# EFFECT OF STARCH PRETREATMENT ON THE MICROBIAL DEGRADATION OF LOW DENSITY POLYETHENE CARRIER BAGS

**BY**

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## DECLARATION

I declare that the work in this Thesis entitled '**EFFECT OF STARCH PRETREATMENT ON MICROBIAL DEGRADATION OF LOW DENSITY POLYETHENE CARRIER BAGS'** 'was performed by me at the Laboratories of the Department of Microbiology, Ahmadu Bello University, Zaria, under the supervision of Dr. S.E. Yakubu, Dr. S.A. Ado and Prof. O.K. Sunmonu.

The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this project report was previously presented for another degree or diploma at any university.

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## CERTIFICATION

This thesis titled '**EFFECT OF STARCH PRETREATMENT ON MICROBIAL DEGRADATION OF LOW DENSITY POLYETHENE CARRIER BAGS'** by TAFIDA, Theresa Aishatu meets the regulations governing the award of the degree of Masters of Science of the Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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## ABSTRACT

In this study, a cassava starch/low density polyethene (LDPE) blend was compounded and the effect of this starch pretreatment on the rate of degradation of synthetic polyethene carrier bags was tested using bacteria and fungi (as singles and as consortia) isolated from dump sites. Weight loss and Fourier Transformed Infra Red Spectroscopy (FTIR) were used as analytical techniques for measuring biodegradation. Fungi was found to degrade both synthetic LDPE and LDPE/Starch blend resulting in a weight loss of 3.54% to 19.68% while that obtained from bacteria gave a weight loss of 1.0% to 18.5% within 30days . Mixed cultures comprising of both bacteria and fungi gave better results than the individual organisms and addition of starch was found to greatly enhance the biodegradation of the low density polyethene carrier bags. FTIR analysis showed the appearance of carbonyl groups in the LDPE residues after microbial treatments between wavelengths of 1703.67$cm^{-1}$ and 1872.83$cm^{-1}$ indicating likely oxidation of the carbon-carbon double bond (C=C) and various oxidation products formed during the biodegradation. This study therefore shows that microorganisms with the ability to degrade low density polyethene carrier bags exist in our natural environment. It has also shown that blending LDPE with natural polymers such as starch can greatly enhance the biodegradation of this polymer and that microbial consortia can accelerate the rate of degradation of this polymer. Therefore finding the right microorganisms and/or their enzymes that can efficiently and completely biodegrade the recalcitrant polyethene into carbon dioxide, water and biomass will go a long way in solving this global threat and studying the synergism between them will give more insight for future efforts towards the biodegradation of these polymers.

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# CHAPTER ONE

# INTRODUCTION

## 1.1 BACKGRUOND

One of the most common items in our modern world is the ubiquitous plastic shopping bag which is made out of polyethene. Polyethene is a member of a large family of polymers called thermoplastic polymers. It consists of long chains of the monomer ethene with its $2CH_2$ groups connected by a double bond, ($H_2C=CH_2$) (Nanda *et al*., 2010).

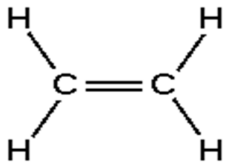



Figure 1.1 Structure of ethene

Polyethene, a waxy chemically inert plastic, is one of the simplest and most inexpensive polymers. It contains the chemical elements, Carbon and Hydrogen, (Suseela and Toppo, 2007; Ramesh and Pramila, 2011). Polyethene is produced by polymerization of ethene gas by allowing the free roaming ethene gas molecules to bond together to form long chains of polyethene in the presence of a catalyst. It can be produced through radical polymerization, anionic addition polymerization, ion coordination polymerization or cationic addition polymerization. Each of these methods results in a different type of polyethene.

## 1.2 HISTORY OF POLYETHENE

Like many of the greatest scientific discoveries, polyethene was first created by accident. But it may be the first one that required two accidents to bring it into wider

use. Polyethene was first synthesized by the German Chemist Hans Von Pechmann by accident in 1898 while heating diazomethane. When his colleagues Eugen Bamberger and Friedrich Tschirner characterized the white, waxy substance he had created, they recognized that it contained long -$CH_2$- chains and termed it polymethylene (Gabriel, 2012). The first industrially practical polyethene synthesis was discovered (again by accident) by Eric Fawcett and Reginald Gibson at the Imperial Chemical Industries (ICI) in Northwick, England in 1933. Upon applying extremely high pressure to a mixture of ethylene and benzaldehyde, they again produced a white waxy material (Sathaananthan, 2004; Arutchelvi *et al.,* 2008). However, since the reaction had been initiated by trace oxygen contamination in their apparatus, the experiment was at first difficult to reproduce and it was not until 1935 that another ICI Chemist, Michael Perrin, developed this serependity into a reproducible high-pressure synthesis for polyethene that became the basis for industrial low density polyethene production beginning in 1939 ( Olley, 2002). Subsequent landmark in polyethene synthesis revolved around the development of several types of catalysts that promote ethylene polymerization at more mild temperature and pressure ( Olley, 2002).

## 1.3 CLASSIFICATION OF POLYETHENE

Polyethene is classified into different types on the basis of their density which in turn depends on the extent and type of branching (Lajeunesse, 2004). Its mechanical property also depends on the extent and type of branching, crystal structure and molecular weight. There are four major classes

1. High Density Polyethene (HDPE)

2. Medium Density Polyethene (MDPE)

3. Low Density Polyethene (LDPE)

4. Linear Low Density Polyethene (LLDPE)

HDPE is defined by a density greater than or equal to 0.941 to 0.970g/cm$^3$. It has low degree of branching and thus stronger intermolecular forces and tensile strength. The lack of branching is ensured by an appropriate choice of catalyst (such as chromium or Ziegler-Natta catalyst) and reaction conditions (Aquart.com.Accessed11/15/2012). MDPE is defined by a density range of 0.926 to 0.940g/cm$^3$. It has good shock and drop resistance properties, less notch sensitive and better stress cracking resistance than HDPE. LDPE is defined by a density range of 0.910 to 0.940g/cm$^3$. It has many more branches than HDPE, which means that the chains do not "fit well" together. It has therefore less strong intermolecular forces as the instantaneous induced dipole-dipole attraction is less. This results in a lower density and tensile strength, increased malleability and faster biodegradation (Ramesh and Pramila, 2011). LDPE is created by free radical polymerization. LLDPE is defined by a density range of 0.915 to 0.925g/cm$^3$. It is a substantially linear polymer with significant numbers of short branches commonly made by co-polymerization of ethylene with short chain alpha-olefins as 1- butene, 1- hexene and 1- octane. It has higher tensile strength than LDPE, exhibits higher impact and puncture resistance than LDPE Aquart.com.Accessed11/15/2012.

## 1.4 USES OF POLYETHENE

The properties of polyethene (light, waterproof, flexible, chemical resistance, chemically inert and most importantly inexpensive and available) give it a wide range of applications. The most common household use of LDPE is in plastic bags; the most common household use of HDPE is in containers for milk and liquid laundry

detergent. Significant uses of MDPE include plumbing fittings and enclosures for inexpensive polyethene consumer devices while LLDPE is used primarily in flexible tubing (Gabriel, 2012).

Polyethene bags are packaged in different ways depending on where they will be used. The most common kind is the white or coloured polyethene bags, which are either transparent or opaque. The printed polymer bags are used as handle bags, plastic shopping bags or merchandise bags. Retail stores use these and print their logos and other information on the bags as additional advertisement. The banking industry uses clear coin bags to package coins according to denominations. These types of polyethene bags are thicker than what suppliers give to retail stores for their shopping bags. Garbage bags are usually coloured black or green, and vary in size and thickness depending on whether they will be used for commercial, industrial or domestic purposes. Reclosable or resealable bags have a plastic ridge that closes and pulls open. They are for dry food storage since they can be refilled and resealed.

Aside from the food industry, textile and clothing industry and wholesale and retail stores, the medical industry also uses polyethene bags for medical supplies packaging, and for medical specimen bags. Courier and mailing services also use polythene bags for mail and packages, to protect them from water.

## 1.5 DISPOSAL OF POLYETHENE

In the past two decades, polyethene and other plastics have become the most favoured materials in food and water packaging industry resulting in their increased proportions in the waste streams (Jayasekara *et. al*., 2005; Fobil and Hogarh, 2006). Most polyethene wastes are discarded on land, incinerated, recycled or disposed off through landfills. The wide use of polyethene and its careless disposal makes it an

important environmental issue. Their persistence in landfills is adding to the growing water and surface waste litter problems which have caused concern about non degradable wastes and promoted increased interest in the development of products that are degradable or in the development of new alternatives for the reduction of wastes (Kumar *et al.,* 2007). Polyethene and plastics in general have received more attention than any other component of the solid waste stream due to their durability and visibility in litter (Shah, 2007).

## 1.6 HAZARDS OF POLYETHENE

Polyethene plays an important role for many 'short-live' applications such as packaging and carrier bags (Shah, 2007). The end result of widespread use and lack of proper disposal system was and is still causing severe damage to the environment. The hazards of discarding polythene are becoming more and more severe (Shah, 2007).

Most of the used polyethene bags are often thrown in the streets, drainage facilities or are blown by wind into the drainages leading to clogging of drains and sewage systems to cause water stagnation and flooding (Ranasinghe, 2002; Aziz and Galib, 2005). Water clogging was believed to be the cause of several outbreaks of dengue fever in Dhaka in the last few years. Blocked drains forced sewer water into drinking water supplies during the floods turning the water virtually poisonous (Aziz and Galib, 2005).

Polyethene discarded on land act as breeding hubs for mosquitoes where they lay their eggs in collected rain water in the bags. This can lead to spread of mosquitoes with subsequent increase in incidence of malaria (Sathaananthan, 2004). Polyethene bags carelessly thrown block rain water from seeping into the soil due to

its impermeability thereby making a loss in the natural underground water storage system. This impermeability also prevents water and air to penetrate into the soil thereby affecting soil fertility (Pramila *et al*., 2012).

When polyethene bags get buried in the soil, they prevent emission of toxic gases and pollutants from the earth. Discarded polyethene bags on land prevent sunlight exposure of the soil thereby destroying the beneficial bacteria causing loss of soil fertility and the capacity of soil to yield agricultural produce. Burying polyethene bags in mountainous regions has been shown to loosen the grip on vegetation and soil binding causing landslides (Aziz and Galib, 2005). Polyethene bags that have been discarded into water bodies threaten life in these water bodies affecting the survival of animals in the aquatic and marine ecosystems. They cause blockage in the intestine of fish, bird and marine mammals resulting in death (Ranasinghe, 2002; Sathaananthan, 2004; Aziz and Galib, 2005). Polyethene bag rumen impaction as a result of environmental pollution is fast becoming a major problem in ruminants in many parts of Nigeria and other parts of the world. These bags are thrown on rubbish dumps, road sides and virtually everywhere posing a great hazard to ruminants that are left to scavenge around (Mohammed and Muhammad, 2007). Many street cows died in many Indian cities and autopsies of dead cows revealed that the reason for death was eating of polyethene. According to official estimates, in Lucknow alone, about 80-100 cows die every day as a result of polyethene consumption. Elsewhere in the state too, the polythene plague is playing havoc with the cattle population. The Animal Husbandry Department of Uttar Pradesh has come up with an alarming discovery: Milk from cows which have polythene bags clogging their stomachs can cause diseases like tuberculosis and cancer (lessplasticmorelife.com. 2012).

Carelessly discarded polyethene bags and other plastics reduce the aesthetic quality of the environment because they become litter. Burning of polyethene bags result in emission of poisonous gases, (dioxin and hydrogen cyanide) which are highly carcinogenic. Inhalation of these gases causes serious health problems in people (Ranasinghe, 2002; Sathaananthan, 2004; Aziz and Galib, 2005).

