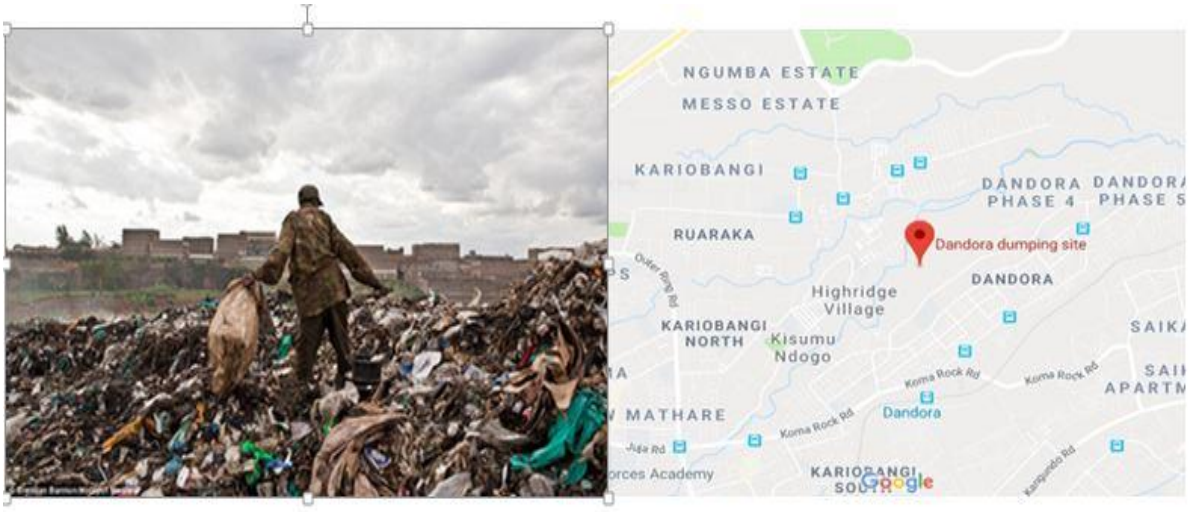


Figure 3.2: A photograph of Dandora dumpsite indicating some of the informal settlements around the site.

3.2: Sample collection and Preparation of medium for LDPE degrading bacteria

A randomized block design was used to identify points for sample collection. Soil samples were collected from five different sampling blocks of the dumpsite. The samples from each sampling block were collected at five randomly selected points of

1meter physical diameter. This resulted in a total of 25 samples collected. Soil was aseptically scooped from and adjacent to buried poly-ethene materials at 5 cm depth below the litter layer. On-the-site temperature was recorded in order to ascertain the in-situ biodegradation conditions. Samples were kept in Ziploc bags and transported to the Institute of Biotechnology Research (IBR) lab at JKUAT in a cool box. Once in the lab, the pH of the samples was also measured and recorded.

3.3: Preparation of artificial media and incubation

1 g of soil sample was added to 50ml of 0.85% autoclaved normal saline solution to prepare the inoculums. The inoculum was kept at 37°C for 2-3 hrs in a shaker incubator before inoculation. Artificial media was prepared as follows: 0.1% $(NH_4)_2 SO_4$, 0.1% $NaNO_3$, 0.1% K_2HPO_4, 0.1% KCl, 0.02% $MgSO_4$ and 0.001% yeast extract in 1000ml distilled water according to Burd, (2009). LDPE powder weighing 2g was added to each 100 ml of artificial medium in order to increase surface area for the microbial attachment hence improve attachment to the poly-ethene sheets. To prepare growth culture for the LDPE degrading microorganisms, 1% of the prepared inoculums was transferred to 200 ml of artificial medium. Thirty and 40 micron poly-ethene sheets (app 3 cm x 3 cm each) were weighed, disinfected in 70% ethanol and air-dried for 15 min in an oven then introduced into the artificial media. Culture flasks for fungal incubation were augmented with 250mg/ml ampicillin to inhibit bacterial growth. All treatments were done in triplicates. Un-inoculated artificial media with LDPE was used as the negative control. All the treatments were incubated in an incubator shaker at 150 rpm for up to 16 weeks.

3.4: Determination of LDPE degrading potential of the bacterial isolates

The LDPE sheets were recovered from the different flasks after 16 weeks incubation. Washing of the sheets was done using 2% SDS to remove the bacterial biomass then dried in an oven overnight then weighed.

At the end of the incubation period, the structural changes in the LDPE surface was investigated using the EQUINOX 55 FT-IR spectrometer at the JKUAT chemistry lab. For each LDPE sheet, a spectrum was taken from 400 to 4000 wave numbers at a resolution of 2 cm^{-1} and over 32 scans (Kapri *et al*., 2010; Lucas *et al*., 2008; Pramilla & Ramesh, 2015). The control LDPE sheet was not subjected to any incubation process.

The Finnigan –Voyager Mass Spectrometer coupled to a Fisons Trio 8000 Gas chromatograph at the Analytical Chemistry Lab in JKUAT was used for GC-MS. The sample solution was filtered using a Whatmann 0.45 µm filter paper and extracted using a C-18 solid phase extraction (SPE) cartridge. The C-18 cartridge was first conditioned using 10 ml methanol, followed by 10 ml distilled water and loaded at a flow rate of 5 ml/minute. Elution of the analytes from the SPE cartridge was finally carried out using 10 ml of n-hexane and reconstituted using 1 ml n-hexane. Samples were then subjected to GCMS analysis according to recommended standard conditions (**Appendix 3**). The results were recorded and analyzed.

3.5: Isolation of LDPE degrading microorganisms

Based on effectiveness of biodegradation demonstrated, bacterial isolation was done from the culture flasks at the end of the incubation period. Isolation was only carried out

from those incubation flasks that had shown indications of biodegradation based on weight changes, FTIR and GC-MS outcomes.

For bacterial isolation, a loopful of culture from the artificial media was put on a nutrient agar plate, a spreader was used to spread it till dry. Incubation was done overnight at 37°C or up to three days for slow growing bacteria. The mixed cultures of bacteria were continually sub-cultured to obtain pure bacterial cultures which were then stored at –20°C and at -80°C in 15% glycerol slants.

For fungal isolation, a drop of the inoculum was put and spread till dry on PDA plates and incubated for five days. The various fungi were isolated based on morphology and sub-cultured continually to obtain pure fungal cultures.

3.6: Characterization and Identification of Isolates

Bacteria isolated based on colony morphologies using standard microbiological criteria, with special emphasis on color, shape, size and form. Biochemical characterization was done based on Catalase test, Citrate utilization, triple sugar iron (TSI) slant, TSI butt, TSI Gas production, methyl red (MR), Voges–Proskauer (VP), Urease test, Gelatin liquefaction, Indole test, Motility test, Nitrate reduction and Starch hydrolysis. Preliminary characterization by Gram staining was done (using safranin) of each of the isolates using the method of Dussault, (1955) and observed under a light microscope at ×100 (Keast *et al*., 1984). The Gram staining technique was used to categorize the bacterial isolates into Gram negative and Gram positive(James, 1988). Fungal isolates

were also isolated using standard microbiological criteria, with special emphasis on color and form. Staining was done using Lactophenol blue dye.

3.7: Bacterial DNA extraction

Total genomic DNA was isolated from the bacterial cultures grown to the late exponential phase by means of a standard protocol (Ausubel *et al*., 2003) as follows: 1.5 ml of the overnight bacterial culture (grown in LB medium) was transferred to a 1.5 ml eppendorf tube and centrifuged at 13000 rpm for 1min to pellet the cells. The supernatant was discarded. The cell pellet in was suspended in 600 µl TE buffer and centrifuged at 13000rpm and the supernatant discarded. The cell pellet was re-suspended in 200 µl TE buffer and the following were added: 5 µl lysozyme (20mg/ml), 5 µl RNAnase (20mg/ml), 10 µl proteinase K (20mg/ml) followed by overnight incubation at 37°C. The next morning the temperature was adjusted to 56°C for one hr and an equal volume of phenol/chloroform (1:1) was added and mixed well by inverting the tube until the phases were completely mixed. Spinning was done at 13000rpm for 15 min at RT. The upper aqueous phase was carefully transferred to a new tube by using 1 ml pipette. This step was repeated twice to ensure all protein had been removed. An equal volume of chloroform-isoamyl alcohol (24:1) was added to the aqueous layer and centrifuged at 13000rpm for 15 min. The aqueous layer was removed into a new tube. This step was also repeated to ensure all phenol is removed. An equal volume of isopropanol was added and stored overnight at -20 °C. The samples were then defrosted and centrifuged at 4°C for 30 min to pellet the DNA. The pellet was washed in 70% ethanol and

centrifuged at 13000rpm for 5 min then the ethanol was carefully pipetted out. The pellet was air dried on the bench for 20 min and the isolated genomic DNA was checked on a 1% agarose gel.

3.8: Amplification and sequencing of Bacterial DNA

Amplification of the 5' end of the 16S rDNA gene was performed with prokaryotic universal primers: forward primer (8-F) 5'-AGAGTTTGATYMTGGCTCAG- 3' and reverse primer: (1942R) 5' - GGTTACCTTGTTACGACTT-3' (Weisburg *et al*, 1991) The PCR was performed on a GeneAmp PCR system 9600 (Applied Biosystems), using 1 µl Taq Polymerase (Applied Biosystems), 1 µl each of 10 pM concentrations of forward and reverse primer, 27 µl sterile deionized water, 8 µl PCR buffer containing dNTPs and $MgCl_2$, and 2 µl DNA template, for a total reaction volume of 40 µl. The cycling program used was as follows: 1 cycle of 94°C for 5 min; 30 cycles of 94°Cfor 30 sec, 55°C for 30 sec, and 72°C for 1.5 min; and a final extension of 72°C for 10 min. The PCR products were visualized through electrophoresis on a 1% agarose gel with ethidium bromide added directly. The 1.5 kbp products were subjected to Sanger di-deoxy sequencing using the forward primer and reverse primers at Macrogen DNA, Inc. (Netherlands). Sequence files were edited using Chromas version 2.6.2 and compared with the GenBank nucleotide database using the Basic Local Alignment Search Tool (BLAST).

3.9: Fungal DNA extraction

Fungal mycelia was grown for 7 days at 55°C on potato dextrose agar. Mycelia was frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. The powder was transferred into 2ml eppendorf tubes and 600µl of preheated extraction buffer added **(Appendix 2**). The contents were incubated in a water bath at 65 ºC for 30 minutes with mixing after every 10 minutes. Two hundred and seventy microliters of 5M potassium acetate was added and centrifuged at 13000rpm for 10 minutes. The supernatant (700µL) was transferred into clean tubes volume and 5µL RNase (10mg) added then incubated for 30 minutes at 37ºC to remove RNA. Chloroform and iso-amyl alcohol was prepared in the ratio of 24:1 and an equal volume added to the mixture. Six hundred microliters of supernatant was pipetted into clean tubes. DNA was then precipitated by adding a tenth of the volume of 3M potassium acetate and two thirds of the volume of isopropanol. It was incubated at -20ºC for 30 minutes then centrifuged at 13000rpm for 10 minutes. The pellet was washed using 70% ethanol followed by 10 minutes of centrifuging then the DNA was eluted in 50µL of RNase-free water and stored at -20ºC.

3.10: Amplification and sequencing of Fungal DNA

Primer pair **F-**566:5' - CAGCAGCCGCGGTAATTCC - 3' and for R- 1200:5'-CCCGTGTTG AGTCAAATTAAGC - 3' which amplify on average a 650 bp long fragment from the V4 and V5 regions were used (Hadziavdic *et al*., 2014). The amplification of fungal DNA was performed on a GeneAmp PCR system 9600 (Applied Biosystems), using 1 µl Taq Polymerase (Applied Biosystems), 1 µl each of 10 pM

concentrations of forward and reverse primers, 27 µl sterile deionized water, 8 µl PCR buffer containing dNTPs and $MgCl_2$, and 2 µl DNA template, for a total reaction volume of 40 µl. The cycling program used were as follows: 1 cycle of 95°C for 5 min; 35 cycles of 95°Cfor 30 sec, 60°C for 45 sec, and 72°C for 40 sec; and a final extension of 72°C for 5 min. The PCR products were visualized through electrophoresis on a 1% agarose gel with ethidium bromide added directly. The products were subjected to Sanger dideoxy sequencing by Macrogen, Inc. (Netherlands). SeqMan Pro was used to assemble both the forward and reverse sequence file (Aarti & Khusro, 2015; Nayarisseri *et al*., 2013) .The sequences thus obtained were compared against the sequences available in the NCBI, database using the basic local alignment tool (BLASTn). The 18S rRNA gene sequences obtained in current study, together with those of the closest neighbor strains were aligned using ClustaX version 2.1. Phylogenetic relationships were inferred from phylogenetic comparison of the 18S rRNA sequences using Mega 7 (Tamura *et al*.,2007) and maximum-likelihood algorithms (dnaml and dnamlk) available in Phylip. Maximum likelihood and parsimony-derived trees were bootstrapped using PHYML (Hall, 2013; Abdennadher & Boesch, 2007).

3.11: Screening for production of Extra-cellular enzymes

Bacterial isolates were screened for their ability to produce extracellular enzymes i.e. laccases, esterases, xylanases and pectinases. The ability of the isolates to utilize respective substrates i.e. lignin, tween 20, xylan and pectin indicate the ability to produce the respective enzymes. Positive results were indicated by the potential of the

respective isolates to produce enzymes that would utilize these substrates while the negative tests were indicated by the presence of the substrate after growth of the isolates (Castro *et al.,* 1993, Cappuccino and Sherman, 2002).

3.11.1: Screening for presence of enzyme laccase

Enzyme laccase is in the group of enzymes that are necessary in the lignin degradation pathway and has been documented to have a role in poly-ethene degradation. The media for selection of lignin modifying fungi was prepared by the use of plain agar and minimal salt media with the incorporation of lignin (to encourage selection of lygninolytic fungi) and Guaiacol, which acts as a colorimetric indicator of the lignin-modifying enzymes laccase or peroxidases (Arora & Sandhu, 1985). All chemicals were obtained from Sigma Chemical Co., St. Louis. The presence of a reddish coloration after 3-5 days of incubation was an indication of laccase activity. The Laccase assay per 1 liter: 400 µl Guaiacol, agar 15g, 2 g Malt extract, 0.5 g KH_2PO_4, 0.001 g $ZnSO_4$, 0.4 g K_2HPO_4, 0.02 g $FeSO_4$, and 0.2 g $MgSO_4$, 0.5g KH_2PO_4, 0.1g NH_4NO_3, 0.1g KCl, 5ml KOH, 0.25g Chloramphenicol (Viswanath *et al.*, 2008), forming a colored zone as a positive result.

3.11.2: Screening for presence of enzyme esterase

The isolates were cultured on basal media (1 % KH_2PO_4, 0.0 1% $MgSO_4.7H_2O$, 0.005 % $CaCl_2.2H_2O$, 4 % NaCl and 1 % Na_2CO_3) supplemented with tween 20 as the sole carbon source. The medium was then thereafter inoculated by the spotting of isolates per plate and incubated for at least 48 hours. The media was observed for zones of

precipitation of calcium crystals around each isolate (Peng *et al*., 2014). Positive isolates for esterases production were indicated by the precipitation of calcium crystals around the colonies.

3.11.3: Screening for presence of enzyme xylanase

The isolates were cultured on basal media (1 % KH_2PO_4, 0.0 1 % $MgSO_4.7H_2O$, 0.005 % $CaCl_2.2H_2O$, 4% NaCl and 1 % Na_2CO_3) supplemented with 1 % xylan (Fluka) as the sole carbon source, by the method described by (Lee *et al*., 2008). The medium was then inoculated with the isolates and incubated for at least 48 hours. These were flooded with 1 % Congo red dye. The dye was then replaced with NaCl (1 M) and subsequently rinsed with distilled water. The plates were observed for halos around the colonies, as indication of positive xylan degradation.

3.11.4: Screening for presence of enzyme pectinase

The isolates were cultured on basal media (1 % KH_2PO_4, 0.0 1 % $MgSO_4.7H_2O$, 0.005 % $CaCl_2.2H_2O$, 4% NaCl and 1 % Na_2CO_3) supplemented with 1 % pectin (Himedia) as the sole carbon source at pH 7, by the method described by (Lee *et al*, 2008). The medium was then inoculated with the isolates and incubated for at least 48 hours. These were flooded with 1 % Congo red dye. The dye was then replaced with NaCl (1M) and subsequently rinsed with distilled water. The plates were observed for halos around the colonies, as indication of positive pectin degradation.

3.11.5: Screening for genes producing alkane degrading enzymes

Primers coding for Alk B genes (**Table 3.1**) responsible production of depolymerization enzymes were used to screen for presence of the genes in the isolates (Belhaj *et al*., 2002).

Table 3.1: Primers for Alk B genes encoding depolymerases responsible for alkane degradation

Primers	**PCR product**	**Reference**
alkB 1 set 1 82 5′ TGGCCGGCTACTCCGATGATCGGAATCTGG3′ 111	870 bp	Kok *et al*.
951 5′ CGCGTGGTGATCCGAGTGCCGCTGAAGGTG3′922		Whyte *et al*.
alkB1 *set 2* 134 5′ CATTTCCCTGGTGATTG 3′ 151 851 5′ CCGTCCTCGCCCTTTCGC3′ 834	718 bp	Stover *et al*
alkB2 134 5′ CCTGGCTGGTGATCAGCG3′151 882 5′ CGAGTGTTCCGGCGTGGTG3′ 864	749 bp	Stover *et al*.

Amplification was done using the sets of primers shown (Table 2). The PCR was performed on a GeneAmp PCR system 9600 (Applied Biosystems), using Taq DNA polymerase (MWG) was added after boiling. A total of 30 cycles of amplification was performed with template DNA denaturation at 94 ˚C for 1 min, primer annealing at 40 ˚C for 1 min and primer extension at 72 ˚C for 2 min (Vomberg & Klinner, 2000). The

PCR products were visualized through electrophoresis on a 1% agarose gel with ethidium bromide added directly.