## 1.7 DEGRADATION OF POLYETHENE

Degradation is defined as any physical or chemical change in polymer as a result of environmental factors (light, heat, moisture) and/or chemical conditions (Orhan *et al*., 2004; Shah, 2007; Pramila *et al*., 2012). Polyethene (as well as other plastics) remains after disposal for thousands of years in the environment. However, polyethene is subject to oxidative degradation by means of light and temperature leading to the deterioration of its mechanical properties and finally to the fragmentation of the product. Because of this, producers amend polyethene with antioxidants to enable processing of the material without danger of its eventual oxidation to preserve a product (Arkatkar *et al.,* 2009).

The non degradability of polyethene is due to its chemical structure, large molecular weight, crystalline nature and hydrophobicity (Hadad *et al*., 2005; Satlewal *et al*., 2008; Shah *et al*., 2009). Abiotic degradation mainly depends on temperature, light exposure, oxygen and moisture availability. Environmental degradation by sunlight and oxygen may result in loss of tensile strength and enbrittlement without commiserate loss of mass while degradation by mechanical forces may simply reduce large pieces of plastics to smaller fragments (Suseela and Toppo, 2007). Degradation can be achieved through photo degradation by the use of light energy, thermal

degradation by the use of heat energy or biodegradation by the use of microorganisms (Shah, 2007).

### 1.7.1 Biodegradation of Polyethene

Biodegradation is defined as any physical or chemical change in a material as a result of biological activities (Shah, 2007). The biodegradability of polymers is predetermined by their chemical and/or physical structure. Of specific importance is the need of having hydrolysable or oxidizable bonds in the polymer backbone. The accessibility of these bonds for enzymes and a sufficient flexibility of macromolecules are subsequent requirements for biodegradation (Sasek *et. al*., 2006). The resistance of polyethene to biological attack is related to its hydrophobicity, water repellence, high molecular weight and its lack of functional groups recognizable by microbial enzymatic systems. Major strategies to facilitate polyethene disintegration and subsequent biodegradation were focused on the direct incorporation of carbonyl groups within the backbone or on their in situ generation by pro-oxidants (Chiellini *et al*., 2003; Ramesh and Pramila, 2011).

Presently, materials under development consists of a well balanced proportion of antioxidants and pro-oxidants such that the material preserves its mechanical and service properties during preset period and after this relatively fast and intensive oxidation occurs. Due to oxidation, the molecular weight of polyethene is dramatically reduced to low molecular weight oxidation products (Arkatkar *et al.,* 2009).

### 1.7.2 Microorganisms Involved in Polyethene Degradation

Microorganisms play a significant role in biological decomposition of materials including synthetic polymers in natural environments. High density and low density Polyethenes are the most commonly used synthetic plastics. They are slow in degradability in natural environments, causing serious environmental problems (Seneviratne *et. al*., 2006; Nayak and Tiwari 2011).

Polymers are potential substrates for heterotrophic microorganisms including bacteria and fungi. Microorganisms such as bacteria, fungi and actinomyces are involved in the degradation of both natural and synthetic plastics (Gu *et al*., 2000). Polyethene considered to be inert can be biodegraded if the right microbial strain is isolated (Hadad *et al*., 2005). A wide variety of Actinomycetes like *Streptomyces* strains, fungi like *Aspergillus* and *Penicillium* are reported active against polyethene (Zheng *et al*., 2005). In May 2008, a sixteen year old young Canadian, Daniel Burd discovered a type of bacteria, *Sphingomonas* that can degrade over 40% of the weight of plastic bags in less than three months (Wylie, 2008 ).

## 1.8 STATEMENT OF THE PROBLEM

Commercial use of polyethene started in the 1980s and successfully took over the jute and paper bag market. Its popularity increased due to its low price and portability. People use and throw polyethene bags carelessly everywhere with no effective disposal system resulting in dire consequences on the environment, man and wildlife (Aziz and Galib, 2005).

The world's annual consumption of plastic materials has increased from about fifty million tonnes in the 1950s to nearly hundred million tonnes today (Bollag *et al.*,

2000; Waste Online, 2006). Today, the use of polyethene has become an unavoidable entity of human life especially in the developing world. As the consumption of polyethene material increased in manifold, its disposal is emerging as a parallel industry. Disposal of polyethene material is a serious problem. Normally, polyethenes are thrown into water bodies as garbage or discarded in landfills to degrade (Suseela and Toppo, 2007). They accumulate in the environment at a rate of twenty five million tons per year (Hadad *et. al*., 2005; Satlewal *et al*., 2008).

The problems of polythene pollution in Nigeria are quite glaring, most of the used polyethene bags are typically thrown in the streets or into drainage facilities or are blown by wind into the drainages leading to clogging of drains and sewage systems, water stagnation and flooding (Okafor *et al.,* 2008). Improperly discarded polyethene bags are harmful to animals when swallowed. Polythene bag rumen impaction as a result of environmental pollution is fast becoming a major problem in ruminants in many parts of Nigeria. This is due to wide spread use and improper disposal of polyethene shopping bags. These bags are thrown on rubbish dumps, road sides and virtually everywhere posing as a great hazard to ruminants that are left to scavenge around (Mohammed and Muhammad, 2007). The practice of burning polyethene bags as a method of disposal causes air pollution and results in emission of poisonous gases; (dioxin and hydrogen cyanide) which are highly carcinogenic. Inhalation of these gases causes serious health problems (Aziz and Galib, 2005). In this part of West Africa, polythene bags and related plastic bottles are generally used by households in almost all consumables. They comprise bags for small pints of water for safe drinking, and small to medium size polythene bags for wrapping consumables and take-away food items. These containers are daily used and dumped or littered around every part, nook and cranny of the environment and hence constitute health

hazard and nuisance to the society (Ibrahim, 2009). Polyethene is non biodegradable but its strength while in water or soil deteriorates with time and during the period, the chemicals with which the polyethene is composed of are gradually released thus polluting the soil or water for up to 40 years (Aziegbe, 2007).

It is not certain when exactly the use of polyethene started in Nigeria but what cannot be disputed is that its usage and the concomitant environmental pollution has been on the increase since the 1970s when rapid urbanization, socialization, cultural integration and development in technology resulted in a shift in the average Nigerian life style from the traditional to the western ways of living and feeding, together with the placement of aesthetics over and above the use of natural leaves as wrappers. This accounts for the continued patronage and use of polyethene in Nigeria. Since polyethene in Nigeria is not adequately disposed off, they are ever present on the landscape and the environment is therefore, filthily coloured with all shades of polyethene resulting in a drastic reduction of environmental aesthetics. Polyethene bags are also capable of holding rain for days, weeks and months. These small pools of water are usually breeding ground for mosquitoes thereby increasing the incidence of malaria in Nigeria (Aziegbe, 2007). Chances are that our environment will become seriously unsustainable in the future if this situation is not checked.

## 1.9 JUSTIFICATION OF THE STUDY

The use of polyethene has become a part of our everyday life. Market mechanism drives people to use polyethene because it is cheap and easily available. Lack of degradability and the closing of landfill sites as well as growing water and land pollution problems have led to concern about plastics. Awareness of the waste problem and its impact on the environment has awakened new interest in the area of

degradable polymers (Shah *et al*., 2008). Biodegradable or degradable polyethene is either starch based (hydrodegradable) or additive based (photodegradable and biodegradable). Degradable polyethene as alternative to conventional polyethene has poor mechanical strength and short shelf life. Their degradation depends on conditions of heat, light, stress, air and can only be composted in a special composting facility. Health and safety concerns have also arisen over potentially hazardous chemical additives to plastics (Waste Online, 2006). The greatest stumbling block is cost; it costs an average of $1.30/lb to produce degradable polymers such as Poly Lactic Acid (PLA) compared to $0.5/lb of polyethene. These biopolymers have not been able to replace polyethene in the packaging industry and will be ludicrous to expect them to fully replace the conventional polymer anytime soon (Shah, 2010).

It is important to consider the microbial degradation of both natural and synthetic polymers in order to understand what is necessary for biodegradation and the mechanisms involved (Shah *et al*., 2008). Hence there is an urgent need to discover efficient microorganisms (and/or their products) capable of utilizing polyethene as their carbon source. Finding microorganisms with the ability to biodegrade polyethene into simple products (carbon dioxide and water) that are safely absorbed into the ecosystem will go a long way in curbing this global problem.

## 1.10 AIM AND OBJECTIVES OF THE STUDY

### 1.10.1 Aim of the Study

This study is aimed at isolating microorganisms associated with buried low density polyethene carrier bags with the ability of degrading polyethene and assessing the effect of starch pretreatment on the degradation of polyethene carrier bags.

### 1.10.2 Specific Objectives

1. To isolate, identify and screen microbial isolates attached to low density polyethene carrier bags buried in refuse dumps with ability to degrade them.

2. To subject synthetic low density polyethene carrier bags and starch pretreated low density polyethene carrier bags to degradation using selected microorganisms.

3. To compare the amount of polyethene degraded by the selected microorganisms using weight loss as an analytical tool.

4. To analyze the degradation of the untreated and starch treated synthetic low density polyethene bags before and after microbial applications using Fourier Transform Infrared Spectroscopy (FTIR).

# CHAPTER TWO

# LITERATURE REVIEW

## 2. 1 POLYETHENE CARRIER BAGS

One of the most common items in our modern world today is the plastic shopping bag. Highly convenient, strong and inexpensive, plastic shopping bags are appealing to both customers and businesses as a reliable way to deliver goods from the store to home. Plastic carrier bags are made out of polyethene which is a synthetic polymer from a hydrocarbon source consisting of the monomer, ethylene, that are engineered, manipulated and then processed for the purpose of bonding together into long polymer chains (Kumari *et al*., 2009; Ramesh and Pramila, 2011; Usha *et al*., 2011).

The use of polyethene has become an unavoidable part of human life (Kumar *et al*., 2007). Market mechanisms drive people to use polyethene because it is cheap and easily available (Suseela and Toppo, 2007). Plastic carrier bags have contributed significantly in creating a sustainable, cost effective, energy efficient packaging system and for carrying, storing and packaging various types of commodities/products. The properties of non toxicity, inertness, chemical resistance, safety in handling due to its non breakability, cheapness and availability has contributed to its popular use as a packaging and carrier bag (Zhang and Carter, 2012).

## 2.2 DISPOSAL METHODS OF POLYETHENE

As the consumption of polyethene has increased manifold, its waste management is emerging as a parallel industry. About 140 million tonnes of synthetic

polymers are produced worldwide annually with their utility escalating at a rate of 12% per annum (Shah, 2007; Nanda *et al.,* 2010; Usha *et al*., 2011). About 3% of plastic materials is recycled while the remainder are left as litter or land filler ( Kumari *et al.,* 2009).

The adoption of a more hygienic mode of packaging food, beverages, 'iced water' and other products has brought about the replacement of the existing cultural packaging methods as leaf wrappers, brown paper and metal cups in cities and towns. The replacement of these cultural methods is due to the unique properties of versatility, inertness, flexibility and cost effectiveness of polyethene carrier bags; because of this, plastics have become the most favoured packaging material (Fobil and Hogarh, 2006). According to Vijaya and Reddy (2008), the drastic rise in the use of non biodegradable plastic materials during the past three decades has not been accompanied by corresponding developmental procedures for safe disposal or degradation of these plastics. These plastics are inert and resistant to microbial attack and therefore remain in the nature without any deterioration for a very long time.

Most plastic wastes are disposed by:

**i. Landfilling**

Millions of tons of plastic wastes including low density polyethene carrier bags and other packaging materials are buried in landfill sites around the world every year. Conventional polyethene products can take longer than a hundred years to degrade; taking up valuable landfill space (Shah, 2007). Unfortunately, this technique can no longer accommodate the present waste disposal problems because of rapid population growth and industrialization which are major factors competing for land. These two factors (population growth and industrialization) have greatly increased the volume of

polyethene generated (Aziegbe, 2007). Wastes deposited in landfills are also faced with strict anoxic conditions thereby making their degradation difficult.