3.12: Determination of growth conditions of bacterial isolates

Bacterial cultures were grown overnight on nutrient agar plates at 37°C. A sterile tip of an inoculating loop was used to pick bacterial cultures by simply touching the tip of the loop to a well isolated colony. This was transferred to 100 mL Luria Bertani (LB) Broth in universal bottles and incubated overnight (18 hr, 250 rpm, 37°C). 5µl of each of the bacterial cultures was transferred into 100ml tubes containing pre-warmed (37°C) LB media with the growth requirements under investigation as shown below. The spectrophotometer was blanked using sterile LB broth. Every 2 hours, a 50 µl aliquot was sampled from each tube culture into the autoclaved well plates whose OD had been recorded. This was repeated for 10 hours at 600nm and the bacterial OD was obtained by subtracting the measured OD from the plate OD.

3.12.1: Growth at different sodium chloride concentrations

Universal bottles containing 100ml pre-warmed (37°C) LB media that had been previously autoclaved were prepared. They were adjusted to different salt concentrations as follows: 10% (10g NaCl in 100ml broth), 5% (5g NaCl in 100ml broth) and 0% (pure broth). 5µl of each of the bacterial overnight cultures at lag phase was transferred into the broth supplemented with the varying salt concentrations. The spectrophotometer was blanked using sterile LB broth. Every 2 hours, a 50µl aliquot was sampled from each tube of incubated cultures into the autoclaved well plates whose OD had just been

recorded. This was repeated 2 hourly for 10 hours. The bacterial OD was obtained by subtracting the blank plate OD from the measured OD.

3.12.2: Growth at various temperatures

Universal bottles containing 100ml pre-warmed (37°C) LB media that had been previously autoclaved were prepared. 5µl of each of the bacterial overnight cultures at lag phase was transferred into the broth. The cultures were incubated in different incubators set at varying temperatures (20°C, 30°C and 40°C). The spectrophotometer was blanked using sterile LB broth. Every 2 hours, a 50µl aliquot was sampled from each of incubated cultures into the autoclaved well plates whose OD had just been recorded. This was repeated 2 hourly for 10 hours. The bacterial OD was obtained by subtracting the blank plate OD from the measured OD.

3.12.3: Effect of pH on growth of the isolates

Universal bottles containing 100ml pre-warmed (37°C) LB media that had been previously autoclaved were prepared. They were adjusted to different pH levels using 1 M HCl and 1 M NaOH as follows: pH 6, pH 8 and pH 10. 5µl of each of the bacterial overnight cultures at lag phase was transferred into the broth adjusted to the varying pH ranges. The spectrophotometer was blanked using sterile LB broth. Every 2 hours, a 50µl aliquot was sampled from each tube of incubated cultures into the autoclaved well plates whose OD had just been recorded. This was repeated 2 hourly for 10 hours. The bacterial OD was obtained by subtracting the blank plate OD from the measured OD.

3.13: Determination of growth conditions of fungal isolates

3.13.1: Growth at different sodium chloride concentration

The ability of fungal isolates to grow at different sodium chloride concentrations was determined using Potato Dextrose Agar augmented with 250mg/ml ampicillin to inhibit bacterial growth and supplemented with NaCl: 0 %, 5% and 10 % sodium chloride. The media was inoculated with each of the fungal isolates and incubated at 28 ^{o}C, then checked for growth after 4 days by observing the extent of growth. The level of growth was scored using the colony diameter, whereby (0 mm) indicated no growth, (1-2 mm) indicated minimal growth, (3-4 mm) indicated average growth (5-7 mm) indicated satisfactory growth while (8-10 mm) indicated excellent growth.

3.13.2: Growth at various temperatures

Fungi, as a group of organisms, exist over a wide range of temperatures. However, individual species can only exist within a narrow range of temperatures as it normally affects the rate of chemical reactions through its effect on cellular enzymes (James, 1988). The aim of the experiment was to determine the optimum temperature for growth of the isolates. Potato Dextrose Agar augmented with 250mg/ml ampicillin to inhibit bacterial growth.at pH 7.0 was prepared, sterilized and dispensed in sterile petri dishes. Each plate was inoculated one fungal isolate and incubated at temperatures 20, 30, and 40^{o}C. Growth of isolates was checked after 4 days of incubation. The level of growth was scored using the colony diameter, whereby (0 mm) indicated no growth, (1-2 mm) indicated minimal growth, (3-4 mm) indicated average growth (5-7 mm) indicated satisfactory growth while (8-10 mm) indicated excellent growth.

3.13.3: Effect of pH on growth of the isolates

Growth and survival of microorganisms is greatly influenced by the pH of the environment, and all fungi and other microorganisms differ as to their requirements. Each species has the ability to grow within a specific pH range, which may be broad or limited, with the most rapid growth occurring within a narrow optimum range (James, 1988). The aim of the experiment was to determine the optimum pH for growth of the isolates. Potato Dextrose Agar was prepared, augmented with 250mg/ml ampicillin to inhibit bacterial growth and pH was adjusted to 6, 8 and 10 using 1 M HCl and 1 M NaOH. This was sterilized and dispensed in sterile Petri dishes. Each medium was inoculated with a fungal isolate and incubated at 28 ^{o}C. Growth of isolates was checked after 4 days of incubation. The level of growth was scored using the colony diameter, whereby (0 mm) indicated no growth, (1-2 mm) indicated minimal growth, (3-4 mm) indicated average growth (5-7 mm) indicated satisfactory growth while (8-10 mm) indicated excellent growth.

3.14: Data Analysis

Data on weight loss of the polymer was recorded in excel sheets. The GenStat software version 12.1.0.338 was used to perform a one way analysis of variance (ANOVA) for all the measured data. The ANOVA graphs, presented as mean ± standard error (SE) in the result section were generated using Microsoft excel. Fisher's Protected Least Significant Difference (LSD) test was used to compare and separate the means of weight changes among samples.

In the analysis of the FT-IR outcomes, EQUINOX 55 FT-IR data points were taken from 400 to 4000 wave numbers at a resolution of 2 cm^{-1} and over 32 scans. The data points were entered into a worksheet in the Origin Pro version 8.5 software where they were used to generate spectra with the control superimposed on the treatment. Genetic affiliations of the screened isolates was deduced from phylogenetic tree generated using MEGA 7 as described earlier.

Data on extra cellular enzymatic experiments was recorded in excel sheets. General Analysis of Variance was performed for all the measured data using the GenStat version 12.1 software. The ANOVA graphs, presented as mean ± standard error (SE), in the result section were generated using Microsoft excel. Tukey's honest significant difference (HSD) test was used to compare and separate the means of diameter of zones of clearance (presented in form of alphabet letters in the tables). Data on the growth of bacterial isolates at varying temperature, pH and salt concentration was recorded in excel sheets and the trend of growth assessed using line graphs. The data (colony diameters) for growth of the fungal isolates was analyzed using the GenStat software and the means for the isolates at various growth conditions were separated and grouped using Fisher's protected Least Significant Difference at ($P<0.05$).

CHAPTER FOUR

RESULTS

4.1: Weight loss outcomes

The weight loss data analysis per bacterial sample for the 30 micron poly-ethene revealed that bacterial sample A5, B2 and E1 exhibited significantly high weight loss as a result of bacterial degradation of poly-ethene (**Fig 4.1**) while fungal samples A5, B2, E1, E3 and D4 exhibited significantly high weight loss following fungal degradation of poly-ethene (**Fig 4.2**). These results are represented as mean % weight loss ± standard error (SE) with the weight loss caused by fungi being generally higher than that caused by bacteria (**Table 4.1**).

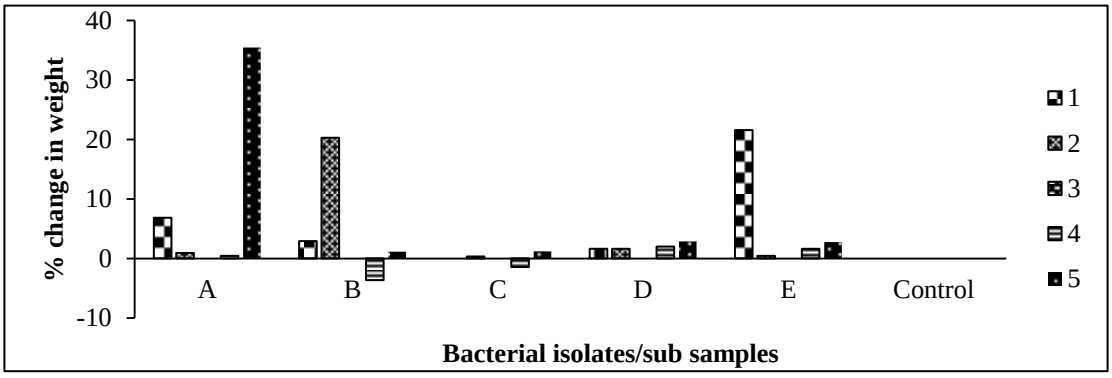


Figure 4.1: The % mean weight reduction of the 30 micron poly-ethene sheet incubated with bacterial samples following incubation at 37 ˚C for sixteen weeks. Each data point represents the average of three replicates ± SE.

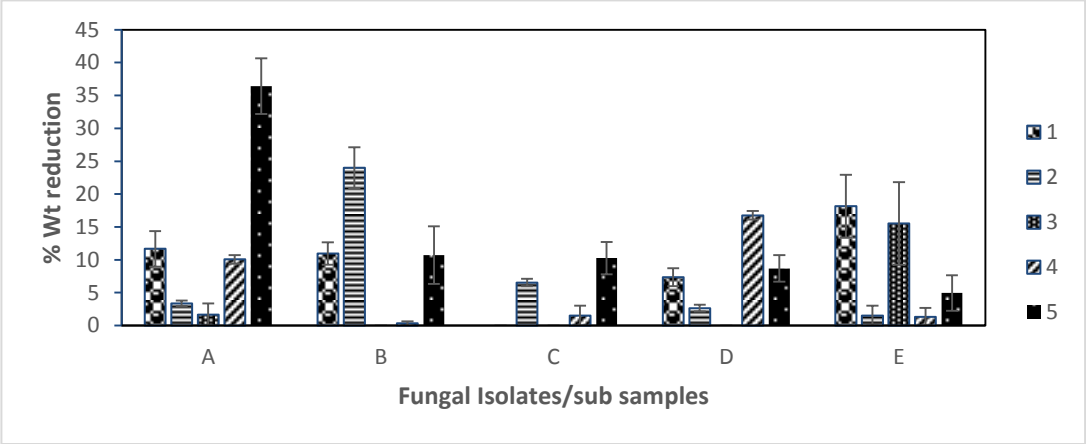


Figure 4.2: The % mean weight reduction of the 30 micron poly-ethene sheets incubated with fungal samples after incubation at 28 ˚C for sixteen weeks. Each data point represents the average of three replicates ± SE

Table 4.1: The 30 micron poly-ethene % reduction in weight (mg) following sixteen weeks incubation with fungal and bacterial isolates at 28˚C and 37 ˚C respectively. A negative mean indicates there was a weight gain instead of loss. Means with same superscript letters within the same column are not significantly different using Fisher's Protected Least Significant test at ($P<0.05$).

No.	Fungal Sample	Mean wt loss %	S.E	Bacterial Sample	Mean wt. loss %	S.E
	A1	11.44^{e}	1.58	A1	6.85^{cd}	0.67
	A2	3.71^{abc}	0.39	A2	0.83^{b}	0.18
	A3	3.38^{abc}	0.56	A3	0.37^{b}	0.37
	A4	10.08^{de}	0.71	A4	0.45^{b}	0.79
	A5	36.40^{g}	5.53	A5	35.72^{g}	4.01
	B1	10.79^{e}	0.99	B1	2.88^{bc}	0.38
	B2	24.10^{f}	3.26	B2	20.28^{e}	2.30
	B3	0.19^{a}	0.19	B3	0.37^{b}	0.06
	B4	0.31^{a}	0.19	B4	-4.88^{a}	1.23
	B5	14.00^{e}	1.79	B5	1.82^{bc}	0.30
	C1	6.55^{bcd}	0.07	C1	0.05^{b}	0.05
	C2	2.28^{ab}	0.24	C2	1.20^{bc}	0.24
	C3	3.37^{abc}	0.28	C3	0.13^{b}	0.13
	C4	0.33^{a}	0.20	C4	2.33^{bc}	0.38
	C5	10.27^{e}	0.98	C5	1.11^{bc}	0.10
	control	0^{a}	0	control	0^{b}	0

D1	2.65^{ab}	0.94	D1	2.48^{bc}	0.40
D2	2.08^{ab}	0.59	D2	2.62^{bc}	0.34
D3	0^{a}	0	D3	0^{b}	0
D4	16.77^{e}	0.57	D4	1.74^{bc}	0.27
D5	8.62^{e}	0.21	D5	4.66^{cd}	0.32
E1	18.43^{f}	2.20	E1	20.05^{e}	4.21
E2	1.564^{ab}	0.17	E2	0^{b}	0
E3	18.15^{f}	2.20	E3	1.01^{b}	0.10
E4	1.55^{ab}	0.80	E4	0.09^{b}	0.10
E5	5.65^{bcd}	1.024	E5	3.57^{bcd}	0.31
TOTAL	**210.92**			**115.58**	

The weight loss data analysis per bacterial and fungal sample for the 40 micron poly-ethene gives an indication that the fungal sample A5, B2, C5 and E1 exhibited significantly high weight loss as a result of fungal degradation of poly-ethene while bacterial samples A5, B1, B2 and E4 exhibited significantly high weight loss following bacterial degradation of poly-ethene (**Table 4.2**).

Table 4.2: The 40 micron poly-ethene % reduction in weight (mg) upon sixteen weeks incubation with various fungal and bacterial isolates at 28˚C and 37 ˚C respectively. Means with same superscript letters within the same column are not significantly different using Fisher's Protected Least Significant test at (P<0.05).

No.	Fungal Sample	Mean wt. loss %	SE	Bacterial Sample	Mean wt. loss %	SE
1	A1	7.73^{d}	0.18	A1	1.15^{ab}	0.23
2	A2	4.08^{bc}	0.12	A2	2.76^{bcd}	0.36
3	A3	4.25^{bc}	0.14	A3	1.93^{abc}	0.33
4	A4	7.43^{d}	0.59	A4	3.65^{cd}	0.41
5	A5	17.00^{h}	0.97	A5	11.43^{h}	0.57
6	B1	10.09^{e}	0.25	B1	10.18^{gh}	0.51
7	B2	14.03^{g}	0.15	B2	8.46^{fg}	1.22
8	B3	0^{a}	0	B3	0^{a}	0
9	B4	7.86^{d}	0.18	B4	2.98^{bcd}	0.30
10	B5	7.03^{d}	0.53	B5	2.94^{bcd}	0.50
11	C1	0^{a}	0	C1	1.12^{ab}	0.62

12	C2	6.67^{d}	0.34	C2	8.27^{fg}	1.13
13	C3	0a	0	C3	0^{a}	0
14	C4	3.44^{b}	0.29	C4	2.77^{bcd}	0.19
15	C5	12.34^{f}	0.27	C5	4.35^{de}	0.24
16	control	0^{a}	0	control	0^{a}	0
17	D1	7.87^{d}	0.94	D1	4.44^{de}	0.55
18	D2	2.94^{b}	0.41	D2	3.28^{bcd}	0.62
19	D3	0^{a}	0	D3	0^{a}	0
20	D4	10.01^{e}	0.45	D4	4.13^{de}	3.26
21	D5	10.41^{e}	0.38	D5	7.24^{f}	0.97
22	E1	11.99^{ef}	0.37	E1	7.55^{f}	0.21
23	E2	4.20^{bc}	0.16	E2	0^{a}	0
24	E3	0^{a}	0	E3	2.09^{abcd}	0.20
25	E4	10.80^{e}	0.36	E4	8.13^{fe}	0.30
26	E5	11.36^{ef}	1.54	E5	6.33^{ef}	0.33

A comparative analysis of the % weight loss of the 30 micron poly-ethene per sampling point. Data for fungal and bacterial samples poly-ethene weight reduction per sampling point indicates that weight loss attributed to fungal samples was generally higher than weight loss attributed to bacterial samples with point A exhibiting the highest loss of 12.65% for fungal and 8.73% for bacterial samples (**Fig 4.3**). The weight loss for the 40 micron poly-ethene per sampling point also gives an indication that the fungal degradation is greater than the bacterial degradation with a highest fungal weight loss of 6.92% and the highest bacterial weight loss of 2.12% (**Fig 4.4**)

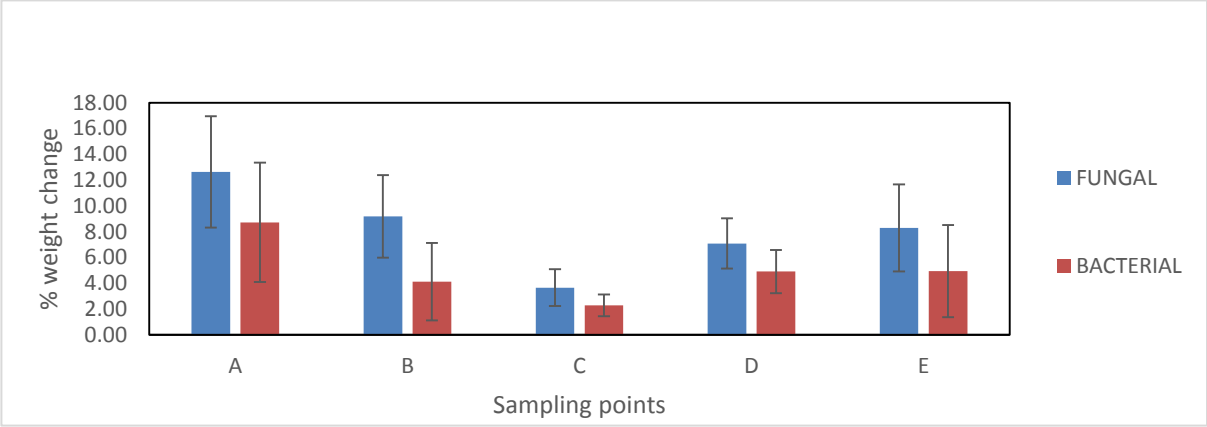


Figure 4.3: A comparison of bacterial and fungal % reduction in mean weight of the 30 micron poly-ethene sheets between sampling points after sixteen weeks incubation at 37 ˚C and 28˚C respectively. Each data point represents the average of three replicates ± SE

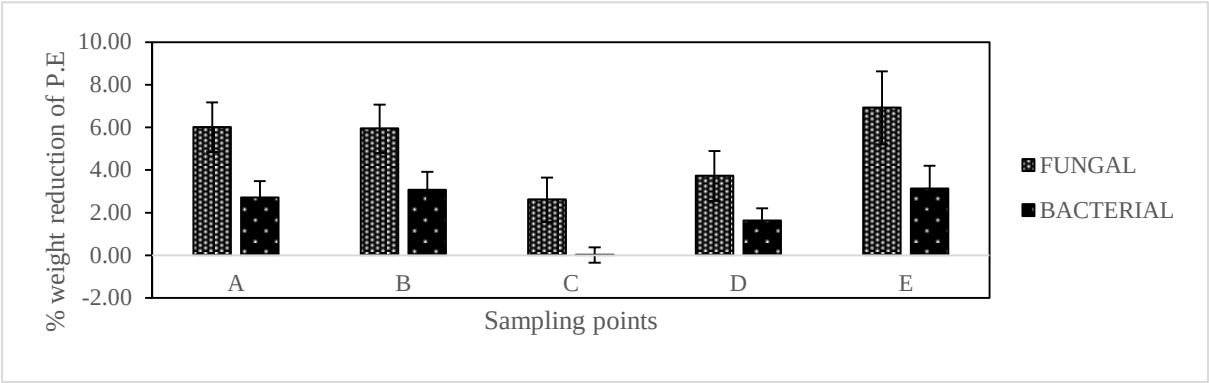


Figure 4.4: A comparison of bacterial and fungal % reduction in mean weight of the 40 micron poly-ethene sheets between sampling points after sixteen weeks incubation at 37 ˚C and 28˚C respectively. Each data point represents the average of three replicates ± SE

The % weight loss from the fungal samples for the 30 micron and 40 micron poly-ethene between sampling points was compared and it indicates that there was a significant difference between the weight loss of the 30 and 40 micron poly-ethene with the 30 micron weight loss being significantly higher than that of the 40 micron at sampling point A (**Fig. 4.5).** The weight loss % data attributed to the bacterial samples was also

compared for both the 30 and 40 micron poly-ethenes and **Fig 4.6** reveals that the weight loss of the 30 micron poly-ethene was significantly higher than that of the 40 micron poly-ethene at sampling point A.