**ii. Incineration**

The burning of polyethene and other plastic materials leads to serious air pollution. It also leads to release of toxic gases like dioxin and hydrogen cyanide which are carcinogenic. Experts have reported that polyethene bags and other plastics if burnt below seven thousand degree Celsius (7000 $^0$C) create a dioxin like poisonous gas which can cause cancer and skin diseases (Sathaananthan, 2004; Aziz and Galib, 2005).

**iii. Recycling**

Other measures introduced to deal with the polyethene waste include the use of the 3Rs, Reduce, Reuse, and Recycle. The advocacy of reducing the usage of polyethene and other plastics can only be plausible when other alternatives are sought and made available, such as reusable cloth, paper or other natural material like banana leaves (Ranasinghe, 2002).

Reuse means using the product in its original form without extra processing other than cleaning (Shah, 2007). The quality of product deteriorates with reuse. In recent reports, it has been mentioned that reuse of plastic bags can cause cross contamination of foods by microorganisms (Gerba *et al.,* 2010). The material to be reused must maintain the original quality.

Recycling provide economic and environmental benefits if the costs of resources used in collection, sorting, cleaning and reprocessing are not greater than the resources saved. Recycling is not economically feasible due to the cost factor and

loss of mechanical properties and the quality of most materials deteriorates with reprocessing (Shah, 2007; Singh *et al.,* 2012). The recycling rates for plastic bags are extremely low, only 1-3% (McKinney and Schoch, 2003; Kumari *et al*., 2009). This is attributed to three reasons:

i. plastics are made from different resins and because they cannot be mixed, they must be sorted and processed separately. Such labour- intensive processing is expensive in high wage countries like the U.S.A and Canada. Most plastics also contain stabilizers that must be removed before recycling.

ii. recovering individual plastic resin does not yield much material because only small amounts of any given resin are used per product

iii. The price of oil used to produce petrochemicals for making plastic resins is so low that the cost of virgin resins is much lower than that of recycled resins. The cost of recovery is very high. The largest problem is the fact that recycling plastic grocery bags will not "pay for itself". Recycling inevitably leads to a reduction of desired physical properties. Recycling and Reuse therefore have limited value in solving the Polyethene waste management problems (Miller, 2005; Shah, 2007).

## 2.3 HAZARDS OF POLYETHENE

There has been growing public concern over environmental deterioration associated with the improper disposal of polyethene and other plastic materials. Discarded low density polyethene bags and other plastics besides being highly visible are rapidly increasing the percentage of solid wastes in landfills, resistant to biodegradation leading to pollution, harmful to the natural environment. These problems have made plastic waste a major focus in the management of solid wastes (Kumar *et al*., 2007). Many negative reports abound in recent time on plastic bags

and their tremendous effect on the environment. These reports have generated public interest to solve the problem of plastic wastes.

### 2.3.1 Environmental Impacts/ Land Pollution

Ninety six percent (96%) of all grocery bags are thrown into landfills (Williamson, 2003). Plastic bags decompose very slowly and can in fact, take up to 1000 years; inhibiting the breakdown of biodegradable materials around or in it (Stevens, 2001). Light weight plastic bags are additionally harmful due to their propensity to be carried away by wind and become attached to tree branches, fill road side ditches or end up in public water ways, rivers or oceans. Burning of these wastes also releases harmful toxic gases thereby polluting the environment

### 2.3.2 Impact on Livestock and Wild Life

Plastic grocery bags are thrown on rubbish dumps and are often blown by wind and get caught in trees or along fences where they are mistakenly eaten by animals that are left to scavenge around leading to suffocation or blockage of digestive tracts and eventually death of the animals (Mohammed and Muhammed, 2007). South Africa, Kenya, Somaliland and India are four nations that report high levels of these problems with as many as 100 cows dying per day in India (Edwards, 2000; World Watch, 2004).

Several hundred thousand tonnes of plastics have been reported to be discarded into the marine environment every year. It has been estimated that one million marine animals are killed every year either by choking on floating plastics or by becoming entangled in plastic debris (Rutkowska *et al*., 2002; Kumar *et al*., 2007).

### 2.3.3 Impact on Human Health

Wind blown plastic bags fill road side ditches or end up in public water ways resulting in clogging of drains and sewage systems which in turn create significant negative health. Blocked drains lead to flooding as has been reported in The Asian News (2005) that Mumbai in India experienced massive monsoon flooding resulting in at least 1000 deaths with additional people suffering injuries. City officials blamed the destructive floods on plastic bags which clogged gutters and drains preventing the rain water from leaving the city through underground systems.

By clogging sewer pipes, plastic grocery bags also create stagnant water which produce ideal habitat for mosquitoes and other parasites with potential to spread a large number of diseases such as encephalitis and dengue fever but notably malaria. Plastic grocery bags also have the potential to leach their chemical components and toxins into soil and water sources which can be passed on to humans resulting in health dangers such as neurological problems and cancers (Environmental Literacy Council, 2005; Aziegbe, 2007).

Other hazards associated with improper polyethene disposal on land include

- blocking of rain water from seeping into the soil thereby affecting not only plant growth but also leads to non recharge of the underground water

- Polyethene buried in mountainous regions loosen the grip on vegetation and soil binding leading to landslides

- prevention of emission of toxic gases and pollutants from the earth

- destruction of beneficial bacteria in the soil causing loss of soil fertility and therefore reduction in the capacity of the soil to yield agricultural produce (Sathaananthan, 2004; Aziz and Galib, 2005; Kumari *et al.,* 2009).

## 2.4 DEGRADATION OF POLYETHENE

Degradation is defined as any physical or chemical change in a material caused by any environmental factor including light, heat, moisture, wind, chemical conditions or biological activities resulting in bond scission, chemical transformations, and changes in material properties such as mechanical, optical or electrical characteristics, cracking, erosion, or formation of new functional groups (Shah *et al*., 2008).

### 2.4.1 Types of Degradation of Polyethene

i. **Photo-degradation**

Photo degradation involves the use of light; it is first initiated by the absorption of light energy by the appropriate group present in the polyethene. This initial action of light results in the scission of the polyethene molecule giving rise to smaller fragments which eventually mix with the dust or the smaller volatile fragments and escape into the atmosphere (Shah *et al*., 2008).

ii. **Thermal degradation**

Thermal degradation is achieved by the use of heat energy in the presence of oxygen, (thermo-oxidative degradation). Thermal degradation rupture bonds of the polyethene resulting in radical sites which react with oxygen present in the air. This also results in smaller fragments and volatiles (Shah *et al*., 2008).

iii. **Biodegradation**

Biodegradation involves the use of microorganisms to cause physical or chemical change in a material (Shah, 2007; Vijaya and Reddy, 2008). It is defined as a process which occurs due to the action of enzymes secreted by living organisms (bacteria and fungi) leading to its chemical decomposition (Torabi and Hagheeghatpadjooh, 2004; Arkatkar *et al.*, 2009; Shah, 2010)

Degradation of polyethene by sunlight or heat may result in loss of tensile strength and enbrittlement without commiserate loss of mass while degradation by mechanical forces may simply reduce large pieces of plastic to smaller fragments. Biodegradation however involves the use of biological agents (microorganisms) with the ability to utilize the polyethene as a substrate for growth and energy resulting in end products (carbon dioxide, water and microbial biomass) that are harmless to the environment.

### 2.4.2 Nondegradability of Polyethene

Nondegradable plastics accumulate in the environment at a rate of 25 million tons/year (Arutchelvi *et al*., 2008; Arkatkar *et al*., 2009; Kumari *et al*., 2009). Hydrophobicity, high molecular weight and water repellence of Polyethene in its natural form account for its non biodegradability thus their use in packaging (**Arkatkar and Doble, 2008;** Ramesh and Pramila, 2011).

The wide spread application of these synthetic polymers is not only due to their favourable mechanical and thermal properties but also due to their stability and durability. These endless applications have resulted in the formation of large quantities of waste leading to their careless dumping in the environment. The

increased cost of waste disposal as well as potential hazards associated with waste incineration has led to serious concerns (Arkatkar *et al*., 2009). Therefore, there is a growing interest in degradable plastics which degrade more rapidly than conventional disposables (Orhan *et al*., 2004).

## 2.5 DEGRADABLE POLYETHENE

A degradable Polyethene is one which is capable of being broken down by mechanisms such as exposure to heat, light, oxygen or bacterial action. There are three types of polymers which scientists are currently focusing on

i. Conventional plastics (class I) which do not biodegrade because microorganisms in soil are not able to penetrate their smooth surfaces; they usually have an impenetrable petroleum based matrix. Examples include polyethene (PE), polypropylene (PP) and polystyrene (PS)

ii. Polymer blends (Class II) where natural fibers surround the conventional matrix. The goal is that these will biodegrade at a rapid rate than the conventional polymers. Microorganisms would be able to digest the natural polymer leaving a weak residue of polymer with rough and open edges. Examples include thermoplastic starch blends.

iii. Class III polymers which will completely biodegrade because the polymer matrix is composed of natural sources. Microorganisms can completely feed on these polymers and eventually result in the generation of carbon dioxide and water as by-products. Examples of this class III polymer include polylactic acid (PLA), polyhydroxybutyrate (PHB) and polycaprolactone (PCL).

However, polymers belonging to class III have their own challenges and shortcomings. PLA for instance has a higher density (1.25g/cc) compared to PP (0.946g/cc) and PS (1.05g/cc). It also has high polarity making it difficult to adhere to surfaces without tie-layers to non polar polyethene and PP in multi-layer structures. It has poorer heat resistance than polyethene and limited barrier against moisture and gases (Zhang and Carter, 2012). The greatest stumbling block in PLA and PCL is cost: an average of $1.30/lb compared to $0.5/lb of polyethene. Both have not been able to replace Polyethene in the packaging industry and it will be ludicrous to expect them to fully replace the conventional polymer anytime soon but they do have a very positive prospect, mainly because of the benefit that they come from renewable resources (Wu, 2003; Pajak *et al.,*2004,; Torabi and Hagheeghatpadjooh, 2004; Shah, 2010).

Research on degradable synthetic polymers began in the early 1980s with a very general estimate of worldwide plastic waste generation amounting to about 57million tons (Orhan *et al*., 2004). There is therefore is the great demand for degradable or biodegradable plastics as a means of reducing the environmental impact related to waste management of plastics (Kumar *et al*., 2007; Vijaya and Reddy, 2008; Mostafa *et al*., 2010). In this context, different degradable plastics have been developed where inertness and resistance to microbial attack is reduced by incorporating additives (starch and pro-oxidants), (Orhan *et al*., 2004).

Efforts have been made to enhance the rate of degradation of these recalcitrant polymers by modifying the polymer or initiating the degradation process by generating free radicals (Vijaya and Reddy, 2008; Arkatkar *et al*., 2009). The degradation rate can be enhanced by

i. Mixing with prooxidants

ii. Carrying out pretreatment which could be thermal, UV, microwave, high energy radiation, or chemicals like nitrous acid.

iii. Blending them with biodegradable natural polymers such as starch or cellulose or with biodegradable synthetic polymers such as (PLA) and (PCL)

iv. Improving the attachment of the organisms on the polymer surface by using surface active agents or inducing the microorganisms to produce surfactant and

v. Through genetic modification of the microorganism.

### 2.5.1 Polyolefin with Pro-oxidant

Additives such as pro-oxidants have been used to increase the rate of biodegradation of polyolefins. Pro-oxidants are transition metals which are added in the form of stearates. They accelerate photo and thermal oxidation in the chain. Examples include Iron (Fe), Cobalt (Co) and Manganese (Mn). The metal ions such as $Fe^{3+}$ initiate the formation of radical during photo oxidation whereas $Co^{2+}$ or $Mn^{3+}$ in the absence of light act as catalyst for decomposition of peroxides leading to chain scission. Materials with time programmed mechanical properties can be prepared by using a balanced mixture of antioxidant and pro-oxidant additives such that during exposure to weather when the antioxidant capacity is used up, there will be a relatively fast loss of mechanical properties of the polymer due to the presence of the pro-oxidant leading to its fragmentation (Arkatkar *et al.,* 2009).

### 2.5.2 Pretreatments

Pre-treatment of polymers using physical or chemical methods prior to biodegradation have been found to enhance the process of biodegradation. These pre-treatment methods include the use of

i. Ultra Violet radiation (UV)

Sunlight is rich in UV radiation and polymer wastes dumped in the open undergo photo initiation process. Photo oxidation, which is controlled by the intensity of the light leads to the formation of radicals which increase the reactivity of the polymer. This pre-treatment leads to a decrease in the average molecular weight of the polymer. Cleaved chains are most frequently terminated by carboxylic groups and other functionalities such as esters, ketones, alcohols and double bonds (Arkatkar *et al.,* 2009).

ii. Thermal pre-treatment

Thermal pre-treatment makes the polymer more potent to microbial attack. It oxidises the chain thereby introducing hydroxyl, carboxyl and hydroperoxyl groups. Formation of oxidised products also makes the polymer more hydrophilic which makes it more conducive for microbial attachment (Arkatkar *et al.,* 2009).

iii. High energy radiation

Electron-beam radiation is a form of ionising energy that is generally characterised by its low penetration and high dosage rate. It is a concentrated and highly charged stream of electrons. High energy electrons up to 10 million electron volts (MeV) can be produced by large scale accelerators. The energy of the electrons impinging on the polymer is absorbed by it thereby bringing in the required changes to it by way of producing radicals which can subsequently initiate several reactions in the polymer. Irradiated polymeric materials become brittle (deterioration) due to reduction in their molecular weight as a result of degradation. Other changes that

could occur include; loss in chain length, decrease in cross linking and modifications in the crystalline domain.

iv Chemical pre-treatment

Chemicals can affect the strength, flexibility, surface appearance, colour, dimension or weight of plastics. Chemicals affect polymers in several ways as follows:

- Attack the chain resulting in the reduction in its physical properties
- React or oxidise the functional groups in or on the chain. Depolymerisation can also take place during this process.
- Form radicals
- Bring about physical changes including absorption of solvents; change in its strength, electrical properties and colour resulting in softening and swelling of the plastic.
- Allow solvent to permeate through the plastic leading to its dissolution and
- Develop stress-cracking due to the interaction of a "stress-cracking agent" with moled-in or external stresses.

### 2.5.3. Polymer Blends

Blending of natural polymers with synthetic ones is one of the strategies being employed in order to enhance the biodegradation of synthetic polymers. These natural polymers include starch, cellulose, lignin, chitosan and chitin but the most popular is starch (Wu, 2003; Pajak *et al*., 2004; Borghei *et al*., 2010). The idea of the use and incorporation of a cheap biodegradable additive into otherwise inert polymers was introduced in 1973 by Griffin (Torres *et al.,* 2008). The starch inside the matrix of low

density polyethene acts as degradable filler and can improve its degradability. The percentage of natural polymer added in the blend affects the physical and mechanical properties of the synthetic polymer. The shelf life of such blends decrease since the rate of degradation of the natural polymer used as filler is several orders of magnitude larger than that of the synthetic polymer (Zheng *et al*., 2005; Arkatkar *et al*., 2009)

### 2.5.4 Surface active Agents; Biosurfactants

Biosurfactants are surface active compounds produced on microbial cell surface or secreted out by the microorganism. They are amphiphiles having both hydrophilic and hydrophobic groups which reduce the interfacial tension at the surface of the liquid or at the interface of two immiscible liquids. They increase the solubility, bioavailability and biodegradation of hydrophobic or insoluble organic compounds. They also play an essential role in the swarming motility of microorganisms, biofilm formation and can complex with heavy metals aiding their removal. Polyethenes have "$CH_2$" groups and therefore hydrophobic which is one of the reasons responsible for their resistance to microbial attack. The prerequisite for polymer biodegradation is the attachment of microorganisms or biofilm formation on the surface of these polymers. It is reported that external addition of synthetic surfactants like Tween 60/80 enhanced biofilm formation and thus rate of biodegradation (Arkatkar *et al.,* 2009).

### 2.5.5 Genetic Modification of Microorganisms

Microorganisms can be genetically modified so that the desired enzyme is made to be secreted more or the process could be directed to follow a specific pathway once the biodegradation pathway has been established. Reducing the long time period required for biodegradation of polymers can be achieved by enhancing the capability of the microorganism to degrade the polymer. This can be attempted by utilising

genetic engineering tools including DNA sequencing, protein sequencing and production of recombinant organisms (Arkatkar *et al.,* 2009).

## 2.6 STARCH AS AN ENHANCER

Starch is an organic filler that can be used in combination with synthetic polymers to make it biodegrade. Relatively inert polyethene could become more degradable by introducing starch as a biodegradable additive (Rutkowska *et al*., 2002; Shah *et al*., 2008; Borghei *et al*., 2010) and has received maximum attention in the preparation of biodegradable plastics. Polymer-starch blends are considered because of their biodegradability and they are environmentally friendly. Starch basically is made up of large and long chains of the monosaccharide glucose. The two main types of molecules present in starch are amylose, (a linear structure) and amylopectin, (a branched structure) irrespective of its source. The amylose is usually present at about 20-25% while amylopectin is about 75% - 80 % (Shah, 2010)

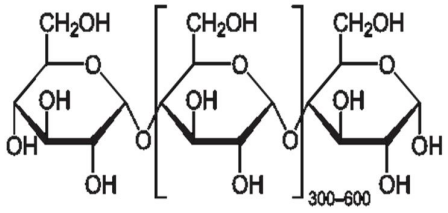



Figure 2.1 Structure of Amylose (Tester *et al*., 2004).

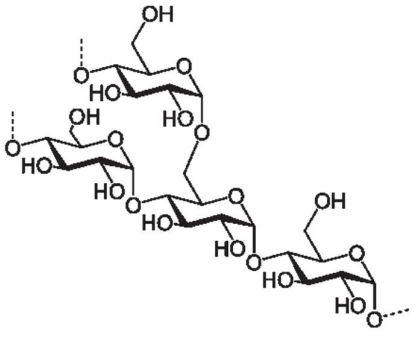



Figure 2.2 Structure of Amylopectin

. Starch is a good biodegradable filler because it possesses satisfactory thermal stability and causes minimum interference with melt-flow properties of most materials used in plastic industry (Fabunmi *et al*., 2007). Starch has many appealing properties,

it is abundant, sustainable/renewable, inexpensive, non hazardous and biodegradable; properties that are increasingly becoming more important in this environmentally conscious and sustainability driven days. Also, legislation affecting chemicals is increasing and the use of hazardous substances is becoming increasingly difficult and restricted. Starch also has potential functionality notably hydroxyl groups to assist adsorption and chemical modification (Borghei *et al*., 2010).

Nigeria is currently the world's largest producer of cassava (Phillips *et al*., 2004). Currently, small and medium scale entrepreneurs are directing their resources into various cassava processing projects across the country. The technology for the processing of cassava starch is relatively inexpensive, and can be sourced within Nigeria. This is because cassava processing machines are currently being locally fabricated (IITA, 2011). The direct incorporation of starch as a natural biodegradable agent to enhance or accelerate the bio degradability of polyethene has been evaluated. The use of starch as a biodegradable agent accelerated the time of degradation in the environment (Chiellini *et al.,* 2003; Borghei *et al*., 2010). Biodegradation of starch based polymers is as a result of enzymatic attack at the glucosidic linkages between the sugar groups leading to a reduction in chain length and the splitting off of the sugar units (mono, di and oligosaccharides) that are readily utilized in biochemical pathways (Mostafa *et al*., 2010; Shah, 2010).

## 2.7 MICROBIAL DEGRADATION OF POLYETHENE

Polyethene, a synthetic polymer from hydrocarbon source is made up of large numbers of small molecular units that are engineered, manipulated and then processed for the purpose of bonding together into large polymer chains. According to Kumari *et al*. (2009), recalcitrant plastics accumulate in the environment at the rate of twenty

five million (25million) tonnes per year. It is estimated that less than 0.5% of polyethene would degrade over 100 years and 1% if exposed to sunlight for two years (Suseela and Toppo, 2007).

High molecular weight, hydrophobicity, water repellence and lack of functional groups recognizable by microbial enzymatic systems accounts for polyethene resistance to biological attack (Chiellini *et al*., 2003). Since polyethene is widely used worldwide, and considering the enormity of its negative effects on the environment and wild life, there is considerable research on the development of biodegradable plastics as well as on the degradation of existing plastics using microorganisms (Kumar *et al.,* 2007).

There are two ways to manage the utility of these polymers in nature; one is to exploit microorganisms in degrading polyethene and two, is to develop artificial polymers susceptible to degradation (Nanda, 2010). Since microorganisms are capable of degrading most of the organic and inorganic materials, there is a lot of interest in the microbial degradation of plastics and polyethene wastes (Kumar *et al*., 2007). Considerable work on the biodegradation of polyethene has been carried out by many researchers such as Bonhomme *et al*. (2003), Kathiresan (2003), Sharma and Sharma (2004), Orhan *et al*. (2004), Shah (2007), Vijaya and Reddy (2008), Shah *et al*. (2008) and Kumari *et al*. (2009).

Microorganisms play an important role in biological decomposition of materials including synthetic polymers in natural environments (Seneviratne *et al*., 2006) and polymers are potential substrates for heterotrophic microorganisms. Biodgradation depends on molecular weight, crystallinity, nature of pretrearment, and type of microorganism. Generally, an increase in molecular weight results in decline of

polymer degradability by microorganisms. Microorganisms such as bacteria, fungi and actinomycetes are involved in degradation of both natural and synthetic polymers (Shah, 2007; **Arkatkar and Doble, 2008;** Shah *et al*., 2008; Ramesh and Pramila, 2011). Kathiresan (2003) in her studies on polyethene and plastic degrading microbes from the mangrove soil found both Gram positive and Gram negative bacteria associated with the degradation of polyethene. Among the bacteria, *Pseudomonas* species degraded 20.54% of polyethene and 8.16% of other plastics in one month period while *Aspergillus glaucus* degraded 28.80% of polyethene and 7.26% of other plastics within same period. Seneviratne *et al*., (2006) found a fungus, *Penicillium frequentans* and a bacterium, *Bacillus mycoides* to be the most effective organisms in their work aimed at isolating and identifying microorganisms associated with the degradation of various polyethene carrier bags in the soil while Burd in 2008 isolated Gram negative bacteria belonging to the genus *Pseudomonas* and genus *Sphingomonas* (Terry, 2008). This is supported by the findings of Sharma and Sharma (2004) who found *Pseudomonas stutzeri* to degrade low density polyethene and polypropylene. Vijaya and Reddy (2008) identified *Bacillus spp, Staphylococcus spp, Streptococcus spp, Diplococcus spp, Micrococcus spp, Pseudomonas spp*. *Moraxella spp, A. niger, A. ornatus, A. nidulans, A. cremeus, A. flavus, A. candidus and A. glaucus* as the predominant species isolated from the sample of polyethene bags in their studies on the impact of soil composting using municipal solid waste on biodegradation of plastics. Shah (2007) in his work on the role of microorganisms in biodegradation of plastics also isolated and identified a number of bacteria, actinomycetes and fungi having the ability to degrade both natural and synthetic plastics. These organisms included *Bacillus spp, Pseudomonas spp*, *Micrococcus spp, Corynebacterium spp, Arthrobacter spp, Aspergillus, Penicillium and Fusarium*

species. Therefore, polyethene considered to be inert can be degraded if the right microbial strain is isolated (Hadad *et al*., 2005).

Factors affecting the rate of biodegradation of polyolefins include

- Lack of active functional group
- High hydropobicity
- High molecular weight
- Distribution of crystalline and amorphous regions
- Structure of the polymer
- Chemical composition of the polymer (blends, presence of additives, uv stabilizers, and antioxidants)
- Presence of microorganisms in the environment in the form of mixed culture.
- Properties of the microorganisms including their ability to produce biosurfactants or other exo-polysaccharides, hydrophobic nature of bacterial cell walls.