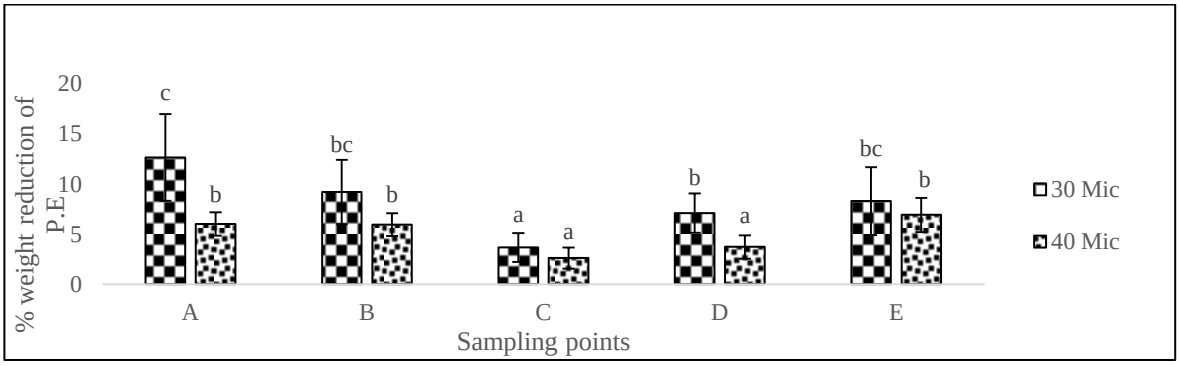


Figure 4.5: A comparison of % mean weight reduction of the 30 micron and 40 micron poly-ethene sheets incubated with fungal inoculums from sampling points A,B, C,D and E at 28 ˚C for sixteen weeks. Each data point represents the average of three replicates ± SE

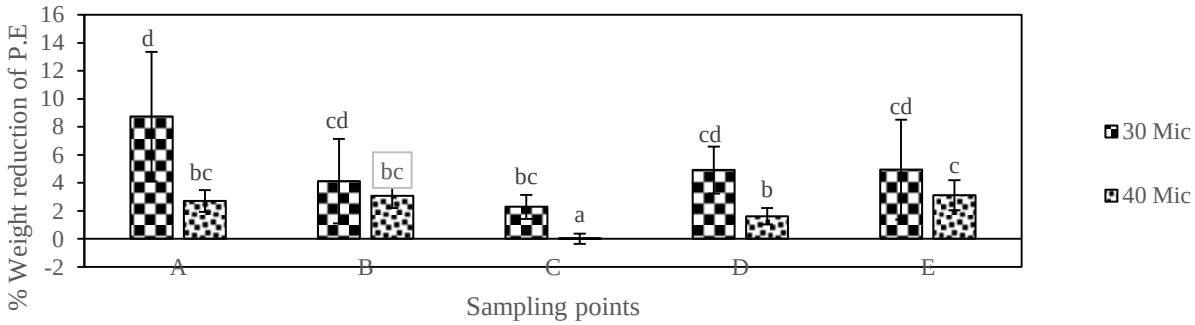


Figure 4.6: A comparison between % mean weight reduction of the 30 micron and 40 micron poly-ethene sheets incubated with bacterial inoculums from sampling points A,B, C,D and E at 37 ˚C for sixteen weeks. Each data point represents the average of three replicates ± SE.

4.2: The FT-IR Outcomes

The Fourier Transform-Infra-Red spectra were superimposed gainst the control samples of the poly-ethene sheets incubated without inoculum. The spectra of the treatments attributed to isolates *Pseudomonas putida* (MG645383), *Bacillus cereus* (MG645253) and *Aspergillus nidulans* (MG779504) (**Fig 4.7, 4.8 and 4.9)** show the apearance of new functional groups in the region between 1650 and 1800 indicative of the formation of aldehydes and/or ketones which are intermediary products of this process. Also in the spectral region from 1000-1300 cm^{-1}, there emerges new functional groups indicatig the possible presence of alcohols, Carboxylic acid, esters and ethers which too are inermediary products of poly-ethene degradation. The huge peaks in the spectral region beween 3000-2850 and 1470-1450 represent alkanes and alkenes which are the main components of the poly-ethene material.In the spectra obtained from poly-ethene powder, (**Figures 4.10 and 4.11)** there is the apearance of new peaks in the region between 1200-900 cm^{-1} indicating possible apearance alcohols, Carboxylic acid, esters and ethers functional groups. In some treatments, there was no appearance of new peaks indicating lack of biodegradation **(Appendix 4).**

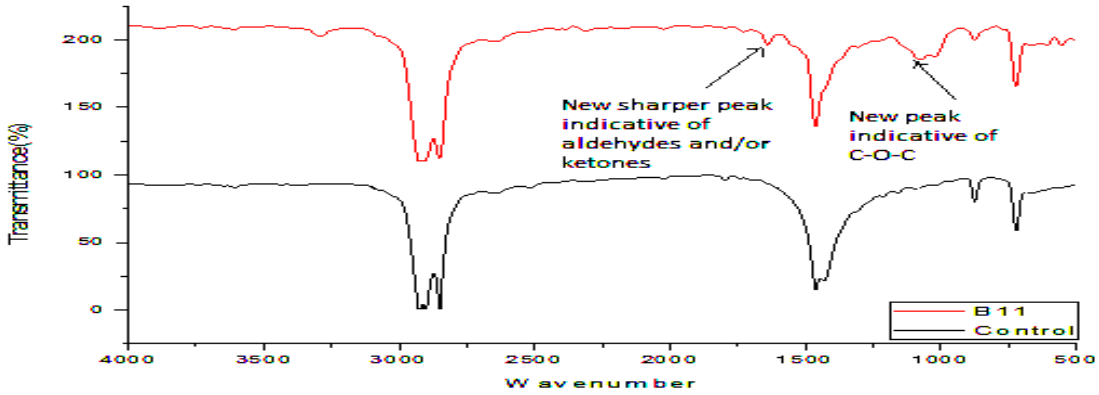


Figure 4.7: The FT-IR spectra of poly-ethene sheet from sample B1, 1 with bacterial inoculum containing *Pseudomonas putida* (MG645383) and the control

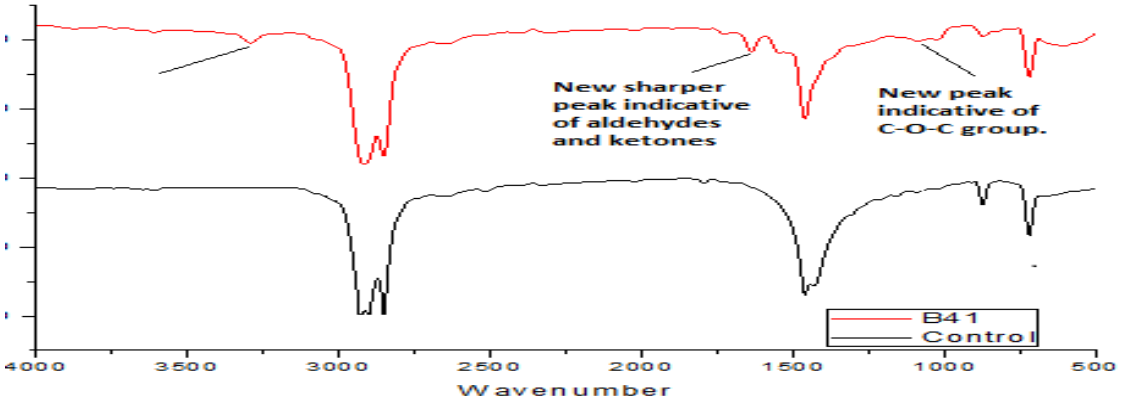


Figure 4.8: The FT-IR spectra of poly-ethene sheet from sample B,4,1 with bacterial inoculum containing *Bacillus cereus* (MG645253) and the control.

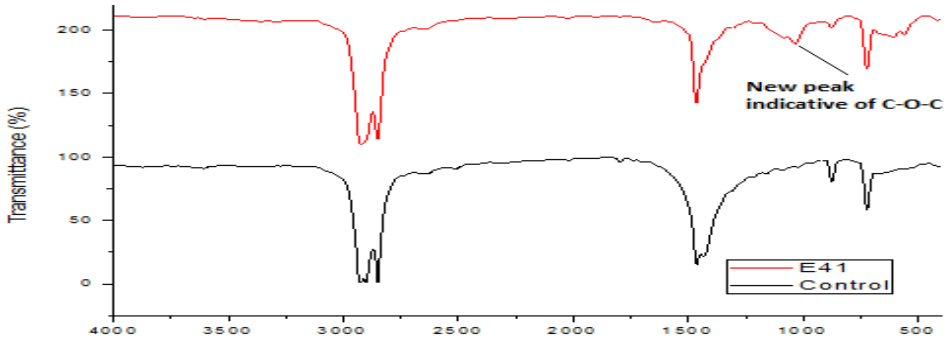


Figure 4.9: The FT-IR spectra of poly-ethene sheet from fungal sample E4,1 inoculum containing *Aspergillus nidulans* strain voucher MF 109 (MG779504)and the control.

Figure 4.10: The FT-IR spectra of poly-ethene powder from sample A5, 1 with fungal inoculum containing *Aspergillus oryzae* strain RIB40 (MG779508 and the Control

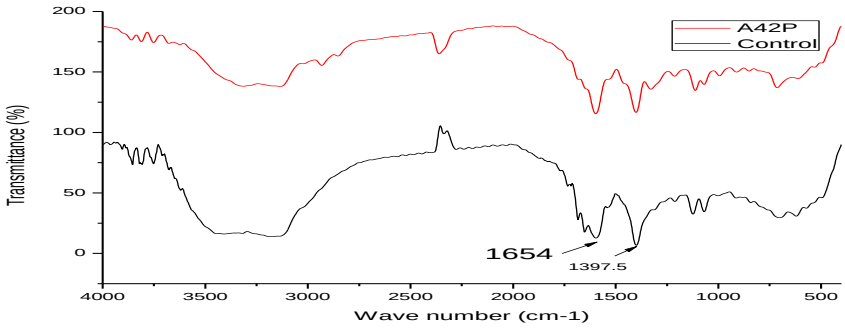


Figure 4.11: The FT-IR spectra of poly-ethene powder from sample A4, 2 with fungal inoculum containing *Aspergillus flavus* strain AD-Jt-1 (MG779506) and the Control

The GC-MS outcomes

The gas chromatogram output from one of the samples indicated the presence of a ketone (4,6-Octadiyn-3-one, 2-methyl) with a retention time of 4.209 min and an alkene 4,6-Octadiyn-3-one, 2-methyl at a retention time of 4.750 min**.** Ketones are intermediary products in poly-ethene degradation.

4.3: Characterization and Identification

Based on the indicators of Poly-ethene biodegradation, the respective incubation flasks containing artificial media plus inoculum from soil were subjected to isolation of bacteria and fungi. A total of 30 bacterial isolates were isolated based on morphology and biochemical tests and gram staining (**Appendix 5**). Among this 7 were Gram negative while 23 were Gram positive. A total of 26 fungal isolates were isolated. Among these 20 were macroscopically and microscopically characterized to belong to the genus *Aspergillu*s while six were characterized as belonging to the genus *Penicillium*. A representative photograph of the outcome of lactophenol blue staining of some of the fungal isolates and a plate culture are shown (**Fig 4.12**).

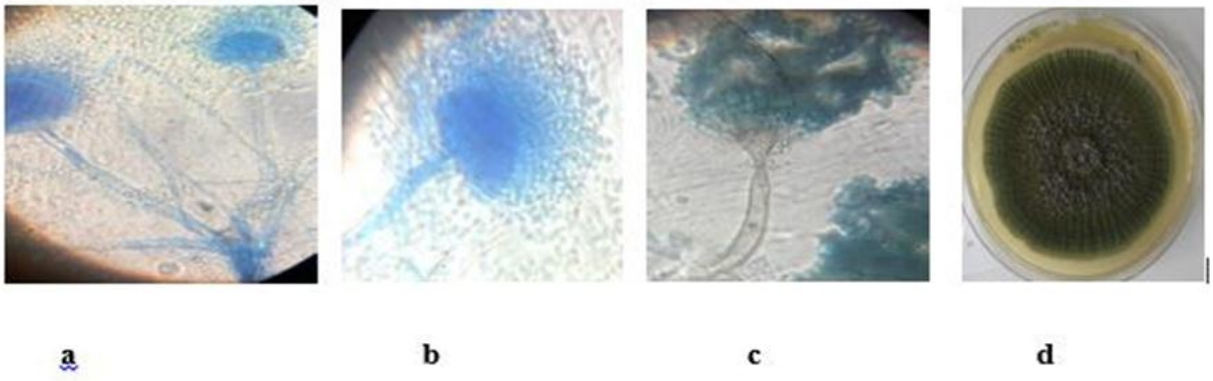


Figure 4.12: Microscopic examination of representative fungal isolates: *a* and *b* are isolates with erect conidia and aerial conidiophores characteristic of genus *Aspergillus*. *C* has a simple conidiophore terminated by flask shaped phialides where spores are produced in chains at

the tip end characteristic of genus *Penicillium* while *d* is a 5-day old fungal growth of isolate B2,2, a.

Amplification of bacterial 16S r DNA using 1492R and 8F universal primers yielded the expected band size of approximately 1420 bps from the PCR products of all the samples (**Fig 4.13**). These products were purified, sequenced and analyzed. The results were used to obtain accession numbers from NCBI GenBank (**Table 4.3**).

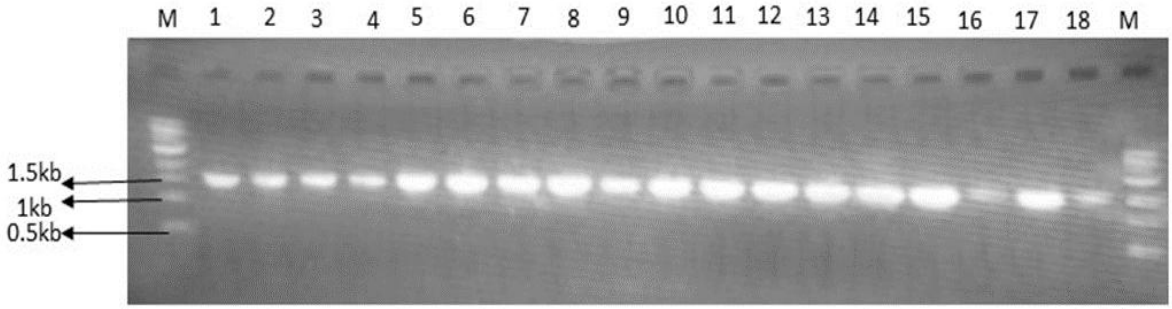


Figure 4.13: Amplification of 16S r DNA for the bacterial isolates 1-18 using 1492R and 8F universal primers. M represents a 1 kb marker. The expected band size amplified is 1420 bps

Table 4.3: Accession numbers and Percentage sequence similarity of bacterial isolates with their closest taxonomic relatives in NCBI GenBank

No.	Sequence -ID	Isolate code	Accession number	Organism	% Similarity
1	1	E1,2a	MG645252	*Bacillus cereus*	99%
2	2	B4,1 pn	MG645253	*Bacillus cereus*	99%
3	3	C2,2a	MG645254	*Brevibacillus parabrevis*	100%
4	4	B4, 2yn	MG645255	*Bacillus cereus*	99%
5	6	A5,a1	MG645256	*Bacillus cereus*	99%
6	7	D4, 1n	MG645257	*Bacillus toyonensis*	98%
7	9	E5,a1	MG645258	*Bacillus thuringiensis*	99%
8	10	B1,2	MG645259	*Bacillus thuringiensis*	99%
9	11	E4,1	MG645260	*Bacillus subtilis*	98%
10	12	D4,yn	MG645261	*Brevibacillus borstelensis*	99%

11	13	E4,1,2	MG645262	*Ochrobactrum pseudintermedium*	99%
12	14	C4,1a	MG645263	*Lysinibacillus macroides*	99%
13	15	A1, a	MG645264	*Bacillus cereus*	98%
14	17	A2,2	MG645265	*Bacillus pseudomycoides*	99%
15	19	C4,1a	MG645266	*Cellulosimicrobium funkei*	97%
16	20	B2,2	MG645267	*Brevibacillus borstelensis*	98%
17	24	B,4,2	MG645268	*Bacillus safensis*	99%
18	25	B,2,2a	MG645269	*Bacillus safensis*	99%
19	26	B1, 1a	MG645383	*Pseudomonas putida*	98%
20	29	C5,1a	MG645270	*Bacillus niacini*	98%

The analyzed sequences were aligned with those of the closest neighbors using ClustaX version 2.1. Phylogenetic relationships were inferred from phylogenetic comparison of the 16S rRNA sequences using Mega 7 and maximum-likelihood algorithms to generate the phylogenetic tree (**Fig 4.14**) shows the phylogenetic relationships among the genera and species. *Brevibacillus, Bacillus* and *Lysinibacillus* are in one major clade while *Pseudomonas, Ochrobactrum* and *Cellulosimicrobioum* are in grouped in another major clade.