### 2.7.1 Biodegradation of Polyethene/Starch Blend

Addition of readily biodegradable compounds such as starch to low density polyethene matrix can enhance the degradation of the carbon-carbon backbone (Shah *et al.,* 2008). The rate of degradation of polymer blends is initially controlled by the degradation of the more readily biodegradable starch component. This initial degradation interferes with the structural integrity of the polymer and increases the surface area considerably for enzymatic attack. Exposure of the remaining polymer to microbes and secreted degradative enzymes is then enhanced. The microbes in turn, release non specific oxidative enzymes that could attack the synthetic polymer. In addition, the gradual degradation of the natural polymer starch leads to increased

surface area by erosion and pitting. This will accelerate the degradation of the synthetic low density polyethene polymer by diffusion of oxygen, moisture and enzymes into the porous polymer matrix (Ismail *et al*., 2011). Starch also provides higher oxygen permeability as it is consumed by microorganisms. Higher permeability helps in the release of degradation products from the sample, thus, making the matrix hollow and increasing the surface to volume ratio (Rutkowska *et al*., 2002). This filler increases the adhesion of the organisms to the surface of the polymer due to the hydrophilic nature of the blend material and the adhesion of the organisms also improve their interaction with the hydrophobic polymer (Chiellini *et al*., 2003; Torabi and Hagheeghatpadjooh, 2004; Arkatkar *et al.,* 2009).

## 2.8 MECHANISM OF MICROBIAL DEGRADATION OF POLYETHENE

Microorganisms can use diverse carbon sources as catabolites. Microbial degradation of plastics is caused by enzymatic activities leading to chain cleavage of the polymer into oligomers and monomers after which they are further metabolised by the microbial cells (Nanda *et al.,* 2010) .The enzymes for metabolising these diverse substrates can be synthesised by a bacterium on a constant basis or else activate the enzyme synthesis when needed or when it is thermodynamically favourable.

Oxidative degradation is the main mechanism for non-hydrolysable polymers such as polyethene and polypropylene which leads to the reduction in their molecular weight. Oxidative enzymes like peroxidases, monooxygenases, manganese peroxidases and dehydrogenases are responsible for the oxidation of ethylenic groups. These extracellular or intracellular enzymes (depolymerases) convert the polymer into monomers, dimmers or oligomers (by a depolymerisation process) which can enter the microbial cell and then be utilized as an energy source (Shah *et al.,* 2008;

Arkatkar *et al.,* 2009). Polyethene is believed to be catabolised by microorganisms at the chain ends. The breakdown caused by microorganisms can be of three different types

i. A biophysical effect in which cell growth can cause mechanical damage
ii. A biochemical effect in which substances from the microorganisms can act on the polymer.
iii. Direct enzymatic action in which enzymes from the microorganisms attack components of the plastic product, leading to splitting or oxidative break down, (Torabi and Hagheeghatpadjooh, 2004).

Fungal and bacterial degradation of polymers may proceed by one or more mechanisms. The extent of degradation is likely to vary depending on the polymer's environment and desired application (Orhan *et al*., 2004; Vijaya and Reddy, 2008). Few reports have been published that elucidate the mechanism of biodegradation of polyolefins. The mechanism of photo degradation of polyethene followed by its biodegradation has been proposed and verified. During photo oxidation, cleavage occurs predominantly at the weak links which have lower bond energies (C-H has bond energy of 98Kcal/mole and C-O has bond energy of 79 Kcal/mole). This leads to the formation of free radicals. Cleavage can also occur due to heat, ionising radiation and mechanical stress. Radicals generated can react further with atmospheric oxygen and trigger the oxidation of the polymer. This reaction continues in a stepwise fashion producing carbonyls, aldehydes, peracid and acids (Arkatkar *et al*., 2009).

In the case of biodegradation, microorganisms can assimilate these abiotic intermediates thus complicating products found in the environment. The rate of degradation is sensitive to microbial population, moisture, temperature and oxygen in

the environment. Photo oxidation enhances the rate of biodegradation of the polymer; it leads to the scission of the main chain in the polymer, thereby leading to the formation of low molecular weight products. This results in the generation of large surface area due to its embrittlement and also a greater degree of hydrophilicity due to the introduction of carbonyl groups. These factors further promote the biodegradation of the polymer (Arkatkar *et al*., 2009).

Biodegradation of polymers involves the following steps:

i. Attachment of microorganisms to the surface of the polymer
ii. Growth of microorganisms utilizing the polymer as the carbon source
iii. Primary degradation of the polymer
iv. Ultimate degradation (Arutchelvi *et al*., 2008).

Microorganisms can attach to the surface if the polymer surface is hydrophilic. Since polyethene has only $CH_2$ groups, the surfaces are hydrophobic. Initial physical or chemical degradation leads to the insertion of hydrophilic groups on the polymer surface making it more hydrophilic. Once the organism gets attached to the surface, it starts to grow by using the polymer as the carbon source. During the primary degradation, the main chain is cleaved leading to the formation of low molecular weight fragments (oligomers, dimmers or monomers). These low molecular weight compounds are further utilized by the microbes as carbon and energy sources. Small oligomers may also diffuse into the organism and get assimilated. The ultimate products of degradation are carbon dioxide, water and biomass under aerobic conditions (Arutchelvi *et al*., 2008; Tokiwa *et al*., 2009).

## 2.9 MEASUREMENT OF BIODEGRADATION OF POLYETHENE

Some studies have assessed the biodegradability of plastic films by measuring changes in physical properties or by observation of microbial growth after exposure to biological or enzymatic treatments. However, in most of the studies, degradation has been quantified by

i. Weight loss of plastic material

ii. Carbon dioxide evolution

iii. Reduction in tensile strength

iv. Accumulation of biomass

v. Oxygen uptake rate

vi. Products of reaction using chemical analysis

vii. Surface changes

viii. Fourier Transformed Infra Red Spectroscopy (FTIR) which is a powerful tool for identifying types of chemical bonds in a molecule by producing an infrared absorption spectrum that is like a molecular "fingerprint". It is the most powerful tool for identifying types of chemical bonds (functional groups). The wavelength of light absorbed is characteristic of the chemical bond. By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined. The spectrum of an unknown can be identified by comparison to a library of known compounds (IR Absorptions, 2012) and

ix. the use of Scanning Electron Microscopy (SEM) which shows changes in mechanical and physical properties of the polymer such as micro cracks and or embrittlement (Shah,2007; Arutchelvi *et al.,* 2008; Vijaya and Reddy, 2008; Kumari *et al*., 2009).

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 SAMPLE COLLECTION

Buried synthetic low density polyethene (LDPE) bag pieces were dug up from a depth of 2cm-10cm from several dumpsites within Samaru community, Zaria. These pieces were collected into factory sterile polyethene bag and transported to the laboratory for further analysis.

## 3.2 MEDIA PREPARATION

Nutrient Agar (NA) and Potato Dextrose Agar (PDA) media used for isolation of the microorganisms from samples were prepared according to the manufacturer's instructions. Twenty eight grams (28g) of NA and thirty nine grams (39g) of PDA were each poured in one litre of distilled water respectively, boiled to dissolve completely and sterilized by autoclaving at 121$^{o}$C for 15minutes at 15psi. NA was used for the isolation of bacteria while PDA to which 1.5g/L of streptomycin (to suppress bacterial growth) was added was used for the isolation of fungi. Approximately 15ml were poured into sterile petri dishes after allowing the media to cool to a temperature of about 45$^{o}$C and allowed to gel.

## 3.3 ISOLATION OF MICROORGANISMS FROM LDPE SAMPLES

Using an electronic weighing balance, twenty five grams (25g) of the collected low density polyethene samples was transferred into a 500ml conical flask containing 225ml of sterile distilled water and the flask was shaken vigorously and allowed to stand for 30minutes. From this stock, 1ml was transferred aseptically into 9mls of sterile distilled water in a test tube using a sterile 2ml pipette to obtain a tenfold

dilution of $10^{-1}$. Further serial dilutions were carried out until a dilution factor of $10^{-6}$ was obtained. Using sterile 1ml pipettes, 0.1ml of prepared dilutions from $10^{-3}$ to $10^{-6}$ were aseptically transferred onto the surface of the solidified NA and PDA media and spread well with a flame sterilized bent glass rod. Inoculated petri dishes with NA were incubated at 37$^{o}$C while the plates with PDA were incubated at 25$^{o}$C (room temperature). NA Plates were observed for bacterial growth after 24 to 48 hours incubation while PDA plates were incubated for 5 to 7days and then observed for fungal growth, all growths were observed and record of colony colour and shape were made. Different colonies observed on both NA and PDA plates were purified by repeated streaking of each distinct colony on appropriate media until pure colonies were obtained. Purified bacterial and fungal isolates were inoculated onto sterile NA and PDA slants respectively and stored in the refrigerator at 4$^{o}$C for future use.

## 3.4 IDENTIFICATION OF MICROBIAL ISOLATES

### 3.4.1 Identification of Bacterial Isolates

Identification of bacterial isolates was done by using a combination of microscopic examination and biochemical tests. The Microgen$^{TM}$ Gram negative A+B identification system (Surrey, United Kingdom) was used for Gram negative isolates while the Microgen$^{TM}$ *Bacillus* identification system (Surrey, United Kingdom) was used for Gram positive isolates.

#### *3.4.1.1 Gram Staining*

A drop of distilled water was placed in the centre of a clean glass slide; using a flamed and cooled wire loop, a single colony from the petri dish was picked and emulsified in the distilled water to form a thin smear and allowed to air dry. Glass

slide with the dried smear was passed twice over a bunsen burner flame in order to fix it after which it was covered with crystal violet, (the primary stain) and left for 30 seconds to 60 seconds. Slide was carefully rinsed with a stream of water from a plastic bottle. Gram's iodine was added and allowed to remain for 10 seconds to 30 seconds and rinsed off. Decolourization was carried out with acetone by allowing it to run over the smear until no purple stain was remaining and rinsed again with water. The secondary stain, Safranin was added and allowed to remain in contact for 30 seconds to 60 seconds, rinsed with water, then blot - dried to remove most of the water and air dried (Johnson and Case, 1998). The stained slides were examined under the microscope using the oil immersion objective (x100).

#### *3.4.1.2 Catalase Test*

A wooden spatula was used to pick a colony from the culture plate and placed in a few drops of 3% hydrogen peroxide on a clean glass slide. Catalase converts hydrogen peroxide to water and oxygen bubbles indicating a positive reaction while absence of oxygen bubbles indicates a negative reaction (Prescott *et al.,* 2005).

#### **3.4.1.3** ***Microgen™ Gram negative A+ B and Microgen™ Bacillus Identification Kit***

After the Gram reaction, catalase test (a requirement for using the identification kit) was performed for all Gram positive isolates. Identification of bacterial isolates associated with buried LDPE was then performed based on biochemical tests using Microgen™ Gram negative (GN) A+B identification kit for Gram negative isolates and Microgen™ *Bacillus* Identification Kit for Gram positive isolates. Procedure for using the GN A+B identification kit was as follows:

A single colony from an 18-24hr culture was emulsified in 5ml of sterile 0.85% saline and mixed thoroughly. Using a sterile pipette, 3 drops of the bacterial suspension was added to each of the twenty four wells of the microwell test strips. After inoculation, wells 1, 2, 3 and 9 (GN A Strip) and 24 (GN B Strip) were overlaid with 3 drops of mineral oil. The strips were sealed back with the adhesive tape, labelled for each organism and incubated at 35 $^{O}$C. Results were read after 48hrs with the aid of the provided colour chart. Additional reagents were added to the following wells after 48hrs incubation. 1 drop of nitrate A + 1 drop of nitrate B reagents were added to well 7 in order to perform the nitrate reduction test after recording the Ortho-nitrophenol-galactosidase (ONPG) result. 2 drops of kovac's reagent was also added to well 8 and result read after 60 seconds. To well 10, a drop each of Voges Proskauer (VP1and VP II) reagents were added and results read after 15-30minutes. Tryptophan deaminase acid (TDA) reagent was also added to well 12 and result read after 60 seconds.