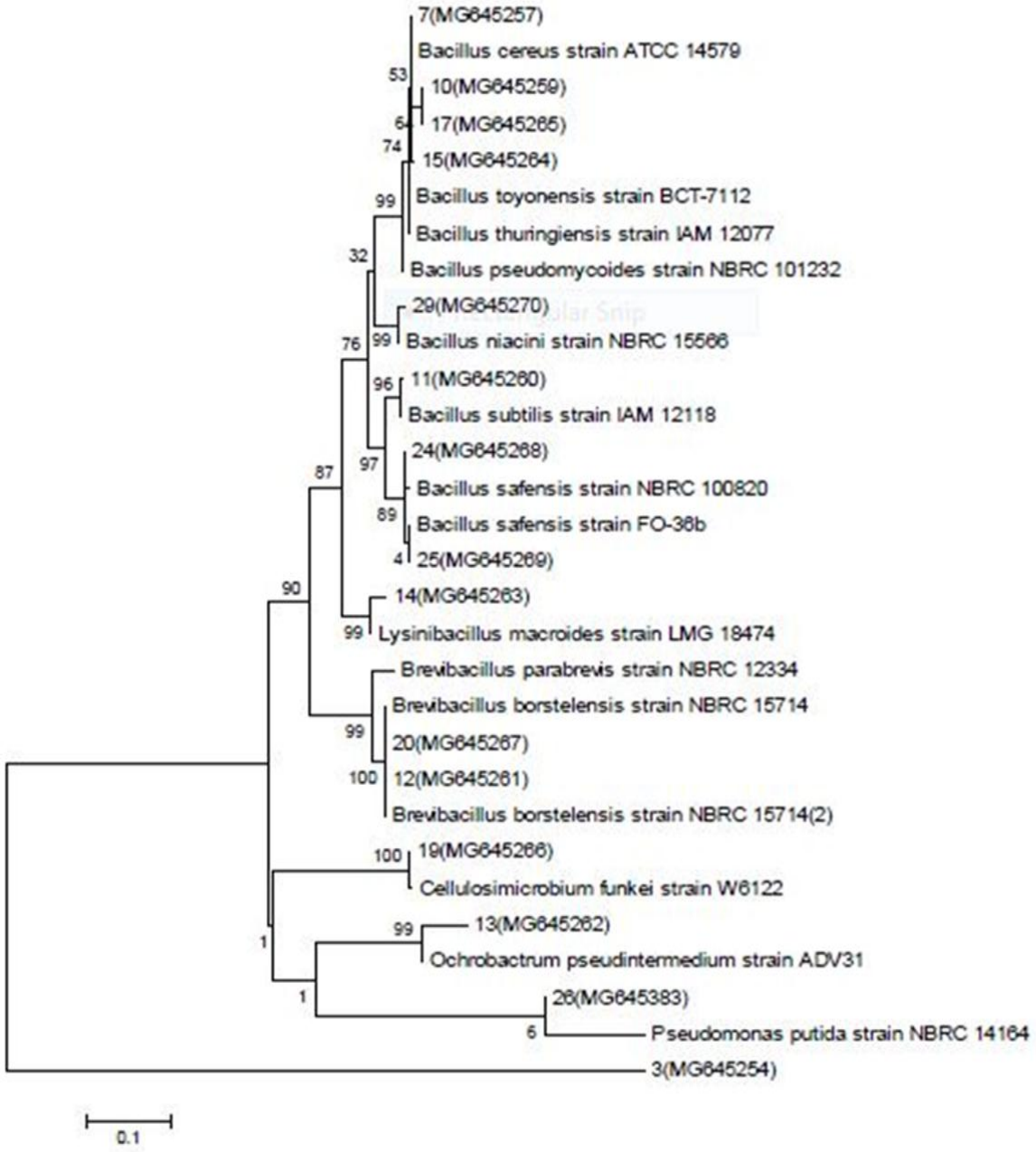


Figure 4.14: Phylogenetic tree generated by MEGA7 for 16S rDNA sequences of the bacterial isolates found to be effective bacterial degraders. All screened bacterial isolates have NCBI accession codes in brackets. The scale bar refers to 0.02 substitutions per nucleotide position. Bootstrap values obtained with 1000 resampling are referred to as percentages at all branches

Amplification of fungal 18S rDNA using 1200R and 566F universal primers yielded the expected band size of approximately 640 bps from the PCR products of the amplified samples (**Fig 4.15**). These products were purified, sequenced and analyzed. The results were used to obtain accession numbers from NCBI GenBank (**Table 4.4**).

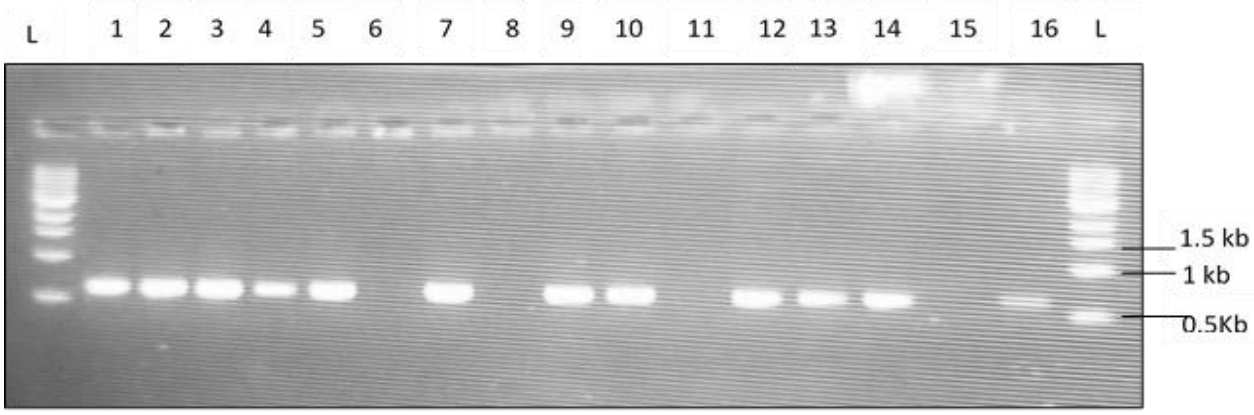


Figure 4.15:The PCR products for the amplification of 18S r DNA for the fungal isolates 1, 2, 3, 4, 5, 7, 9, 10, 12, 13, 14 and 16 using 1200R and 566F universal primers. L represents a 1 kb ladder. The expected band size amplified is 640 bps.

Table 4.4: Accession numbers and Percentage sequence similarity of Fungal isolates with their closest taxonomic relatives in NCBI GenBank

No.	**Sequence -ID**	**Isolate code**	**NCBI Accession number**	**Organism**	**% Similarity**
1	2	E4,1	MG779504	*Aspergillus nidulans* strain voucher MF 109	100**%**
2	4	A1,1	MG779505	*Aspergillus insuetus* strain JAU1	100**%**
3	5	A4,2	MG779506	*Aspergillus flavus* strain AD-Jt-1	100**%**
4	6	C5,1	MG779507	*Aspergillus nidulans* strain Ya10	99**%**
5	8	A5,1	MG779508	*Aspergillus oryzae* strain RIB40	99**%**
6	10	D5,2	MG779509	*Aspergillus flavus* strain Ya1	100**%**
7	12	B5, 1	MG779510	*Aspergillus neoflavipes* strain AJR1	100**%**
8	14	E1,2	MG779511	*Aspergillus nidulans* strain FGSC A4	100**%**
9	15	D4,2	MG779512	*Aspergillus terreus* strain BTK-1	99**%**
10	16	B2,2	MG779513	*Aspergillus fumigatus* strain T3	99**%**

The analyzed sequences were aligned with those of the closest neighbors using ClustaX version 2.1. Phylogenetic relationships were inferred from phylogenetic comparison of the 18S rRNA sequences using Mega 7 and maximum-likelihood algorithms to generate the phylogenetic tree (**Figure 4.16**) which shows the phylogenetic relationships among the various *Aspergillus* species. The tree displays four clades in which the isolates have been clustered.

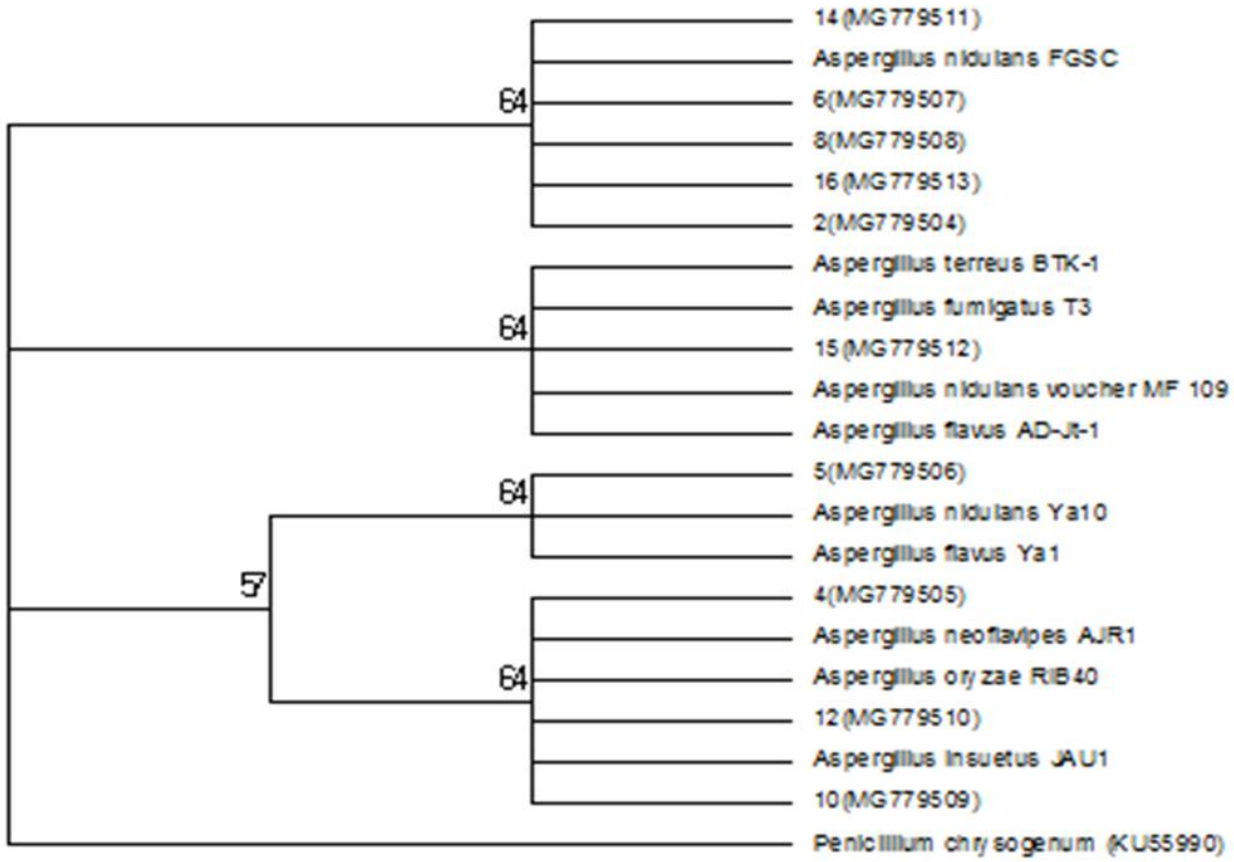


Figure 4.16: Phylogenetic tree of fungal isolates based on 18S rDNA sequences. All screened fungal isolates have NCBI accession codes in brackets. The scale bar refers to 0.007 substitutions per nucleotide position. Bootstrap values obtained with 1000 resampling are referred to as percentages at all branches.

4.4: Screening for enzyme production

Bacterial isolates were screened for production of enzymes laccase and esterase which are among the enzymes implicated in LDPE degradation. Bacterial isolates *Brevibacillus borstelensis* strain B2,2 (9.0±0.51), *Bacillus safensis* strain B4,2 (10±1.15) and *Pseudomonas putida* strain B1,1(9.6±1.20) exhibited the highest laccase activity. Only two isolates-*Bacillus. toyonensis* and *Bacillus. macrolides* were negative for laccase activity(**Figure 4.17**). Esterase activity was highest in isolates *Bacillus safensis* strain B4,2 (10±1.15470), Bacillus niacin strain C5,1a (9.67±1.2) and Bacillus cereus strain A5,a1 (9.33±0.33).

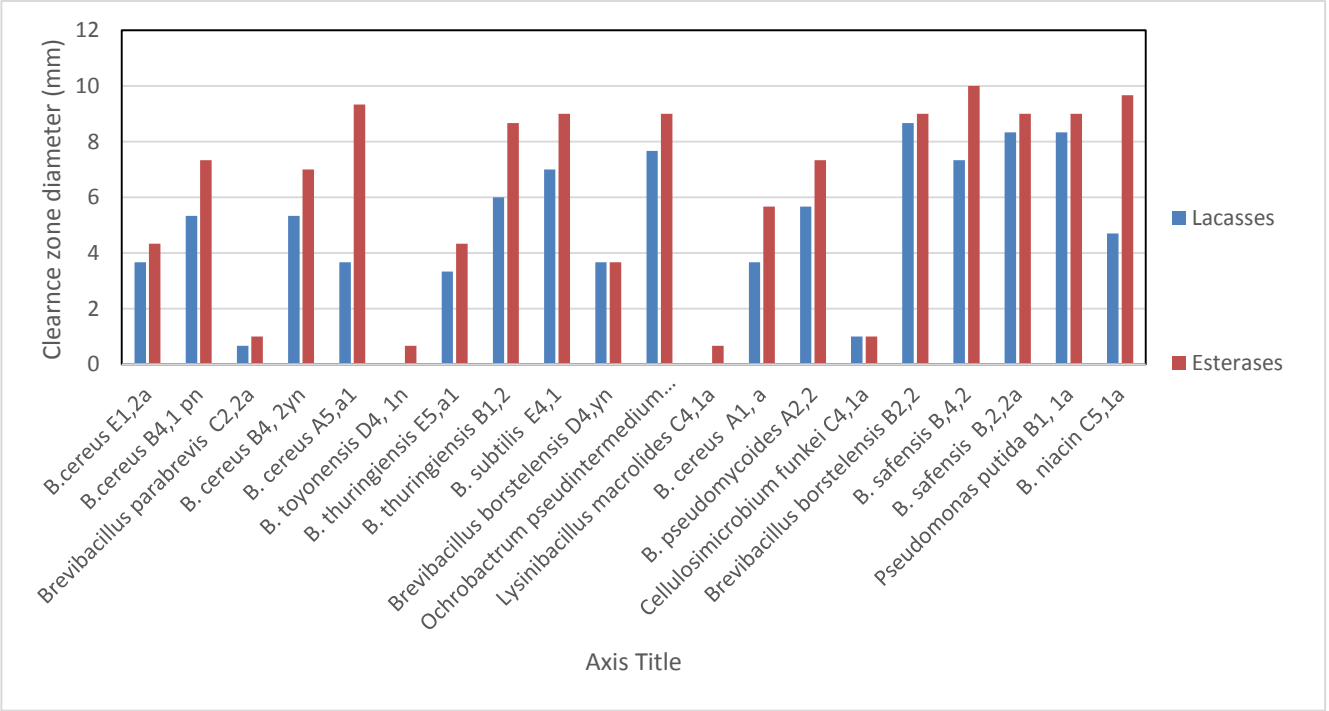


Figure 4.17: Presence of enzymes Laccase and Esterase among the bacterial isolates. Growth of bacterial isolates was measured as colony diameter (mm). Means were grouped using Tukey's Honest Significant Difference test at ($P<0.05$).

Fungal isolates were screened for production of enzymes laccase, esterase, xylanase and pectinase. Isolates B2, 2-*Aspergillus fumigatus*, A5,1-*Aspergillus oryzae* and A4,2a-*Aspergillus flavus* exhibited the highest laccase activity (**Fig 4.18**). The Highest esterase activity was attributed to fungi *Aspergillus Oryzae*.