For the Gram positive, catalase positive organisms, the *Bacillus* identification strips were used as follows: Bacterial suspensions corresponding to 2.0 MacFarland Standard were prepared using provided *Bacillus* suspending medium and mixed thoroughly. Each well of the strip was then inoculated with 4 drops of the bacterial suspensions using sterile pipette, and the strip sealed back and incubated at 35$^{O}$C with well 21(Arginine) overlaid with mineral oil. Results were read after 24hrs for wells 1-18 and again for all wells at 48hrs. Additional reagents were also added to certain wells after 48hrs: well 19- Kovac's reagent, well 34, VPI, VPII reagents and well 20, nitrate A + nitrate B reagents. All results were read and recorded accordingly using the colour chart as a guide. The Microgen$^{(TM)}$ identification system generated an octal code that was used to determine the identity of the isolates using the Microgen$^{(TM)}$ Identification System Software.

### 3.4.1.4 Molecular Identification of Bacterial Isolates

**DNA Extraction from Bacterial Isolates:** Two bacterial isolates were identified by molecular method as follows: Pure bacterial isolates were inoculated into sterile Lauryl Boefni (LB) medium and incubated at $37^{O}$C for 18 to 24hours. Using a micropipette, 500µl of each bacterial isolate was transferred from the LB medium into 1.5ml sterile microtube. To this, was added 400µl of lysis buffer and 4µl of proteinase K and mixed thoroughly. All labelled tubes were then incubated at $55^{O}$C for 3 hours in a Robo Cycler Gradient 40 (Stratagene). With a micropipette, 200µl of 5M NaCl was then added to each tube and centrifuged at 13,000rpm for 10minutes at $4^{O}$C using an ALC High speed centrifuge (PM 180R). After centrifuging, 400µl of supernatant from each tube was decanted into fresh tubes and 800µl of 100% cold ethanol was added and then centrifuged at 13,000rpm for 30minutes at $4^{O}$C. Supernatants were then decanted into fresh microtubes and 400µl of 75% ethanol was added to tubes and spinned as before for 5minutes and then again for 2minutes. Thereafter, the tubes were further spinned for 30seconds to completely remove the ethanol and NaCl. Extracted DNA pellets were dried by leaving the microtubes opened for 5minutes after which they were resuspended in 20µl sterile water, (molecular biology grade). Using micropipette, 8µl of extracted DNA from each isolate was mixed with 5µl of loading dye and loaded into wells along with 10µl of molecular marker on 1.5% Agarose gel and electrophoresed (Electrophoresis Power Supply; model 250:GIBCO, BRL; Life Technologies) at 100volts for 60minutes. The Agarose gel was removed from the Tris Acetyl EDTA (TAE) buffer and visualised in a UV transilluminator (Gel Doc2000; BIO-RAD) and gel documentation of DNA bands carried out.

### 3.4.2 Identification of Fungal Isolates

Identification of fungal isolates included a combination of macroscopic and microscopic examination as follows: using a sterile needle, portions of colonies were removed from the culture and placed on clean glass slides with lactophenol cotton blue stain, teased apart and covered with cover slips and then observed under microscope using the x40 objective (Beneke and Rogers, 1980). Spores and other structures were observed and isolates identified according to the identification keys of Raper and Fennel (1965).

#### 3.4.2.1 Molecular Identification of Bacterial Isolates

**DNA Extraction from Fungal Isolates*:*** Fungal isolates were grown on Potato Dextrose Agar (PDA) for 7 days after which about 200mg (wet weight) were transferred from the culture plates into 1.5ml capacity micro centrifuge tubes. To each of the tubes, was added 400µl of lysis buffer and 4µl of proteinase K and pounded continuously for one hour and then incubated at $55^{O}$C overnight. Equal volumes (700µl) of phenol: chloroform was added to each tube and vortexed briefly to mix. Tubes were then centrifuged at 10,000 rpm at 4 $^{O}$C for 5minutes. Using a sterile micropipette, the top layer was removed and transferred into new tubes and the aqueous DNA layer re-extracted again using the phenol:chloroform. DNA was then precipitated by addition of 70µl of 0.3M Sodium Acetate and mixed well. With the help of a micropipette, 700µl of isopropanol was then added and inverted 5-6 times, then freezed on ethanol dry ice bath for 10minutes. Tubes were then centrifuged again at 10,000rpm, $4^{O}$C for 5minutes and the supernatant removed. Washing of DNA pellets was carried out by addition of 1ml of 70% ethanol and vortexing briefly followed by centrifugation for 5minutes at 10,000rpm. The supernatant was then

removed and tubes left opened at room temperature in order to dry out the ethanol. Dried DNA pellets were then dissolved in 50µl TAE buffer. Eight microlitre of extracted DNA from each isolate was mixed with 5µl of loading dye and loaded into wells along with 10µl of molecular marker on 1.5% Agarose gel and electrophoresed (Electrophoresis Power Supply; model 250:GIBCO, BRL; Life Technologies) at 100volts for 60minutes. The Agarose gel was removed from the TAE buffer, visualised in a UV transilluminator (Gel Doc2000; BIO-RAD) and gel documentation of DNA bands carried out.

**Polymerase Chain Reaction (PCR) for Bacterial and Fungal Isolates*:*** The PCR reaction for isolated bacterial and fungal DNAs was performed with AccuPower$^{(R)}$HotStart PCR Premix (BIONEER) kit according to manufacturer's instructions as follows:

The primer solutions were thawed and template DNA prepared. Appropriate volumes- 0.5µl of diluted primer mix were distributed into each AccuPower$^{(R)}$ HotStart PCR premix. One and half microlitre (1.5µl) of template DNA were added and 18µl of deionised water to make up the total volume to 20µl. A negative control without DNA template was also included. The lyophilized blue pellets were dissolved by vortexing and spinning for about 1minute. The thermo cycler - GeneAmp PCR System 2400 ( PERKIN ELMER) was programmed as follows: pre – denaturation step at 94$^{O}$C for 5minutes followed by 40 cycles of denaturation at 94$^{O}$C for 1minute, annealing at 34$^{O}$C for 1minute and extension at 72$^{O}$C for 1minute and a final extension was at 72$^{O}$C for 5minutes. The PCR tubes were placed in the thermo cycler and the cycling programme started. The amplified PCR products were resolved together with the ladder in 1.5% Agarose gel at100volts for 60 minutes and then visualized in a UV transilluminator (Gel Doc 2000; BIO-RAD) and gel documentation carried out.

Universal 16s ribosomal subunit primers with the following sequences were used for bacterial isolates

GGACTACAGGGTATCTAAT-3' Ribose 1

AGAGTTTGATCCTGG-3' Ribose 2 (789bp)

DNA amplicons were then excised from the gel into1.5ml micro tubes and stored at $4^{O}C$ for further use.

**Gel Purification Using the Accuprep$^{(R)}$ Gel Purification Kit *(*BIONEER*):*** To the excised DNA fragments, 600µl of buffer 1, (gel binding buffer) provided in the kit was added to each tube and then incubated at $60^{O}C$ for 10minutes with vortexing every 2-3minutes during incubation in order to dissolve the gel. The dissolved mixtures were then transferred into the DNA binding filter columns and centrifuged for 1minute at 13,000 revolutions per minute (rpm). The flow through was then poured off and the DNA binding filter column reassembled with the 2.0ml collection tube. 500µl of buffer 2 (washing buffer) was then added to the DNA binding filter column and centrifuged at 13,000 rpm for 1 minute in order to remove salts and soluble impurities in the DNA binding column tube. The flow through was poured off and the DNA binding filter column reassembled again. This washing step was repeated again and dried by additional centrifugation at 13,000 rpm for 1minute to remove residual ethanol. The DNA binding filter columns were then transferred into new 1.5ml micro tubes and centrifuged as before. A final elution step was carried out by the addition of 30µl of buffer 3 (eluting buffer) to the centre of the DNA binding filter column and allowed to stand for at least 1 minute at room temperature for elution**.**

**Sequencing of Bacterial and Fungal Amplicons:** Sequencing reaction was prepared in a 2.0ml tube using the Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit. All reagents were kept on ice while preparing the sequencing reactions and were added in this order

DNA template 10.0µl, Primer (reverse), 2.0µl and DTCS Quick start master mix, 8.0µl. The sequencing reaction in the PCR machine was programmed as follows: Denaturation at 96 $^{O}$C for 20seconds, annealing at 50$^{O}$C for 20seconds and amplification at 60$^{O}$C for 4minutes for 30 cycles.

**Ethanol Precipitation:** Sterile 0.5ml tube for each sample was prepared and labelled. To each of the labelled tubes, 5µl of the stop solution/glycogen mixture comprising of 2µl of 3M Sodium acetate, 2µl of 100mM $Na_2$-EDTA and 1µl of 20mg/ml of glycogen (provided in the kit) was added per sequencing reaction. The sequencing reaction was transferred to each of the appropriately labelled tube and mixed thoroughly. Sixty microlitres of cold 95%(v/v) ethanol from -20 freezer was added and mixed thoroughly then immediately centrifuged at 14,000rpm at 4$^{o}$C for 15min and the supernatant carefully removed with a micropipette. The pellets were rinsed with 200µl 70%(v/v) ethanol from -20 freezer, centrifuged at 14,000rpm at 4$^{O}$C for a minimum of 2minutes and all the supernatants were carefully removed with a micropipette. Pellets were vacuum dried for 10min and resuspended in 40µl of the sample loading solution (provided in the kit).

**Sample Preparation for Loading in to the Instrument:** Resuspended samples were transferred to the appropriate wells of the sample plate (PN 609801) and overlaid with one drop of mineral oil from the kit. The sample plate was loaded into the instrument, CEQ2000XL DNA Analysis System (BECKMAN COULTER, U.S.A) and started.

## 3.5 SCREENING OF MICROBIAL ISOLATES FOR POLYETHENE DEGRADATION ABILITY

Bacterial and fungal isolates being screened for their ability to use Polyethene as their source of carbon and energy were grown in a basal medium containing (g/ $l^{-1}$ of distilled water) $NH_4NO_3$, 1.0; $MgSO_4.7H_2O$, 0.2; $K_2HPO_4$, 1.0; $CaCl_2.2H_2O$, 0.1; KCl, 0.15; yeast extract, 0.1 and 1.0mg/$l^{-1}$ of each of the following micro elements: $FeSO_4.6H_2O$, $ZnSO_4.7H_2O$ and $MnSO_4$, (Hadad *et al.,* 2005). 100ml of the basal medium were dispensed into 250ml conical flasks and sterilized by autoclaving at $121^0$C for 15minutes at 15psi.

Low density Polyethene pellets obtained from Eleme Petrochemical Company, Port Harcourt, Rivers State, Nigeria were melted at $130^0$C using a two roll mill No. 5183 (Reliable, Rubber and Plastics Manufacturing Company, North Bergen, New Jersey, USA) and then hot pressed at $130^0$C and 3 tons for 5minutes to 10minutes (Hot Press model 3851-0. Carver Inc. USA) into sheets using 150 x 150 moulds at the Polymer Laboratory, National Institute of Leather and Science Technology, (NILEST), Samaru-Zaria, Nigeria. Sheets were cut into thin strips, dried overnight in an oven at $60^0$C. Half gram of the dried strips were disinfected for 30minutes in 70% ethanol and added to the conical flasks. One millilitre containing 3.0 x $10^8$ CFU/ml of bacterial isolates (corresponding to McFarland Standard No. 1) and 1ml containing 5 x $10^6$ spore/ml of fungal isolates were added to the above conical flasks and labelled appropriately. All flasks were prepared in duplicates. Flasks with bacterial isolates were shake - culture incubated at $37^0$C for 30 days at 150 revolutions per minute (rpm) in an orbital shaker (Lab-Line Environ- shaker 18. No. 3527-1/34, Melrose Park, ILL) and all flasks with fungal isolates were also shake – culture incubated at ambient temperature ($25^0$C) for 30days using an orbital shaker (Stuart Orbital Shaker,

SSL1) at 150 rpm. After 30days of incubation, remaining polyethene strips were removed from each flask, washed with 2% sodium dodecyl sulphate (SDS) for 4 hours in order to remove bacterial and fungal biofilm, rinsed with distilled water, placed on filter paper sheets and dried in an oven overnight at $60^0$C. Each dried strip was weighed using an electronic weighing balance (FA2004, U.S.A) and resulting weights recorded accordingly.