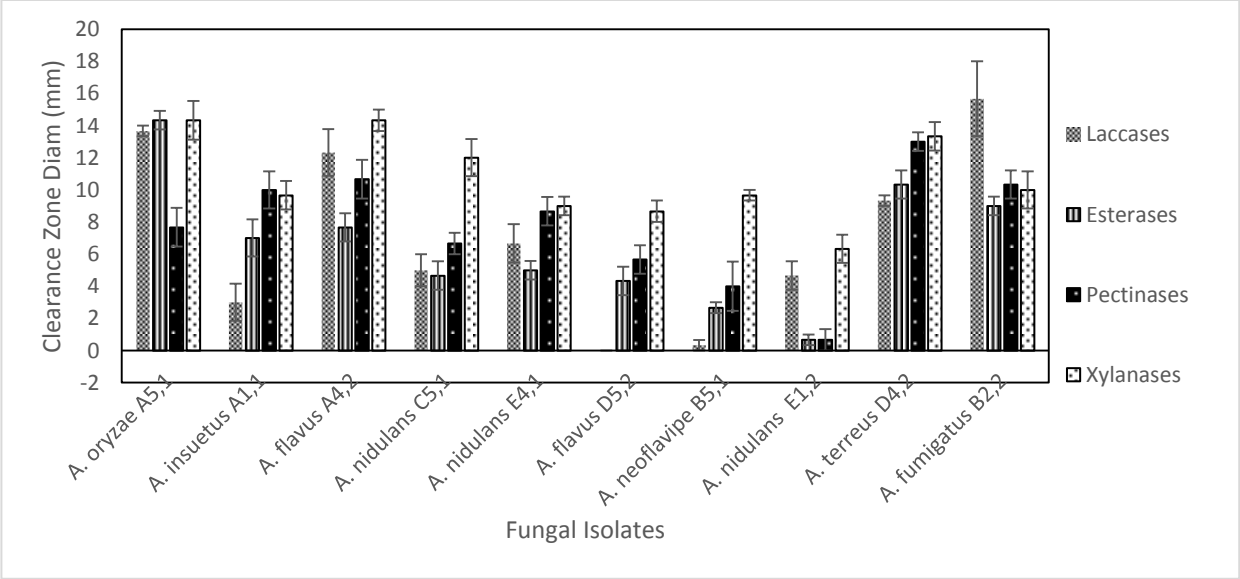


Figure 4.18: Presence of enzymes Laccase, Esterase, Pectinase and Xylanase among the fungal isolates. Growth of fungal isolates was measured as colony diameter (mm). Means were grouped using Tukey's Honest Significant Difference test at ($P<0.05$).

The appearance of the microbial colonies on agar plates for various enzyme tests is illustrated. Esterase presence on fungal plates after 3 days incubation at 28˚C was exhibited by the clearance zone (whitish) around the colony (**Fig 4.19**). Laccase presence on fungal plates after 5 days incubation at 28˚C was exhibited by the reddish zone around the colony (**Fig 4.20**). Xylanase presence on fungal plates after 3 days incubation at 28˚C was indicated through formation of the halo around the colony (**Fig**

4.21). Presence of enzyme pectinase on fungal plates was exhibited by formation of a clearance zone around the colony which was in form of a ring (**Fig 22**).

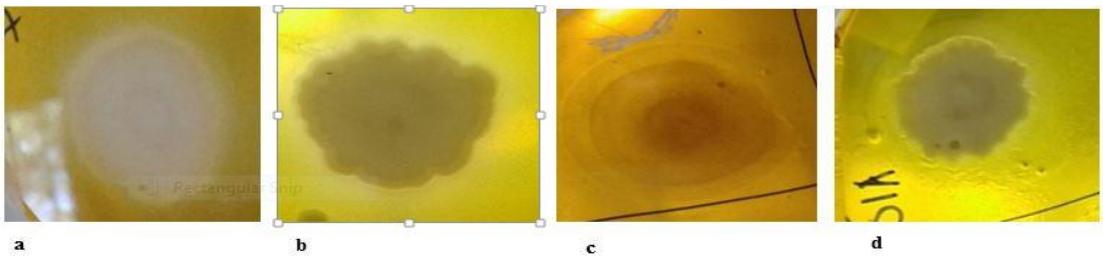


Figure 4.19: Presence of enzyme esterase in fungal samples a.E4,1, b.A1,1, c.A4,2 and d.A5,1 The clearance zone (whitish) around the inoculum indicates esterase activity.

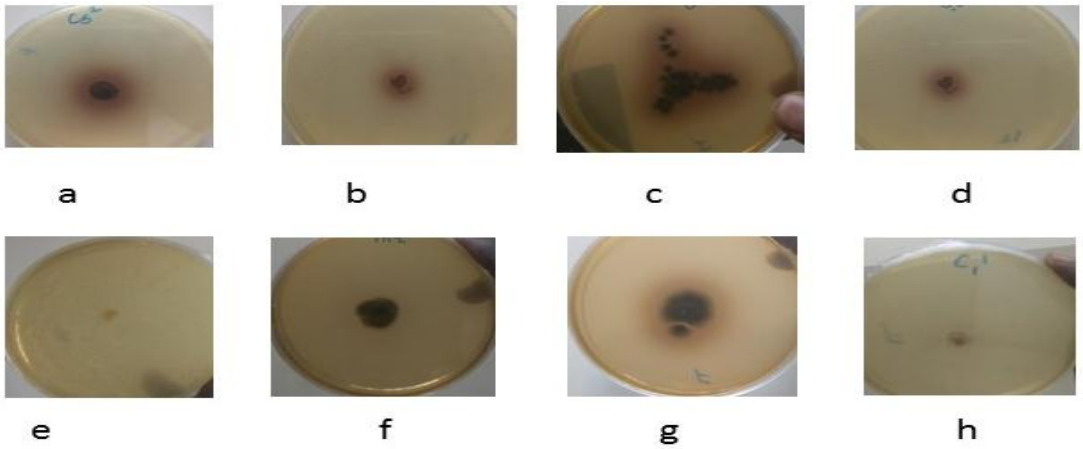


Figure 4.20: Presence of enzyme laccase in fungal samples a.C5,1, b.A4,2, c.A5,1, d.D5,2, e.B5, 1, f.E1,2, g.D4,2 and h.B2,2The reddish zone around the inoculum indicates laccase activity.

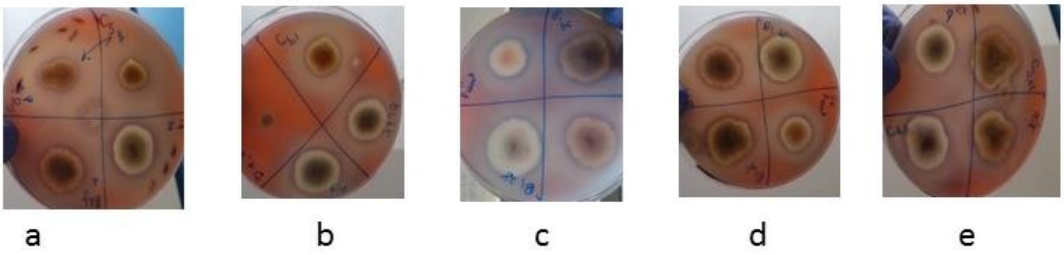


Figure 4.21: Presence of enzyme xylanase in fungal samples: a.A4,2, b.C5,1, c.A5,1, d.D5,2 and e.B5, 1,The clearance zone around the inoculum indicates xylanase activity.

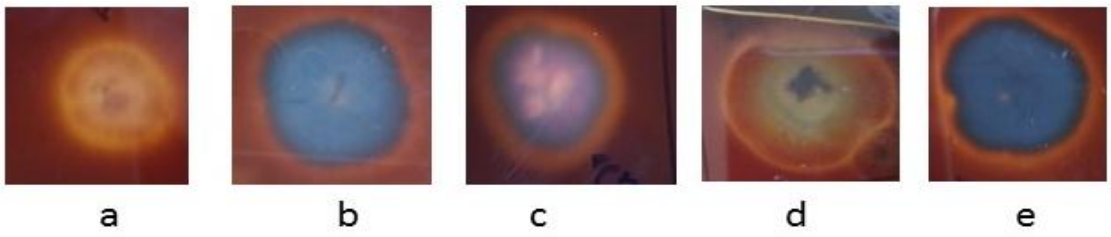


Figure 4.22: Presence of enzyme pectinase in fungal samples a.A4,2, b.C5,1, c.A5,1, d.D5,2 and e.B5, 1. The clearance zone around the inoculum indicates pectinase activity

4.5: Screening for AlkB genes producing alkane degrading enzymes

PCR to amplify Alk B genes was done using three sets of Alk B primers (**Table 3.1**). Only one set of the Alk B primers-AlkB 1 set 1 was able to amplify the Alk B gene producing a fragment of size 870 bps. Alk B genes are responsible for production hydrolase enzymes which are responsible for alkane degradation. The gene was amplified in all the selected fungal samples (**Fig 4.23**) while only 4 bacterial samples were amplified (**Fig 4.24**).

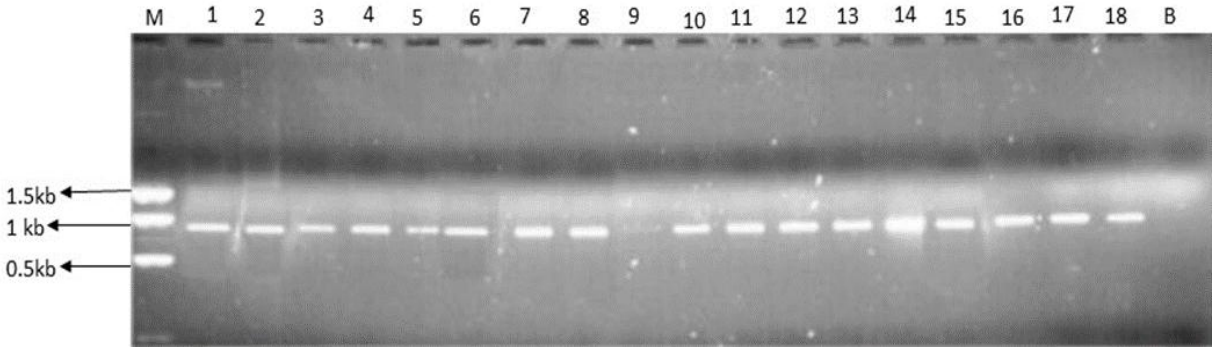


Figure 4.23: The PCR products for the amplification of Alk b for the fungal isolates 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17 and 18 using Alk B1 set 1 primers. Lane 1 represents a 1 kb ladder. The expected band size amplified is 870 bps.

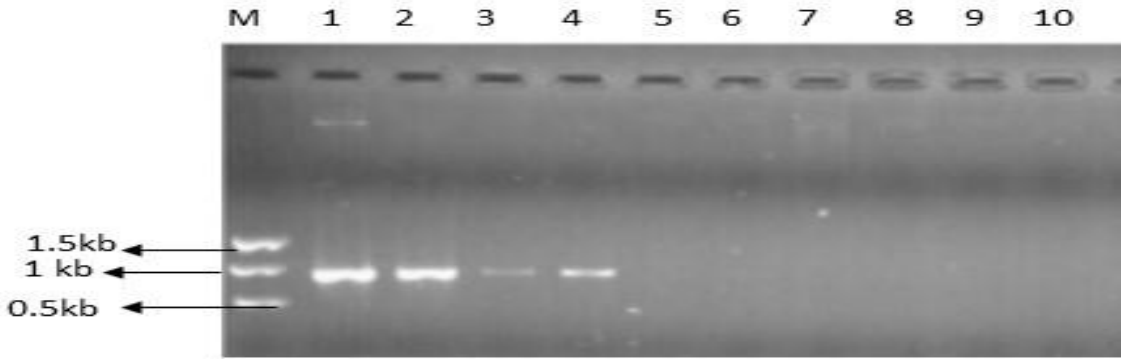


Figure 4.24:The PCR products for the amplification of Alk b for the bacterial isolates 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 using Alk B1 set 1 primers. Lane 1 represents a 1 kb ladder. The expected band size amplified is 870 bps.

4.6: Determination of growth conditions of bacterial isolates

Optical density of the bacterial isolates was taken at different pH levels for selected isolates. At pH 10, there was a general decline in the optical density of all the isolates (**Fig 4.25**). At pH 8, there was a steady but slow increase in the optical density for the isolates (**Fig 4.26**). At pH 6, the optical density increased for the isolates with isolate 2,2 exhibiting the highest final O.D of 0.89 (**Fig 4.27**).

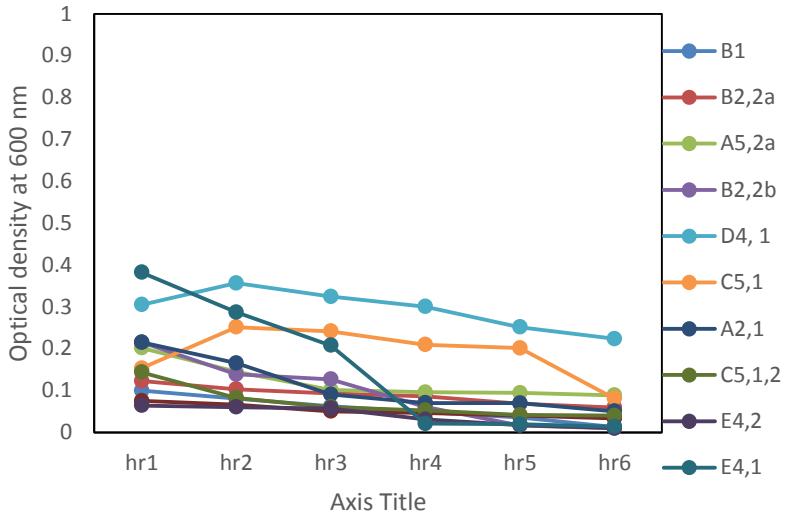


Figure 2.25: Growth rate of bacterial isolates at pH 10

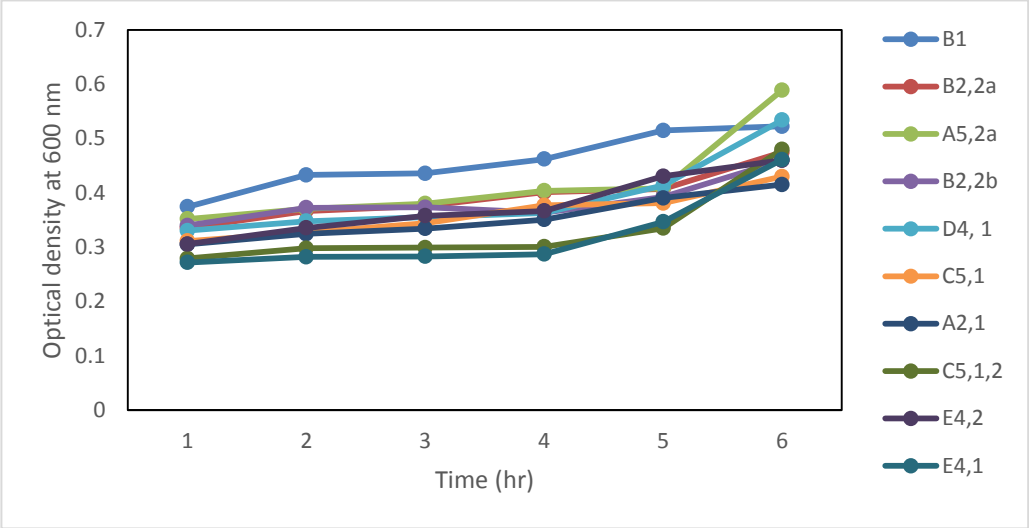


Figure 2.26: Growth rate of bacterial isolates at pH 8

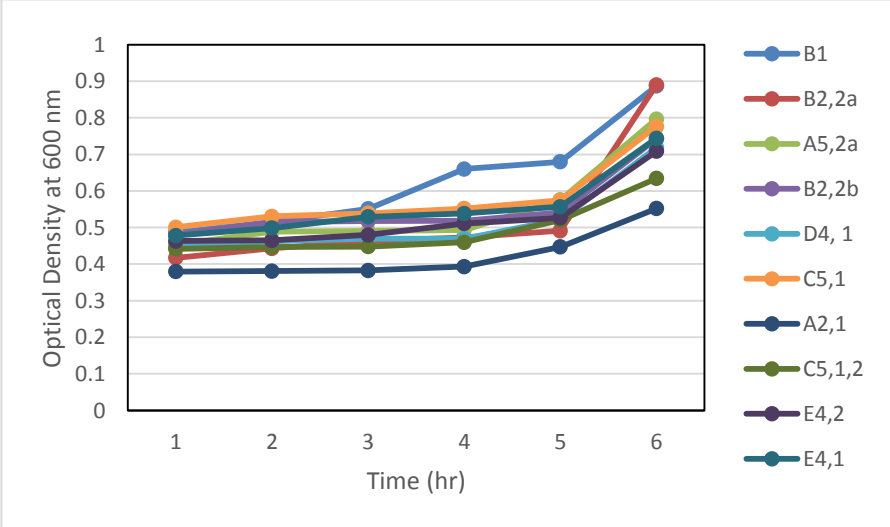


Figure 4.27: Growth rate of bacterial isolates at pH 6

Optical density of the bacterial isolates was taken at different temperatures for selected isolates. At 30˚C and 40˚C, there was a steady increase the optical density of all the

isolates (**Fig 4.28 and 4.29**). The highest OD of 0.753 was recorded at 40˚C for isolate C5,1. At pH 20˚C, there was a decline in OD in most of the isolates (**Fig 4.30**)