### 3.6 PREPARATION OF POLYETHENE/STARCH BLEND

Cassava starch bought from Samaru market was placed in a polyethene bag, wrapped with foil paper and sterilized by autoclaving at $121^0$C for 15minutes at 15psi in order to destroy the inherent microorganisms in the starch. Twenty grams of cassava starch was added gradually to 50g of low density polyethene while being melted at $130^0$C with a two roll mill No. 5183 (Reliable, Rubber and plastics manufacturing company, North Bergen, New Jersey, USA) and mixed continuously until a well mixed starch/polyethene blend was obtained. This blend was then hot pressed (Hot Press model 3851-0. Carver Inc. USA) at $130^0$C and thirty tonnes for 10minutes using 150 x150 moulds at the Polymer Laboratory, National Institute of Leather and Science Technology, (NILEST), Samaru-Zaria, Nigeria. The polyethene/starch blend sheets were kept for further use.

### 3.7 BIODEGRADATION OF POLYETHENE BAGS USING SELECTED INDIVIDUAL BACTERIAL AND FUNGAL ISOLATES

Bacterial isolates and fungal isolates which gave a weight reduction of polyethene strips of 0.1% to 3% were used to carry out biodegradation studies on synthetic low density polyethene carrier bags and the prepared polyethene/starch blend. Synthetic low density polyethene carrier bags purchased from Samaru market

and the prepared polyethene/starch blend were cut into thin strips (1cm x 3cm), and dried overnight at $60^0$C. Half of a gram of both types of polyethene was sterilized for 30minutes in 70% ethanol and placed into 250ml conical flasks containing 100ml of sterilized basal medium. One millilitre containing 3.0 x $10^8$ CFU/ml of bacterial isolates (corresponding to McFarland Standard no. 1) and 1ml containing 5 x $10^6$ spores/ml of fungal isolates were added to the above conical flasks and labelled appropriately. All flasks were prepared in duplicates. Flasks with bacterial isolates were incubated at $37^0$C for 30 days at 150rpm in an orbital shaker and all flasks with fungal isolates were incubated at ambient temperature ($25^0$C) for 30days using an orbital shaker at 150 rpm. After 30days of incubation, the remaining polyethene strips were removed from each flask, washed with 2% sodium dodecyl sulphate for 4 hours in order to remove bacterial and fungal biofilm, rinsed with distilled water, placed on filter paper sheets and dried in an oven (Memmert) overnight at $60^0$C. Each dried strip was weighed using an electronic weighing balance (FA2004, U.S.A) and resulting weights recorded accordingly.

### 3.7.1 Biodegradation of Polyethene Bags Using Bacterial and Fungal Consortium

Synthetic low density polyethene carrier bags purchased from Samaru market and the prepared polyethene/starch blend were cut into thin strips (1cm x 3cm), and dried overnight at $60^0$C. Half gram of both types of low density polyethene was sterilized for 30minutes in 70% ethanol, placed into 250ml conical flasks containing 100ml of sterilized basal medium. Bacterial and fungal consortia comprising a combination of two different organisms were also prepared. One millilitre containing 3.0 x $10^8$CFU/ml of bacterial consortium (corresponding to McFarland Standard no. 1) and 1ml containing 5 x $10^6$ spore/ml of fungal consortium were added to the basal

medium in the conical flasks and labelled appropriately. All flasks were prepared in duplicates. Flasks with bacterial isolates were incubated at $37^0$C for 30 days at 150 rpm in an orbital shaker and all flasks with fungal isolates were incubated at ambient temperature ($25^0$C) for 30days using an orbital shaker at 150 rpm. After 30days of incubation, the remaining polyethene strips were removed from each flask, washed with 2% sodium dodecyl sulphate for 4 hours in order to remove the bacterial and fungal biofilm, rinsed with distilled water, placed on filter paper sheets and dried in an oven overnight at $60^0$C. Each dried strip was weighed using an electronic balance and resulting weights recorded.

## 3.8 ANALYTICAL TECHNIQUES FOR DETERMINATION OF EXTENT OF BIODEGRADATION OF POLYETHENE BAGS BY THE BACTERIAL AND FUNGAL ISOLATES

### 3.8.1. Weight Loss

Half gram (0.5g) of all polyethene samples were used as the initial weight before degradation for 30 days and after 30 days, remaining strips were collected, washed with sodium dodecyl sulphate for 4 hours, rinsed with distilled water and dried overnight at $60^0$C and final weights taken using an electronic weighing balance. Percentage weight loss of each polyethene strip was then calculated as

$$\% \text{ weight loss} = \frac{\text{Weight loss (g)}}{\text{Initial weight (g)}} \times 100$$

Weight loss = Initial weight (g) – final weight (g)

### 3.8.2 Fourier Transform Infra Red Spectroscopy (FTIR)

FTIR analysis was done using the Nicolet IR 100 FTIR (Thermo, U.S.A) machine to determine the degradation of the low density polyethene after culturing with microorganisms in basal medium. The LDPE pieces were milled with potassium bromide (KBr) to form a fine powder. This powder was then compressed into a thin pellet which was fixed to the FTIR sample plate. Spectra were then taken at 400-4000 wave numbers for each sample.

# CHAPTER FOUR

# RESULTS

## 4.1 IDENTITY OF BACTERIAL AND FUNGAL ISOLATES WITH ABILITY TO DEGRADE LOW DENSITY POLYETHENE CARRIER BAGS

Microorganisms isolated from buried low density polyethene (LDPE) carrier bags comprised of Gram positive bacilli, Gram negative bacilli, and fungal isolates. Preliminary Gram reaction and catalase results for bacteria are shown in Table 4.1. Bacterial isolates were identified as *Acinetobacter baumannii, Bacillus brevis, Bacillus firmus, Bacillus cereus group* (*B. cereus, B. thuringiensis, B. mycoides and B. weihenstephanensis)* and *Bacillus sphaericus.* All organisms were citrate negative except *A. baumannii* while *B. sphaericus* was negative for all biochemical tests. Their biochemical characteristics are presented in Tables 4.1 and 4.2. *Lysinibacillus fusiformis* and *Ps. aeruginosa E2* were identified using molecular methods. Their PCR results are presented in Plates I –II and sequencing profiles at 4.1.2.

Fungal isolates were identified as *Aspergillus niger-1, Aspergillus flavus, Aspergillus niger-2, Aspergillus glaucus and Fusarium* species. Their colonial morphologies on Potato Dextrose Agar (PDA) at day 5 were characterised by concentric rings and radial fissures with some having smooth and entire edges while their microscopic morphology revealed them as having globose vesicles and long hyaline conidiophores except for *Fusarium* species which was composed of a mesh of short hyphae and 2-3 celled micro conidia. Details are presented in Table 4.3 while their macroscopic and microscopic morphologies are shown in Appendix IV, Plates VI-XV.

**Table 4.1 Biochemical Characteristics and Identification of Gram Negative (rods) Bacterial Isolates Using the Microgen$^{(TM)}$ - ID System**

| | Gram | Catalase | GN A Wells | | | | | | | | | | | | | | GN B Wells | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | reaction | test | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Isolate Code | | | | Lysine | Ornithine | $H_2S$ | Glucose | Mannitol | Xylose | ONPG | Indole | Urease | V.P | Citrate | TDA | Gelatin | Malonate | Inositol | Sorbitol | Rhamnose | Sucrose | Lactose | Arabinose | Adonitol | Raffinose | Salicin | Arginine | Octal code | Identity |
| BA | - | Rods | + | + | - | - | + | - | + | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | + | 45020001 | *Acinetobacter baumannii* |

Key: + Positive Result, - Negative Result, $H_2S$- Hydrogen Sulphide, ONPG- Ortho-nitrophenol- galactosidase, VP- Voges Proskauer, TDA-Tryptophan deaminase Acid, GN- Gram Negative, ID- Identification

**Table 4.2 Biochemical Characteristics and Identification of Gram Positive (rods) Bacterial Isolates Using the Microgen(TM) Bacillus - ID System**

| Isolate Code | | *Bacillus* strip 1 | | | | | | | | | | | | *Bacillus* strip 2 | | | | | | | | | | | | | Octal code | Identity |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Gram Reaction | Catalase Test | Arabinose | Celobiose | Inositol | Mannitol | Mannose | Raffinose | Rhamnose | Salicin | Sorbitol | Sucrose | Trehalose | Xylose | Adonitol | Galactose | Mdm | Mdg | Inulin | Mlz | Indole | ONPG | Arginine | Citrate | VP | Nitrate | | |
| BB 48hrs | +rods | + | - | - | - | - | + | - | - | - | - | + | + | - | - | - | - | - | - | - | - | - | - | - | + | - | 02060002 | *B. firmus* |
| BC 48hrs | + rods | + | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + | 20000003 | *B. brevis* |
| BE 48hrs | + rods | + | - | - | - | - | - | - | - | + | - | - | + | - | - | - | - | - | - | - | - | - | - | - | + | + | 00220003 | *B.cereus group* |
| BG 48hrs | + rods | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 00000000 | *B. sphaericus* |
| BH 48hrs | + rods | + | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | + | 00020003 | *B. brevis* |

Key: + Positive Result, - Negative result, Mdm-Methyl-D-Mannoside, Mdg- Methyl-D-Glucoside, Mlz- Melezitose, ONPG- Ortho-nitrophenol- galactosidase, VP- Voges Proskauer, ID-Identification

**Table 4.3 Identification of Fungal Isolates with Ability to Degrade Low Density Polyethylene**

| Fungal code | Colour on PDA Surface/reverse | Growth rate | Conidiophore/ hyphae | Vesicle | Conidial head | Conidial Size (µm)/ colour | Radial fissure | Identity |
|---|---|---|---|---|---|---|---|---|
| **F1** | Brown/cream | rapid | Long, smooth, hyaline | Globose | Radiate/ uniseriate | 2.5 brown | + | *A. niger* |
| **F2** | Yellowish Green/cream | rapid | Long, smooth hyaline | Semi globose | Radiate | 1.75 green | + | *A. flavus* |
| **F3** | Black/cream | rapid | long, smooth hyaline | globose | Radiate biseriate | 5 black | + | *A. niger* |
| **F4** | Ash/smoky grey with dark color seeping through | rapid | hyphae, smooth, hyaline with short nodes | - | - | 12.5x5 Blackish brown | - | *Fusarium spp* |
| **F5** | Green | slow | Long, hyaline | | Loosely columnar | 5 green | + | *A.glaucus* |