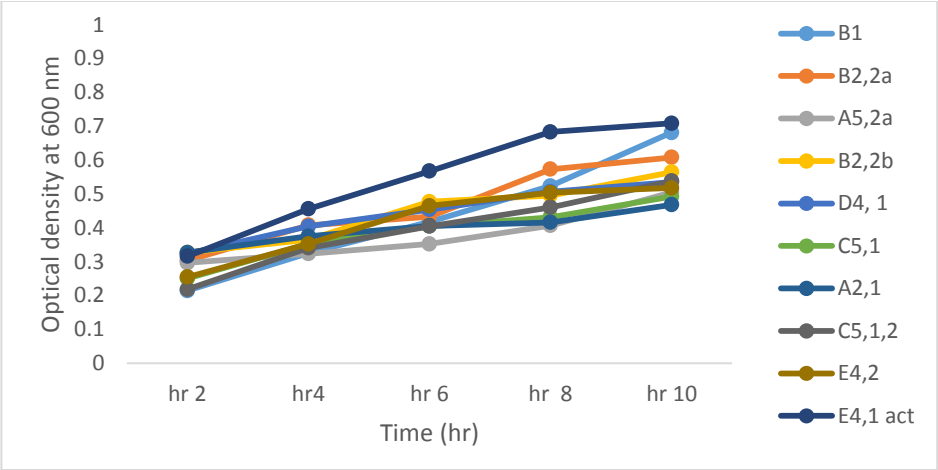


Figure 4.28: Growth rate of bacterial isolates at 30˚C

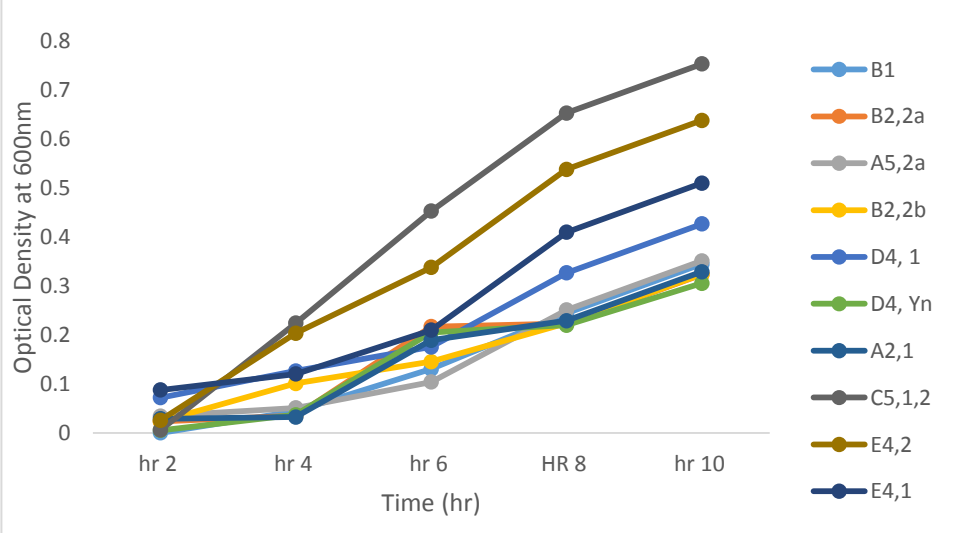


Figure 4.29: Growth rate of bacterial isolates at 40˚C

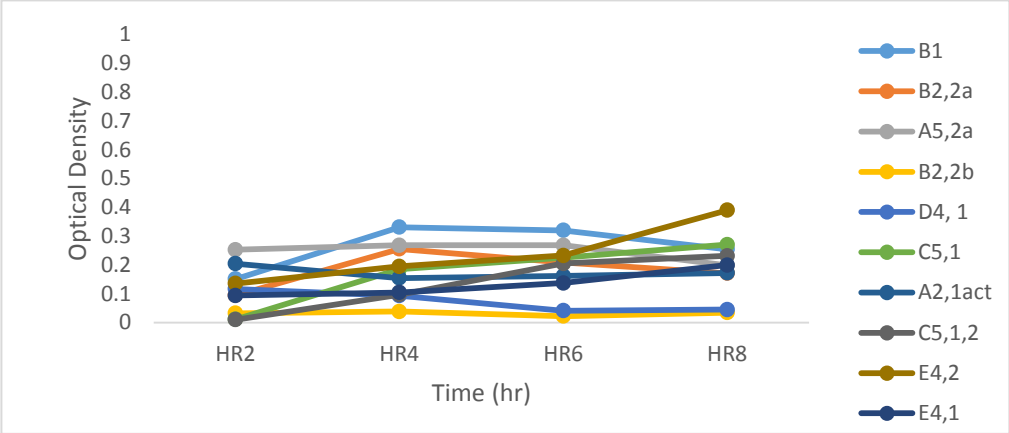


Figure 4.30: Growth rate of bacterial isolates at 20˚C

Optical density of the bacterial isolates was recorded at different NaCl concentrations for selected isolates. At 10% NaCl concentration, there was a decline in the optical density of all the isolates (**Fig 4.31**). At 0% NaCl concentration, there was a rise in the optical density of the bacterial isolates (**Fig 4.32**).

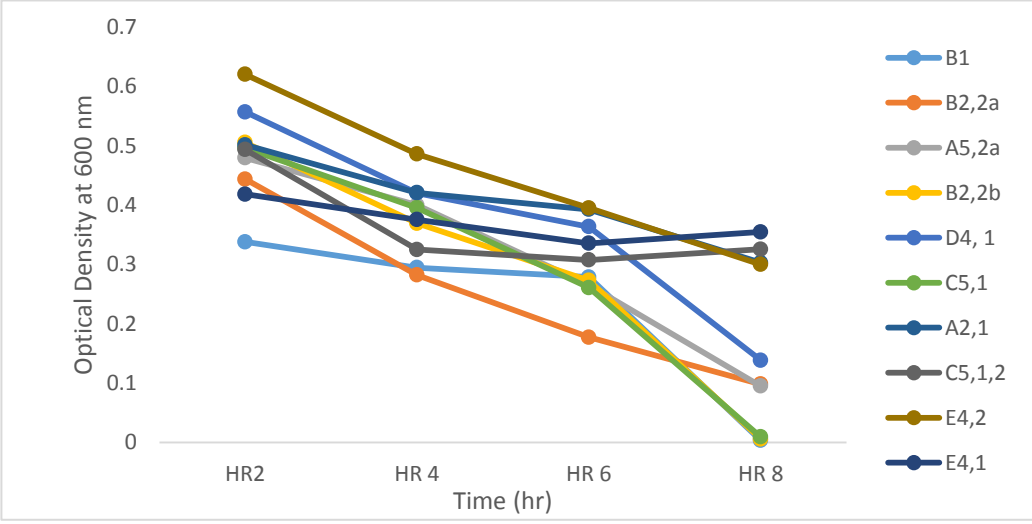


Figure 4.31: Growth rate of bacterial isolates at 10% NaCl concentration

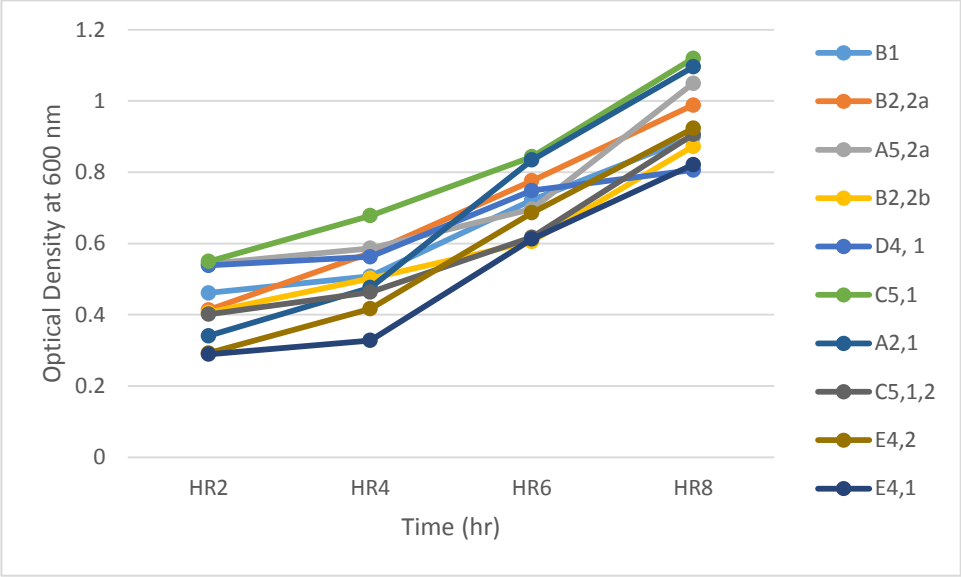


Figure 4.32: Growth rate of bacterial isolates at 0% NaCl concentration

4.7: Determination of growth conditions of fungal isolates

Fungal isolates were screened for their growth at varying pH, Temperature and salt concentration. The growth of fungi at different pH levels (**Fig 4.33**) gives an indication that 8 out of the 10 selected isolates grew optimally at pH 8 with the exception of *A. neoflavipes* strain B5,1 and *A. nidulans* strain E4,1 both which grew optimally at pH 10. *A. fumigatus* strain B2,2 exhibited a significantly high rate of growth (10±0.33) at pH 8 (**Table 4.5**). The slowest growth was recorded at pH 6 in all the isolates with the exception of *A. flavus* strain A4,1 which exhibited a growth of 7±0.57.

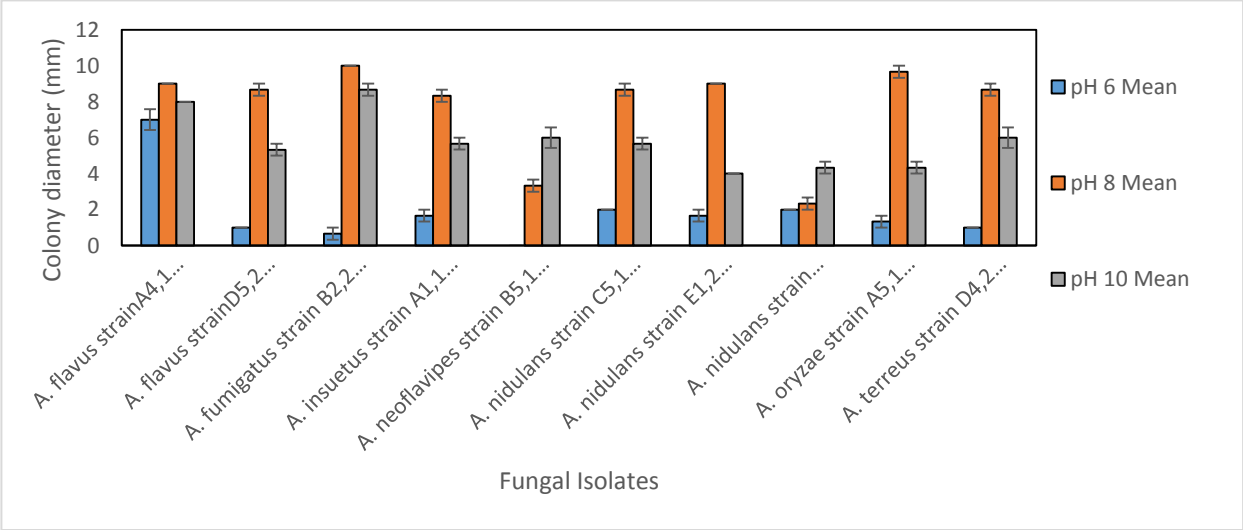


Figure 4.33: Growth of fungal isolates at pH 6, pH 8 and pH 10 respectively.

Table 4.5: Growth of fungal isolates at pH 6, pH 8 and pH 10 respectively measured as colony diameter (mm). Means with same superscript letters within the same column are not significantly different and vice versa using Fisher's Protected Least Significant test at (P<0.05)

Isolates_ID	pH 6 Mean	S.E	pH 8 Mean	S.E.	pH 10 Mean	S.E
A. flavus strainA4,1 (MG779506)	7e	0.5774	9cd	0	8d	0
A. flavus strainD5,2 (MG779509)	1bc	0	8.667c	0.3333	5.333bc	0.3333
A. fumigatus strain B2,2 (MG779513)	0.667ab	0.3333	10e	0	8.667d	0.3333
A. insuetus strain A1,1 (MG779505)	1.667cd	0.3333	8.333c	0.3333	5.667c	0.3333
A. neoflavipes strain B5,1 (MG779510)	0a	0	3.333b	0.3333	6c	0.5774
A. nidulans strain C5,1 (MG779507)	2d	0	8.667c	0.3333	5.667c	0.3333
A. nidulans strain E1,2 (MG779511)	1.667cd	0.3333	9cd	0	4a	0
A. nidulans strain E4,1(MG779504)	2d	0	2.333a	0.3333	4.333ab	0.3333
A. oryzae strain A5,1 (MG779508)	1.333bcd	0.3333	9.667de	0.3333	4.333ab	0.3333
A. terreus strain D4,2 (MG779512)	1bc	0	8.667c	0.3333	6c	0.5774

The growth of fungi at different temperatures (20˚C, 30˚C and 40˚C) as shown **Fig 4.34**) revealed that growth at 30˚C was significantly higher than growth at 20˚C and 40˚C with *A. oryzae* strain A5,1 having the highest growth (10±0) (**Table 4.6**). However isolate E4, 1-*A. nidulans* grew optimally at 40˚C.

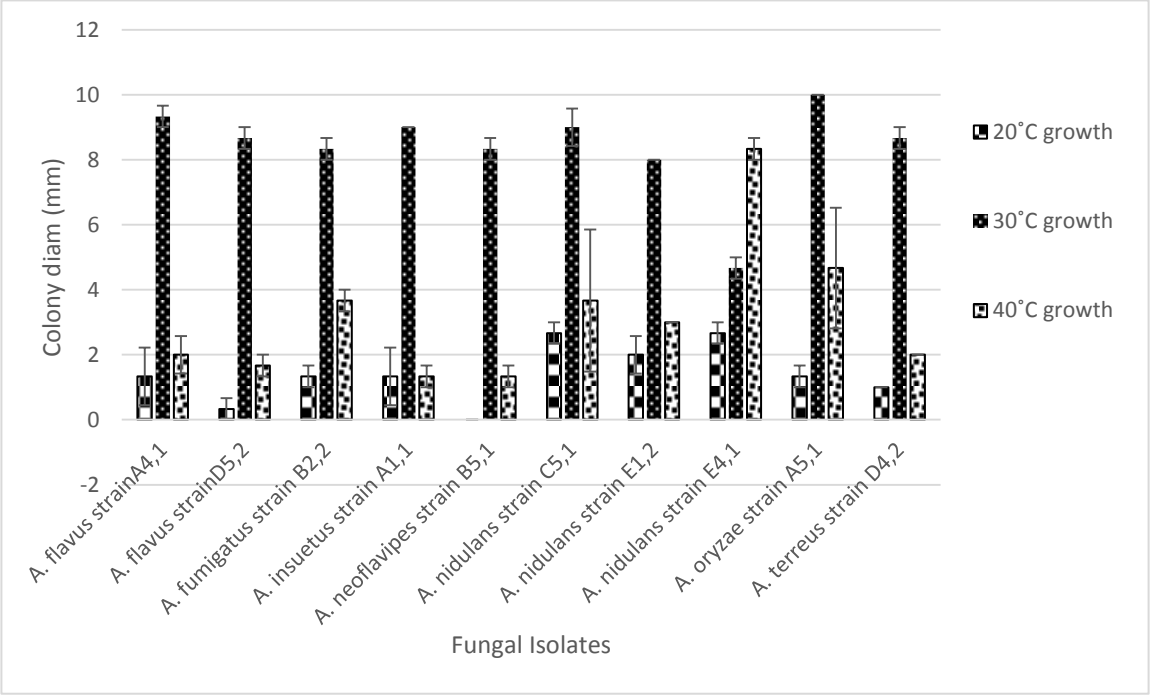


Figure 4.34: Growth of fungal isolates at 20 ˚C, 30 ˚C, and 40˚C respectively.

Table 4.6: Growth of fungal isolates at 20 ˚C, 30 ˚C, and 40˚C respectively measured as colony diameter (mm). Means with same superscript letters within the same column are not significantly different and vice versa using Fisher's Protected Least Significant test at ($P<0.05$)

	20˚C		**30 ˚C**		**40 ˚C**	
Isolates_ID	**Mean**	**S.E.**	**Mean**	**S.E**	**Mean**	**S.E**
A. flavus strainA4,1 (MG779506)	1.333^{abc}	0.8819	9.333^{de}	0.3333	2^{ab}	0.5774
A. flavus strainD5,2 (MG779509)	0.333^{a}	0.3333	8.667^{bcd}	0.3333	1.667^{a}	0.3333
A. fumigatus strain B2,2 (MG779513)	1.333^{abc}	0.3333	8.333^{bc}	0.3333	3.667^{ab}	0.3333
A. insuetus strain A1,1 (MG779505)	1.333^{abc}	0.8819	9^{cd}	0	1.333^{a}	0.3333
A. neoflavipes strain B5,1 (MG779510)	0^{a}	0	8.333^{bc}	0.3333	1.333^{a}	0.3333
A. nidulans strain C5,1 (MG779507)	2.667^{c}	0.3333	9^{cd}	0.5774	3.667^{ab}	0.1858
A. nidulans strain E1,2 (MG779511)	$2b^{c}$	0.5774	8^{b}	0	3^{ab}	0
A. nidulans strain E4,1(MG779504)	2.667^{c}	0.3333	4.667^{a}	0.3333	8.333^{c}	0.3333
A. oryzae strain A5,1 (MG779508)	1.333^{abc}	0.3333	10^{e}	0	4.667^{b}	1.8559
A. terreus strain D4,2 (MG779512)	1^{ab}	0	8.667^{bcd}	0.3333	2^{ab}	0

NaCl concentration was also analyzed as a factor influencing the rate of growth of the fungi. The fungi were incubated at various NaCl concentrations of 0%, 5% and 10% .It is evident from the results (**Fig 4.35**) that the fungal isolates do not depend on salt to thrive as their rate of growth at 0% NaCl concentration was significantly high and the rate of growth at 10% NaCl concentration was significantly low (**Table 4.7)**

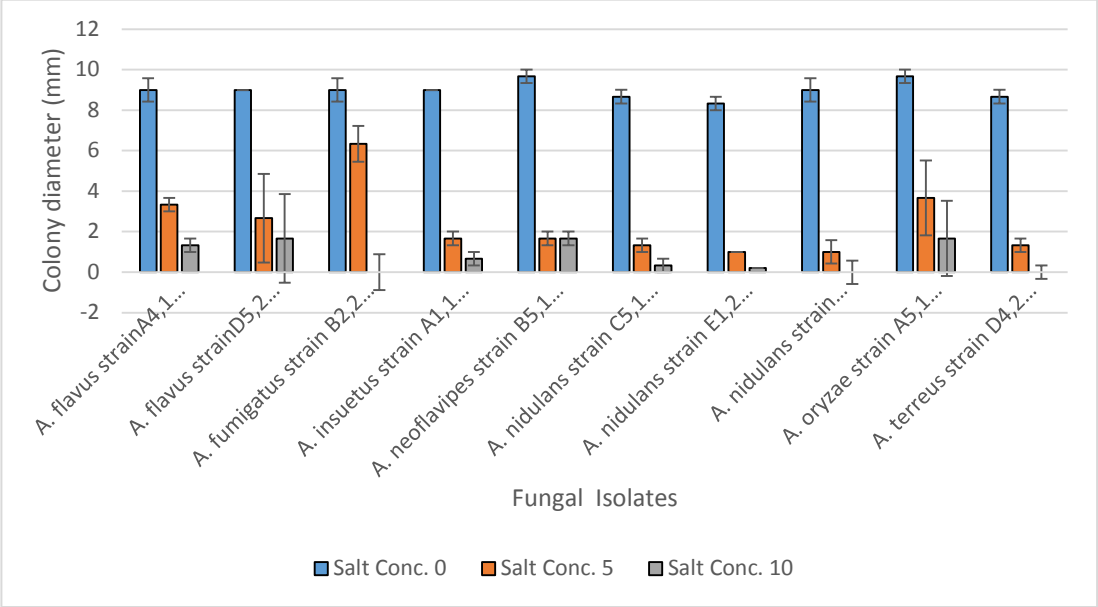


Figure 4.35: Growth of fungal isolates at varying NaCl concentrations

Table 4.7: Growth of fungal isolates at varying NaCl concentrations. Means with same superscript letters within the same column are not significantly different and vice versa using Fisher's Protected Least Significant test at (P<0.05)

Isolates_ID	NaCl Conc 0 Mean	S.E	NaCl Conc 5 Mean	S.E	NaCl Conc 10 Mean	S.E
A. flavus strainA4,1 (MG779506)	9^{a}	0.5774	4.333^{bc}	0.3333	4.333^{bc}	0.3333
A. flavus strainD5,2 (MG779509)	9^{bc}	0	2.667^{ab}	2.1858	2.667^{ab}	2.1858
A. fumigatus strain B2,2 (MG779513)	9^{a}	0.5774	6.333^{c}	0.8819	6.333^{c}	0.8819
A. insuetus strain A1,1 (MG779505)	9^{c}	0	1.667^{ab}	0.3333	1.667^{ab}	0.3333
A. neoflavipes strain B5,1 (MG779510)	9.667^{c}	0.3333	1.667^{ab}	0.3333	1.667^{ab}	0.3333
A. nidulans strain C5,1 (MG779507)	8.667^{bc}	0.3333	1.333^{a}	0.3333	1.333^{a}	0.3333
A. nidulans strain E1,2 (MG779511)	8.333^{b}	0.3333	1^{a}	0	1^{a}	0
A. nidulans strain E4,1(MG779504)	9^{bc}	0.5774	1^{a}	0.5774	1^{a}	0.5774
A. oryzae strain A5,1 (MG779508)	9.667^{c}	0.3333	3.667^{abc}	1.8559	3.667^{abc}	1.8559
A. terreus strain D4,2 (MG779512)	8.667^{bc}	0.3333	1.333^{a}	0.3333	1.333^{a}	0.3333

CHAPTER FIVE

DISCUSSION

5.1: Isolation of LDPE degrading microorganisms and determination of level of biodegradation

Isolation of plastic degrading microorganisms was done in this study by using LDPE sheets and Poly-ethene powder as the carbon sources. The microorganisms isolated after the incubation period were assumed to have utilized LDPE as their carbon source. Most studies employ the use of various materials that have a similar structure as that of LDPE in isolating these microorganisms. Pramilla & Vijaya, (2015) isolated LDPE degrading bacteria by preparing an artificial media in which pretreated poly-ethene was used as the sole carbon source. Hadad *et al*., (2005) screened for poly-ethene degrading microbes by first growing them on liquid waxes. They plated soil samples from a waste disposal site on synthetic medium supplemented with 10 ml of a mixture containing intermediate-size poly-ethene oligomers in the form of liquid waxes. Production of clear zones around the colonies of the wax-degrading bacteria was used as the indicator of the extent of degradation. These colonies were further tested for their ability to grow in SM medium containing poly-ethene powder as the sole carbon source. Determination of the extent of biodegradation of the LDPE was done by assessment of the LDPE sheets using various techniques such as weight loss, FT-IR and GC-MS. Bacteria and fungi have been greatly implicated in the degradation of both natural and artificial polymers (Gu, 2003).

According to Gajendiran *et al*., (2016), weight loss is the simplest and quickest way to assess biodegradation of polymers. After the incubation period, LDPE films were treated

with SDS as surfactant that denatures the microbial cells and washes them off from the surface. The bacterial inoculum of *Bacillus cereus* strain A5,a (MG64264) and *Brevibacillus borstelensis* strain B2,2 (MG645267) produced a mean weight loss of 35.72±4.01 % and 20.28±2.30 ($P<0.05$) respectively on the 30 micron poly-ethene sheets which was significantly higher than the other bacterial samples. This is in agreement with the results recorded by Hadad *et al*, (2005) in which *Brevibacillus borstelensis* strain 707 after 30 days at 50°C reduced the gravimetric and molecular weights of poly-ethene sheets by 11 and 30% respectively. *Bacillus cereus* strain also led to a significantly high weight loss of the LDPE under investigation hence proved to be a promising candidate for this process. According to Pramilla & Ramesh, (2015), *Bacillus cereus* was able to degrade LDPE causing an introduction of new functional groups, a reduction in tensile strength and destruction of the poly-ethene surface. The inoculum sample of *Pseudomonas putida* strain B1, 1a (MG645383) gave 2.80±0.38% mean weight loss on the 30 micron poly-ethene. This was however a lower rate compared to the study done by Nanda & Sahu, (2010) where *Pseudomonas sp* was subjected to LDPE biodegradation alongside three other genera of bacteria and it was the most effective bio-degrader. Bacterial sample B4,1 however recorded an increase in weight at the end of the incubation period rather than a decrease. This could be attributed to the fact that before any microbial activity, there is attachment to the polymer surface which in some cases involves biofilm formation. Biofilm removal may not be done completely even after cleaning with the SDS detergent hence the traces of bacterial biofilm are a possible cause of the weight increase (Gajendiran *et al*., 2016).

Fungal mean weight reductions were generally higher than bacterial with the significantly high mean weight reduction of 36.4±5.53%, 24±3.26% and 18±2.20% being attributed to isolates *Aspergillus oryzae* strain A5,1(MG779508), *Aspergillus fumigatus* strain B2,2(MG779513) and *Aspergillus nidulans* E1,2 (MG779511) respectively. *Aspergillus* genus is a group of fungi that have found immense application in the biodegradation of many compounds. The importance of this group of fungi in the biodegradation of many industrial and domestic by-products cannot be underscored as it is has found use in the elimination of many recalcitrant compounds due to their ability to produce a wide variety of enzymes (Mahalakshmi *et al*., 2012). A comparative study where three different fungal species were investigated for their ability to degrade LDPE, 35 % weight loss of LDPE films was observed after 90 days of incubation with *A. clavatus* strain JASK1, 11.11 % with *Aspergillus japonicas* and *A. niger* degraded 5.8 % in 1 month (Gajendiran *et al*., 2016) in addition to other indicators of biodegradation such as surface deterioration and appearance of new functional groups as a result of microbial action. Structural changes and surface erosions on the LDPE surface were observed upon 2 months incubation with *Aspergillus spp* (Mahalakshmi *et al*., 2012).

In this study, isolate A5,1: *Aspergillus oryzae (*MG779508) resulted in a weight loss of 36.4±5.53%, which was the highest. *Aspergillus oryzae* is a promising biodegrader of poly-ethene as it was able to degrade 30% of poly-ethene in 200 days (Indumathi *et al*., 2016) in addition to formation of micro cracks and increased embrittlement of the LDPE surface upon SEM analysis. In a study done using untreated LDPE incubated with *A. oryzae*, 5% weight loss was recorded compared with control (untreated and unexposed) (Konduri *et al*., 2011). When

they investigated *A. oryzae* poly-ethene degrading capacity under different treatments, they confirmed an 18% weight loss; whereas biotically untreated LDPE incubated with *A. oryzae* showed 5% weight loss. Isolate. *Aspergillus fumigatus* strain B2,2 (MG779513) recorded a weight reduction of 24±3.26% which was the second highest. *Aspergillus fumigatus* is also among the species that have been investigated for their ability to degrade poly-ethene and other polymers. In a study, three fungal species were investigated for their ability to degrade poly-ethene and *A. fumigatus* was the best degrader compared to *A. terreus* and *F. solani* following an analysis of the LDPE surface by SEM and FTIR (Zahra *et al*., 2010).

Other fungi implicated in this study included *Aspergillus nidulans, A flavus, A. terreus and A. neoflavipes* which resulted in weight loss of the LDPE sheets. These have also been previously found to be candidates for degradation. *A. nidulans* was isolated from garbage soil in Libya and found to degrade 12.6% of LDPE in six months (Usha *et al*., 2011). Fungal isolates were isolated from plastics buried in soil for ten months and identified as *Aspergillus terreus* which were found to cause corrosion and pits on the plastic surface indicating a possible utilization of the plastic as their carbon source (Shah *et al*., 2008). *Aspergillus flavus* has also been studied and known as a plastic bioremediation candidate. In a study by Deepika & Madhuri, (2015) it was implicated as the cause of poly-ethene weight reduction of 5.69% to 16.45%. LDPE degradation by *Aspergillus* and *Bacillus* was recorded by Esmaeili *et al*., (2013). However the mean weight reductions per sampling point were lower which is an indication that biodegradation of materials varies by location depending on the microbial composition

of the particular point. Use of weight reduction as a measure of the extent of poly-ethene biodegradation has been widely accepted and used by many authors (Ojha *et al*., 2017, Sheik *et al.*, 2015, Deepika & Jaya, 2015,). These outcomes are in agreement with Pramilla and Ramesh, (2015) who reported the ability of microorganisms to degrade virgin poly-ethene. The LDPE of 30 microns was better degraded than the 40 micron one due to its lower molecular weight.

The total mean weight loss by fungi (210.92) was higher than the total mean weight loss bacteria (115.58) indicating that fungi are generally better biodegradation candidates compared to bacteria. Fungi and bacteria were isolated from mangrove soil and investigated for poly-ethene degradation and the fungi isolates (*Aspergillus glaucus* 28.80 ± 2.40 7, *Aspergillus niger* 17.35 ± 2.00) were found to have better efficacy of LDPE degradation compared to the bacterial isolates (*Pseudomonas sp.* 20.54 ± 0.13, *Staphyloccoccus sp*. 16.39 ± 0.01).(Kathiresan, 2003). This can be attributed to the fact that fungi can utilize a wider range of substrates than bacteria and they also can produce a wide range of enzymes.

Analysis of the poly-ethene spectral figures indicate formation of new peaks at the region between 1650 and 1800. Also new peaks can be seen in the region between 1000 and 1100. The new and increased peaks at 1650-1800 are as a result of carbonyl groups (C=O) indicative of formation of aldehydes, ketones or carboxylic acids which are intermediate products of biodegradation of poly-ethene. The region of increased peak absorbance and new peaks in the 1000-1200 cm-1 region of the FTIR spectrum

correlates with primary and secondary alcohols. The main bands of the studied LDPE sheets consist of a band situated about 2900 cm-1 assignable to CH2 as an asymmetric stretching, a band around 1461–1466 cm-1 revealing a bending deformation, and another band at 720–724 cm-1 which indicates a rocking deformation (Ibiene *et al*., 2013). Intensity of the bands at 1650 cm−1 increased in the powder samples relative to the control. From this, it is suggestive that substantial changes in the functional groups of poly-ethene test samples occurred after incubation with the selected both bacterial and fungal inocula for sixteen weeks under laboratory conditions. These outcomes agree with the findings of Gulmine *et al*., (2003) who confirmed appearance of an absorption band around 1714 cm-1, which could be assigned to the C=O stretching vibration of a ketone group and which grew in intensity with extended aging. Additional bands were also seen at the same time indicating formation of more than one oxidation product. After two months incubation of LDPE with fungi, it was found that some new peaks arose and they could be assigned to specific peaks, such as carbonyl group (1720 cm-1), CH3 deformation (1463 cm-1) and C=C conjugation band (862 cm-1) (Mahalakshmi *et al*., 2012). These results are however in contrast to the report by (Orhan & Bu, 2000; Sudhakar *et al*., 2007 & Feng *et al*, 2009) who concluded that microorganisms can only degrade chemically or physically pre-treated poly-ethene. Presence of 4, 6-Octadiyn-3-one, 2-methyl which is a ketone in addition to 4,4-Dimethyl-2-pentene which is an alkene in the culture supernatant through GC-MS detection can be attributed to the process of biodegradation of the polymer with ketones as part of the intermediary products. This was observed in just one of the samples that had been incubated bacterial

sample for degradation. This outcome is in agreement with a previous study by *(Mahalakshmi et al.,* 2012)who reported that large number of different aldehydes, ketones and carboxylic acids were identified in smoke generated on sheet extrusion of LDPE in an extrusion coating process. In the present study, the degraded products were determined by GC-MS analysis.

5.2: Molecular Identification and Characterization of LDPE degrading bacteria and fungi

Both fungi and bacteria have been implicated as the main agents of polymer biodegradation (Balasubramanian *et al*., 2010, Payal & Himanshu, 2016). They do so when they attach to polymers in their environment and at the right conditions, secrete extracellular enzymes (Gu, 2003) that interact with the polymer during the process of depolymerization to break it down into smaller sub-units (oligomers, dimers and monomers) (Mueller, 2006). The continuous interaction of the polymer sub units with the microbes ultimately leads to production of intermediate biodegradation products like esters, alcohols, carboxylic acids, aldehydes, ketones, ether groups and many others which are can then undergo mineralization (Sahadevan *et al*., 2013). Bacteria has been widely used in bioremediation studies and has been found to have good potential for polymer degradation.

In this study, bacteria of the genera *Bacillus, Brevibacillus, Ochrobactrum, Lysinibacillus, Cellulosimicrobium* and *Pseudomonas* were identified as effective poly-ethene degraders. Bacterial isolates A5, 1a-*Bacillus cereus* (MG645256) produced the highest degradation effectiveness in terms of weight loss i.e. 35.2% followed by isolate

B2,2-*Brevibacillus borstelensis* (MG645267)-20.28%. Isolate B1,1a *Pseudomonas putida* (MG645283)-2.88% and D4,yn-*Brevibacillus borstelensis* strain (MG645261)-6.8%.

The genus *Bacillus* was the most frequently identified among the LDPE biodegrading genera in this study with 44.8% frequency. Species identified under this genus include *Bacillus cereus, Bacillus toyonensis, Bacillus thuringiensis, Bacillus subtilis, Bacillus pseudomycoides, Bacillus safensis* and *Bacillus niacini. Bacillus* is a gram-positive genus of rod-shaped bacteria that are obligate aerobes or facultative anaerobes and include more than 60 species. Various studies have been done to investigate the efficacy of genus *Bacillus* in poly-ethene degradation and various species under this genus have been found to have potential to degrade poly-ethene (Ibiene *et al*., 2013; Vimala & Mathew, 2016; Harshvardhan & Jha, 2013; Ojha *et al*., 2017). In this study isolate A5,1a-*Bacillus cereus* (MG645256) and isolate B2,2-*Brevibacillus borstelensis* (MG645267) degraded poly-ethene resulting into a weight loss of 35.20% and 20.28% respectively. These were the highest mean weight loss for bacteria. *Bacillus cereus* has been found to be a good bioremediation candidate in the biodegradation of poly-ethene due to its ability to produce enzymes laccase and manganese peroxidase (Sowmya & Thippeswamy, 2014). In a comparative study, *B. cereus* was found to be more effective than *B. sphericus* in degrading photo-oxidized and thermos-oxidized LDPE (Suresh *et al*., 2011). According to Hadad *et al*., (2005), *Brevibacillus borstelensis*-Accession number- AY764129 was able to degrade 11% of nonirradiated poly-ethene by weight in 30 days . Two bacterial isolates *Bacillus amyloliquefaciens* (BSM-1) and *Bacillus amyloliquefaciens* (BSM-2) were isolated from municipal soil and used for polymer degradation studies and were found to produce significant changes on LDPE in terms of

weight loss, reduction of tensile strength and appearance of new functional groups (Das & Kumar, 2015).

Four different species of *Pseudomonas* were assessed for their ability to degrade LDPE and the weight loss outcomes were as follows, 20% in *Pseudomonas aeruginosa* (PAO1) (B1), 11% in *Pseudomonas aeruginosa* (ATCC) strain (B2), 9% in *Pseudomonas putida* (B3), and 11.3% in *Pseudomonas syringae* (B4) strain (Myint & Ravi, 2012). They also caused significant surface changes on the LDPE sheets from the results of SEM, FT-IR and GC-MS. Pseudomonas genus has been widely implicated in this process of polymer degradation. A novel strain of Pseudomonas; *Pseudomonas citronellolis* EMBS027, GenBank Accession number KF361478 was isolated by Bhatia *et al*., (2014) from a municipal landfill in Indore, India and it degraded 17.8% of poly-ethene in 4 days. Different species of Pseudomonas were analyzed for their ability to degrade poly-ethene and upon incubation for 120days *Pseudomonas putida* resulted in a weight loss of 9% (Myint & Ravi, 2012). Upon elution, a wide variety of both volatile and semi volatile compounds were eluted by GC–MS was performed for samples incubated with *Pseudomonas aeruginosa* such as long chain fatty acids, esters, hydrocarbons and ether groups. According to (Balasubramanian *et al*., 2010), the main reason why *Pseudomonas* bacteria are more likely to degrade poly-ethene compared to other bacteria is due to their high level of hydrophobicity. An experiment on hydrophobicity demonstrated that Pseudomonas genus are more hydrophobic hence readily adheres to the polymer surface.

Lysinibacillus genus was identified as one of the bacterial isolates that with biodegradation potential in this study through identification of *Lysinibacillus macrolides* strain. This genus was also identified as a cause of poly-ethene degradation through an analysis of the outcomes of SEM, FTIR and weight loss (Esmaeili *et al*., 2013). *Lysinibacillus fusiformis* led production of a variety of intermediary degradation products (1-trimethylsilylmethanol and hexadecanoic acid) from LDPE analyzed through GCMS (Shahnawaz *et al*., 2016).

Alkane hydroxylases are alkane-degrading enzymes that are distributed among many different species of bacteria, yeast, fungi, and algae. A common feature of many alkane degraders is that they contain multiple alkane hydroxylases with overlapping substrate ranges (Abd El-Rahim *et al*., 2009). Depending on the chain-length of the alkane substrate, different enzyme systems are required to introduce oxygen in the substrate and initiate biodegradation. A*lkB* and *alkB* related genes code for an alkane degrading enzyme, alkane hydroxylase (Gyung Yoon *et al*., 2012). The analysis of the fungal and bacterial samples revealed presence of AlkB 1 gene in 17 of the fungal samples and 4 of the bacterial samples. Among the fungal isolates that were positive for this gene was *A. oryzae* which was the highest degradation potential. Bacterial isolates that were positive for alkB 1 gene were D4 yn-*Brevibacillus borstelensis*, B1,1-*Pseudomonas putida* and A5,a1-*Bacillus cereus.* This genetic information is an indication of the genetic ability of the microorganisms to degrade long- chain alkanes through production of this enzyme. Alkane biodegradation is initiated through terminal oxidation to the corresponding primary alcohol, which is further oxidized by dehydrogenases to fatty acids which can enter the TCA cycle (Van Beilen *et al*., 2003).

5.3: Screening for Extracellular Enzymes

Production of extracellular enzymes plays an important role in polymer degradation through depolymerization where the polymer is broken down into smaller sub units (Müller, 2005) which are then enzymatically degraded into intermediary products that can be assimilated into microbial cells (Sahadevan *et al*., 2013) and utilized as carbon sources leading to production of energy, water, carbon dioxide and methane in the case of anaerobic respiration (Hamilton *et al*., 2014). In this study, production of extracellular enzymes esterase, laccase, pectinase and xylanase were investigated. Laccases are an important group of extracellular enzymes that ordinarily have a role in lignin degradation in combination with manganese peroxidases (Lucas *et al*., 2008) and they have been studied for their application in bioremediation of many industrial and domestic products (Viswanath, 2014, Iiyoshi *et al*., 1998) including poly-ethene. Fungal and bacterial isolates in this study were scrutinized for their ability to produce laccase enzyme and isolate B2, 2: *Aspergillus fumigatus* (MG779513) which had a LDPE degradation effectiveness of 24%, had the highest diameter of coloration due to laccase production. This could be attributed to its ability to produce higher amounts of laccase and other extracellular enzymes which are believed to play a role in poly-ethene degradation. According to El-morsy & Ahmed, (2017), the production of this enzyme increases when the microbes are in close proximity with the poly-ethene. Sowmya *et al*., (2014) were able to extract crude laccase enzyme which was incubated with poly-ethene and led to degradation as was evidenced through weight loss, FTIR and SEM. Esterases, also known as lipolytic enzymes, catalyze the cleavage of ester bonds (Zhang *et al*.,

2015) of short-chain triglycerides or esters (Liu *et al*., 2013). They concluded that the enzymes responsible for poly-ethene degradation from *Trichoderma harzianum* were as laccase and manganese peroxidase.

Esters have been identified as part of the intermediary products produced during poly-ethene degradation when the post incubation culture media is subjected to GC-MS analysis that can be assimilated into the microbial cells, undergo hydrolysis to give rise to the subsequent carboxylic acid and alcohol that ultimately undergo respiration to produce energy (Mahalakshmi *et al*., 2012; Zhang at al., 2004). Isolate A5,1- *Aspergillus oryzae (*MG779508) with a weight loss of 36.4% had a high presence of enzyme esterase of 10%. This could have contributed to its high degradation potential compared to other fungal isolates which had lower degradation rates.

5.4: Growth requirements for LDPE degrading bacteria and fungi

Microorganisms grow optimally at certain ranges of various conditions which may vary for different microbes (Lodhi *et al*., 2011). In this study, the conditions that were investigated were temperature, pH and salt concentration. Microbial activities are controlled by enzymes which work optimally at various conditions. Determination of growth rate of fungal isolates was studied using the colony diameter measurements while the bacterial growth rate was studied using the spectrophotometric optical density readings at 600nm. The general optimal growth parameters for bacteria were: temperature 30-40˚C, pH 6-8 and salt concentration of zero. At temperature 40 ˚C, isolate C5,1 had the highest OD of 0.97 at 600nm followed by isolate A2,2 with an O.D

of 0.932 while isolate B1,1-*Pseudomonas putida* recorded the highest OD at 30 ˚C. These two bacterial isolates C5,1a (MG645270) *Bacillus niacin* and A2,2a *Bacillus pseudomycoides* (MG645265) which are also degraders of poly-ethene. According to (Kashimozhi & Perinbam, 2010), the observed optimum pH and temperature range esterase production by *Pseudomonas sp* was 8.0-8.5 and 37-42 °C, respectively. Al-Jailawi1 *et al*., (2015) also pointed out that the optimum temperature for growth of *Pseudomonas putida* S3A, a poly-ethene degrader was 37 °C. The growth rate of the bacterial isolates at 20°C was the slowest compared to 30°C and 40°C. According to Skariyachan *et al*., (2015), *Pseudomonas spp* was able to utilize plastic as a sole source of carbon and showed 20–50% weight reduction over 120 days and this rate was optimally achieved at 37 °C in pH 9.0. This could be attributed to the fact that the sampling site for these bacteria was from a dumpsite where the temperatures were generally ambient and hence favoring the growth of mesophilic bacteria (**Appendix 1**).

The fungal optimum growth conditions were: temperature 30 ˚C, PH 8.0 and salt concentration of zero with some exceptions. Laccase production by fungi for instance is influenced by culture conditions such as type and concentration of carbon sources, pH and temperature (Khalil *et al*., 2014). The optimum temperature for activity of laccase enzyme by fungi was found to be between 25˚C and 30°C as laccase activity reduces with higher temperatures although this can vary highly with the fungal strains. This is a clear indication that the optimum pH conditions for biodegradation of LDPE vary depending on the microbial strain and source but generally for this study, a pH of around 8.0 was responsible for the highest fungal activity. In a study done by (Pawar *et*

al., 2013), it was observed that soil pH of 7.5 was most suitable for the degradation of all the PAHs as 50% degradation was observed at pH 7.5 and *Aspergillus species* was also found to be more prevalent at this pH (7.5-8.0). Safiye *et al*., (2015) recommended liming as a strategy to improve the bioremediation capacity of white rot fungi as they were found to have optimum bioremediation capacity at a pH of 7.4. The application of *Aspergillus niger* in textile dye bioremediation indicated the percentage of removal after 72 hr incubation was 95.2, 96.8 and 97.3% at pH 7, 8 and 9, respectively which also emphasizes the role of a slightly alkaline pH in fungal bioremediation (Abd El-Rahim *et al*., 2009).

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

The aim of this study was to identify and characterize fungi and bacteria from Dandora dumpsite which have the potential to degrade LDPE. The present work indicates that naturally occurring soil bacteria and fungi isolated from the dumpsite show potential of degrading poly-ethene. This is the first study on the isolation of local bacteria and fungi that can degrade LDPE which is the most common plastic in terms of statistics in Kenya. Fungi of genus *Aspergillus* were identified as effective degraders while bacteria of genus *Bacillus* exhibited significant degradation potential. The isolated bacteria and fungi have the ability to produce extra-cellular enzymes associated with poly-ethene degradation. It was ascertained that the microorganisms are capable of producing enzymes laccase and esterase both which have been confirmed to play a role in degradation of poly-ethene. The isolates possess the alkane hydroxylase producing gene (Alk B) which is the molecular explanation for the degradation of LDPE under investigation. Alkane hydroxylase enzyme has been identified as one of the enzymes responsible for the initiation of the biodegradation of the alkane which is a major component of LDPE. The culture conditions for the microorganisms are an important factor to consider especially in application of bioaugmentation strategies. The fungi in this study were found to grow optimally at a pH of 8.0 and temperature of 30˚C while bacterial isolates grew optimally at 40 ˚C and within a pH range of 6-8.

Recommendations for Further Studies

There is need to further optimize the culture conditions to maximize microbial biodegradation especially in cases where co-culturing is done. This knowledge will enable the improvement of biodegradation through combination of isolates.

Region-wise bioprospecting for LDPE degrading microbes will increase the pool of identified microorganisms which have potential to degrade LDPE. This may lead to identification of better degraders that can be applied on a larger scale

Further the screening of genes present in the identified microorganisms that are responsible for producing different polymer degrading enzymes will inform the combination of the right microbial strains in biodegradation processes and eventually enhance the level of degradation.

The use of recombinant DNA technology to insert genes that produce LDPE degrading enzymes into good degraders will serve to further improve their degrading potential.

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APPENDICES

Appendix 1: Sampling Site Temperature and pH

Sample no.	Sampling point	Soil pH	Soil temperature (˚C)
1	A,1	9.0	35
2	A,2	8.0	36
3	A,3	8.1	30
4	A,4	9.2	30
5	A,5	8.7	32
6	B,1	7.0	38
7	B,2	7.5	36
8	B,3	7.3	35
9	B,4	7.5	37
10	B,5	7.0	35
11	C,1	8.2	37
12	C,2	8.0	36
13	C,3	8.2	36
14	C,4	8.0	35
15	C,5	7.8	38
16	D,1	8.4	38
17	D,2	8.0	36
18	D,3	8.0	35
19	D,4	7.8	36
20	D,5	8.0	37
21	E,1	7.0	29
22	E,2	7.4	28
23	E,3	7.8	28
24	E,4	7.6	30
25	E,5	7.0	30

Appendix 2: Fungal DNA extraction buffer

0.1M Tris-HCl pH 8,

10Mm EDTA pH 8,

2.5M NaCl,

3.5% CTAB,

150 µL 20mg/ml proteinase K)

Appendix 3: GC-MS Conditions

Temperature ramp: 60 ^{0}C (2 min); @8 ^{0}C/min to 250 ^{0}C (3 min)

Column: DB-XLB (standard non-polar)

Injection volume: 0.5 µL

Injection mode: Split, 100:1

Mass range: 40 – 470 u

Source temp: 200 ^{0}C

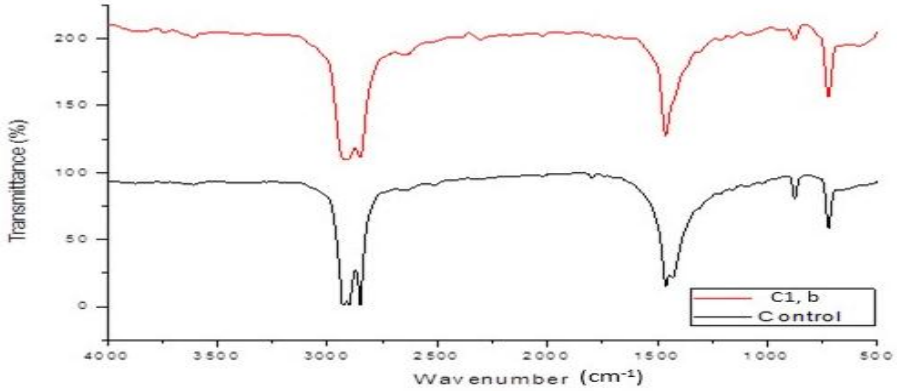
Interface temp: 250 ^{0}C

Carrier gas: He, 99.999% purity

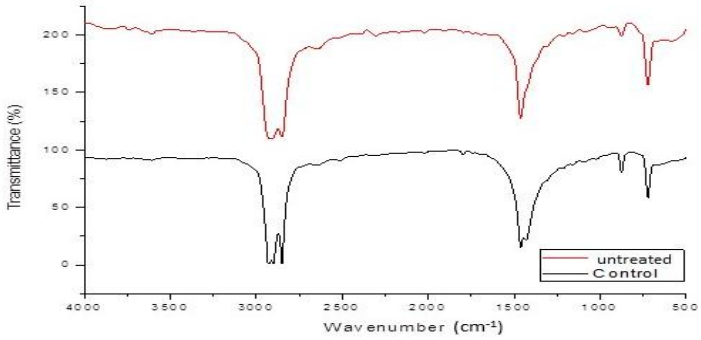
Flow rate: 1 ml/min

Pressure: 8 kPa

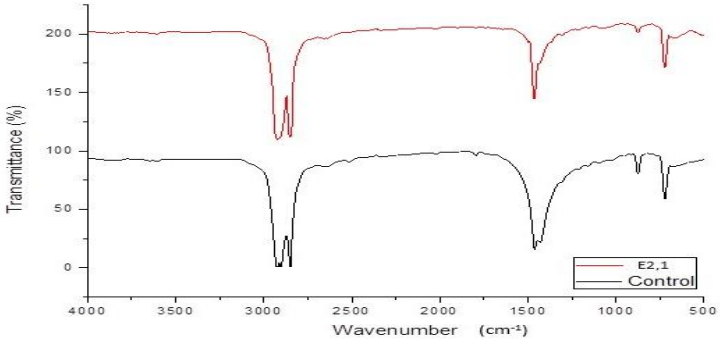
Appendix 4: The FT-IR Negative Outcomes



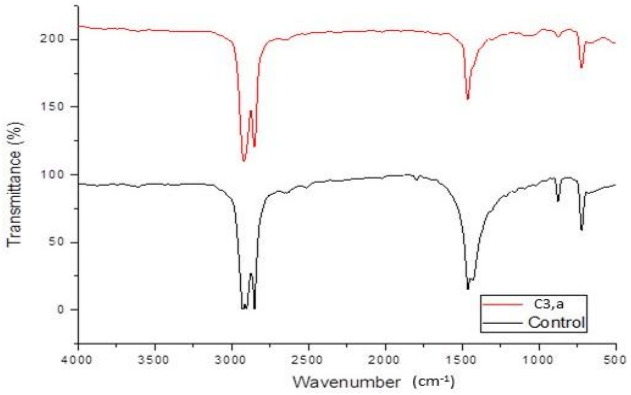
Spectra for LDPE sheet from bacterial sample C, 1 and the control



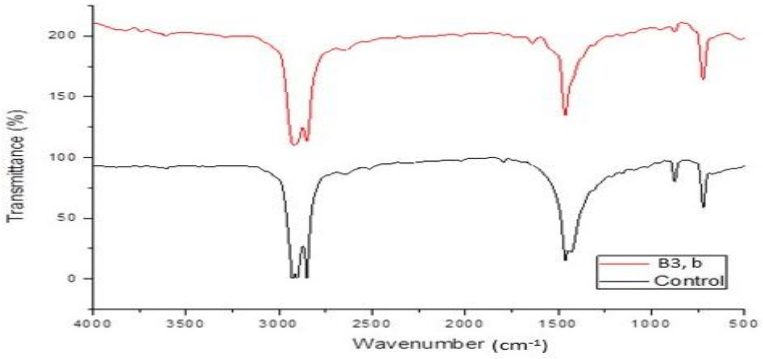
Spectra for LDPE sheet from untreated sample and the control



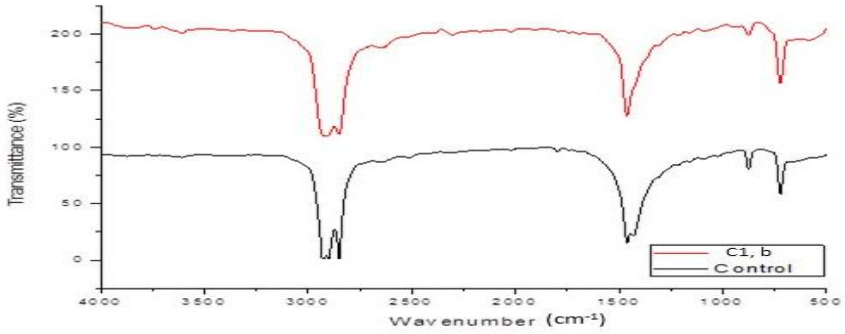
Spectra for LDPE sheet from bacterial sample E2,1 and the control



Spectra for LDPE sheet from fungal sample C3,a and the control



Spectra for LDPE sheet from fungal sample B3,b and the control



Spectra for LDPE sheet from fungal sample C1,b and the control

Appendix 5: Biochemical tests of the bacterial isolates

Isolate code	Gram stain	Catalase test	Citrate utilization	TSI slant	TSI butt	MR	VP	Urease test	Gelatin liquefaction	Indole test	Motility test	Nitrate reduction	Starch hydrolysis	Accession Number	Isolate ID
E1,2a	+ rods	+	+	K	A	-	+	-	+	-	+	+	+	MG645252	*Bacillus cereus*
B4,1 pn	+ rods	+	+	K	A	-	+	-	+	-	+	+	+	MG645253	*Bacillus cereus*
C2,2a	+ rods	+	+	K	A	-	+	-	+	-	+	+	+	MG645254	*Brevibacillus parabrevis*
B4, 2yn	+ rods	+	+	K	A	-	+	-	+	-	+	+	+	MG645255	*Bacillus cereus*
A5,a1	+ rods	+	+	K	A	-	+	-	+	-	+	+	+	MG645256	*Bacillus cereus*
D4, 1n	+ rods	+	+	K	A	-	+	-	+	-	+	+	+	MG645257	*Bacillus toyonensis*
E5,a1	+ rods	+	+	K	A	+	-	-	+	-	-	+	+	MG645258	*Bacillus thuringiensis*
B1,2	+ rods	+	+	K	A	+	-	-	+	-	-	+	+	MG645259	*Bacillus thuringiensis*
E4,1	+ rods	+	+	K	A	-	+	-	+	-	+	+	+	MG645260	*Bacillus subtilis*
D4,yn	+ rods	+	+	K	A	-	+	-	+	-	+	+	+	MG645261	*Brevibacillus borstelensis*
E4,1,2	- cocci	+	-	A	A	-	-	-	-	-	+	+	-	MG645262	*Ochrobactrum pseudintermedium*
C4,1a	+ rods	+	+	K	A	-	+	+	+	-	+	+	+	MG645263	*Lysinibacillus*

															macroides
A1, a	+ rods	+	+	K	A	-	+	-	+	-	+	+	+	MG645264	*Bacillus cereus*
A2,2	+ rods	+	+	K	A	-	+	-	+	-	+	+	+	MG645265	*Bacillus pseudomycoides*
C4,1a	+ rods	+	+	K	K	+	-	+	+	-	+	+	+	MG645266	*Cellulosimicrobium funkei*
B2,2	+ rods	+	+	K	A	-	+	-	+	-	+	+	+	MG645267	*Brevibacillus borstelensis*
B,4,2	+ rods	+	+	K	A	-	+	-	+	-	+	+	+	MG645268	*Bacillus safensis*
B,2,2a	+ rods	+	+	K	A	-	+	-	+	-	+	+	+	MG645269	*Bacillus safensis*
B1, 1a	- rods	+	+	A	A	-	-	-	-	-	+	+	-	MG645383	*Pseudomonas putida*
C5,1a	+ rods	+	+	K	A	-	+	-	+	-	+	+	+	MG645270	*Bacillus niacini*