Key: + Present, - Absent, PDA- Potato Dextrose Agar, µm- micro metre

### 4.1.1 Polymerase Chain Reaction (PCR) Amplicons of Bacteria and Fungi

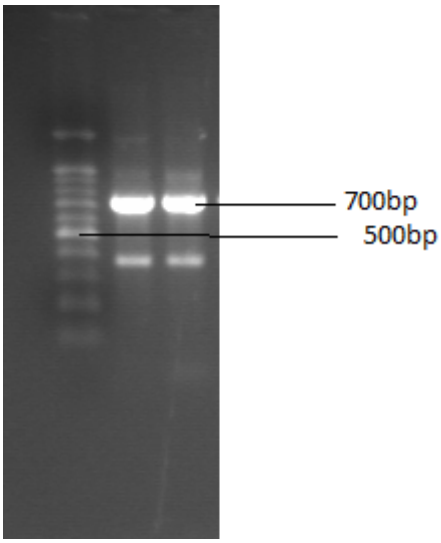



Plate I: Amplicons of the 16s Ribosomal DNA of Bacterial Isolates

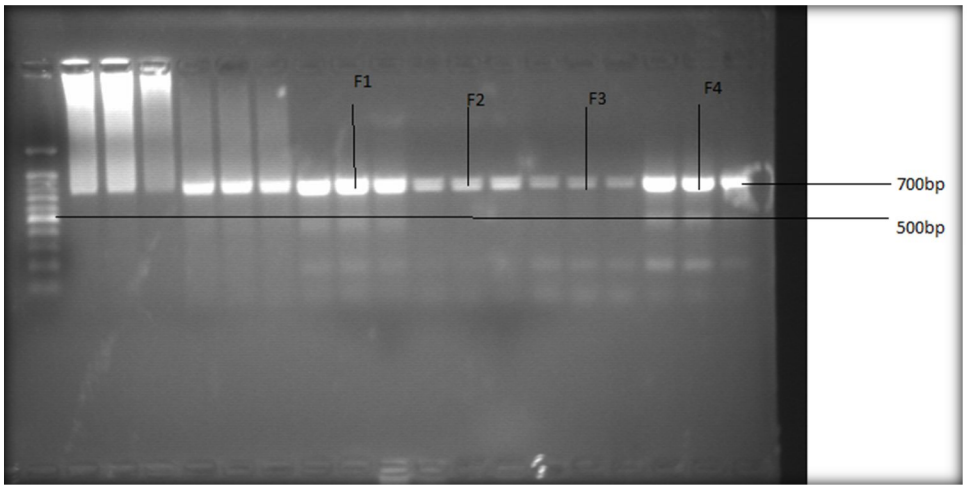



Plate II: Amplicons of the 16s Ribosomal DNA of Fungal Isolates

Key: bp - base pairs
F1-F4- Fungal Isolate codes

### 4.1.2 Sequencing Profiles of Bacteria

Sequencing Profile of *Lysinibacillus fusiformis*

CGCGCCTCAGTGTCAGTTACAGACCAGATAGTCGC
CTTCGCCACTGGTGTTCCTCCAAATCTCTACGCATTTCACCGCTACACTT
GGAATTCCACTATCCTCTTCTGCACTCAAGTCTCCCAGTTTCCAATGACC
CTCCACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAGAAACCACCTGC
GCGCGCTTTACGCCCAATAATTCCGGACAACGCTTGCCACCTACGTATTA
CCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTAATAAGGTACCGTC
AAGGTACAGCCAGTTACTACTGTACTTGTTCTTCCCTTACAACAGAGTTT
TACAAACCGAAATCCTTCTTCACTCACGCGGCGTTGCTCCATCAGGCTTT
CGCCCATTGTGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCC
GTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATC
GTCGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGCC
CATCCTATAACGACAGCCAAAACCGTCTTTCAATATTTCACCATGAGGTG
AAACAGATTATTC

Sequencing Profile of *Peudomonas aeruginosa E2*

CTTTATTGTCGCCATTGCTAATGGTTATGATATATTTTTTAGTTGTT
ATTGCTGTCTAGCTTATTTTTCTCAAATTCTTCTCTTACTGGCCTGGGAA
CTCTCCTGATCTTTGATCAGAGATGTAGTACAACGTCGTTTCAGTCTGAG
ATAATCTTCTGAACTGGTGGGAGCAGTCCTAGTGGATTCACTGACAGATA
TAAATTGTTTTTCTCCTGTTGAACCAGACAAAAGAGCTTGGGTATTTATC
AATGCAAGTTCTTCGTCAGCTATTGAATTACTTTCCAAAAGAGAAATTTC
ATTGAATTTTTTAAATGGAGTCATCTGAGGAGAATTCAGTTCTTTTTTCT
TTATGGGTGTTTCGTATTTGGTGCAACAACTCTTTGGTGGCTGAAATGCC

TTCTGTGCAGCCGGAGAAACAAATGTATAAATGGGACTAACAGGTGGAGG
AAAAGGCAGTCTACTCAAGAAATCCAAGGCTCTTCTCTTTTTGCAGTTCT
TTT

### 4.1.3 Sequencing Profiles of Fungi

Sequencing profiles of fungal isolates are not presented in this thesis this is because blast results from Genebank identified the fungal isolates as bacteria such as *Shigella flexneri* and *Vibro cholerae.* Further investigation revealed that the primers used were universal for bacterial species and not fungi. New primers specific for fungal species with the following sequences (Forward primer: NS1 GTAGTCATATGCTTGTCTC and Reverse primer: ITS4 TCCTCCGCTTATTGATATGC, Plaza *et al*., 2004) were therefore obtained and further work is on-going. Results obtained from this work will subsequently be published in journals.

## 4.2 SCREENING OF BACTERIAL AND FUNGAL ISOLATES FOR THE ABILITY TO DEGRADE LOW DENSITY POLYETHENE (LDPE)

All bacterial and fungal isolates were screened for their ability to degrade low density polyethene (LDPE). *B. brevis, B. cereus group, Lysinibacillus fusiformis and Ps. aeruginosa E2* had weight loss percentages ranging from 0.18% to 0.22% with *Lysinibacillus fusiformis* having the highest value of 0.22% (Table 4.4). Among the fungal isolates, *Aspergillus niger*-1 had the highest value of 3.38% while *Aspergillus niger-2, Fusarium species and Aspergillus flavus* had percentage weight loss of 0.10% to 0.12% (Table 4.5). These bacterial and fungal isolates were therefore selected and subjected to further biodegradation studies.

## 4.3 BIODEGRADATION OF UNTREATED AND STARCH TREATED SYNTHETIC LOW DENSITY POLYETHENE (LDPE)

Biodegradation result of untreated synthetic LDPE carrier bags by the identified individual bacterial isolates showed that *Ps. aeruginosa E2* achieved a weight loss reduction of 0.86% while *B. cereus group* achieved a 1.00% weight loss (Table 4.6). For the starch treated LDPE carrier bags, *Ps. aeruginosa E2* had the highest weight loss of 9.16% followed by *B. cereus group* (4.72%) while *L. fusiformis* had the lowest weight loss of 0.08% (Table 4.7). For the untreated synthetic LDPE carrier bags, bacterial consortium of *B. cereus* group + *Lysinibacillus fusiformis* had the highest weight loss of 1.04% followed by *Lysinibacillus fusiformis* + *B.brevis* (1.02%). However, the consortium of *B. brevis* + *Ps. aeruginosa E2* had no effect on the untreated synthetic carrier bags (Table 4.8).

**Table 4.4 Screening of Bacteria for LDPE Degradation Ability**

| | Biodegradation Ability | | | |
|---|---|---|---|---|
| Organism | Initial weight (g) | Final weight (g) | Weight Loss (g) | Weight Loss (%) |
| *A. baumannii* | 0.5000 | 0.4994 | 0.0006 | 0.12 |
| *B. firmus* | 0.5000 | 0.4996 | 0.0004 | 0.08 |
| *B. brevis* | 0.5000 | 0.4992 | 0.0008 | 0.16 |
| *Lysinibacillus fusiformis* | 0.5000 | 0.4989 | 0.0011 | 0.22 |
| *B. cereus group* | 0.5000 | 0.4990 | 0.0010 | 0.20 |
| *Ps. aeruginosa E2* | 0.5000 | 0.4991 | 0.0009 | 0.18 |
| *B. sphaericus* | 0.5000 | 0.4992 | 0.0008 | 0.16 |
| *B. brevis* | 0.5000 | 0.4991 | 0.0009 | 0.18 |
| Control | 0.5000 | 0.5000 | 0.0000 | 0.00 |

Key: Control- LDPE in basal medium without Microorganism, LDPE- Low Density Polyethene,

**Table 4.5 Screening of Fungi for LDPE Degradation Ability**

| | Biodegradation Ability | | | |
|---|---|---|---|---|
| Organism | Initial weight (g) | Final weight (g) | Weight Loss (g) | Weight Loss (%) |
| *A. niger-1* | 0.5000 | 0.4831 | 0.0169 | 3.38 |
| *A. flavus* | 0.5000 | 0.4995 | 0.0005 | 0.10 |
| *A. niger-2* | 0.5000 | 0.4994 | 0.0006 | 0.12 |
| *Fusarium spp* | 0.5000 | 0.4994 | 0.0006 | 0.12 |
| *A. glaucus* | 0.5000 | 0.4999 | 0.0001 | 0.03 |
| Control | 0.5000 | 0.5000 | 0.0000 | 0.00 |

Key: Control- LDPE in basal medium without Microorganism LDPE- Low Density Polyethene

**Table 4.6 Biodegradation of Untreated Synthetic LDPE Carrier Bags Using Individual Bacterium**

| | Biodegradation Ability | | | |
|---|---|---|---|---|
| Organism | Initial Weight (g) | Final weight (g) | Weight Loss (g) | Weight Loss (%) |
| *Lysinibacillus fusiformis* | 0.5000 | 0.5000 | 0.0000 | 0.00 |
| *B. cereus group* | 0.5000 | 0.4950 | 0.0050 | 1.00 |
| *Ps. aeruginosa E2* | 0.5000 | 0.4957 | 0.0043 | 0.86 |
| *B. brevis* | 0.5000 | 0.5000 | 0.0000 | 0.00 |
| Control | 0.5000 | 0.5000 | 0.0000 | 0.00 |

Key: Control- LDPE in basal medium without Microorganism, LDPE- Low Density Polyethene

**Table 4.7 Biodegradation of LDPE/Starch Blend Using Individual Bacterium**

| | Biodegradation Ability | | | |
|---|---|---|---|---|
| Organism | Initial weight (g) | Final weight (g) | Weight Loss (g) | Weight Loss (%) |
| *Lysinibacillus fusiformis* | 0.5000 | 0.4996 | 0.0004 | 0.08 |
| *B. cereus group* | 0.5000 | 0.4764 | 0.0236 | 4.72 |
| *Ps. aeruginosa E2* | 0.5000 | 0.4542 | 0.0458 | 9.16 |
| *B. brevis* | 0.5000 | 0.4970 | 0.0030 | 0.60 |
| Control | 0.5000 | 0.5000 | 0.0000 | 0.00 |

Key: Control- LDPE in basal medium without Microorganism, LDPE- Low Density Polyethene

**Table 4.8 Biodegradation of Untreated Synthetic LDPE Carrier Bags Using Bacterial Consortia**

| Biodegradation Ability | | | | |
|---|---|---|---|---|
| Organisms | Initial weight (g) | Final weight (g) | Weight Loss (g) | Weight Loss (%) |
| *Lysinibacillus fusiformis +B.Cereus group* | 0.5000 | 0.4948 | 0.0052 | 1.04 |
| *Lysinibacillus fusiformis +Ps.aeruginosa E2* | 0.5000 | 0.4984 | 0.0016 | 0.32 |
| *Lysinibacillus fusiformis +B. brevis* | 0.5000 | 0.4949 | 0.0051 | 1.02 |
| *B.Cereus group+ Ps. aeruginosa E2* | 0.5000 | 0.4977 | 0.0023 | 0.46 |
| *B. Cereus group + B. brevis* | 0.5000 | 0.4979 | 0.0021 | 0.42 |
| *Ps. aeruginosa E2+ B. brevis* | 0.5000 | 0.5000 | 0.0000 | 0.00 |
| Control | 0.5000 | 0.5000 | 0.0000 | 0.00 |

Key: Control- LDPE in basal medium without Microorganism, LDPE- Low Density Polyethene

Biodegradation of the starch/LDPE blend using bacterial consortia as presented in Table 4.9 showed a very high weight loss of 18.50% by *B. cereus group* + *Ps. aeruginosa* E2 while again the consortium of *B. brevis* + *Ps. aeruginosa E2* had no effect on the LDPE/Starch blend

Results obtained from fungal biodegradation of the untreated synthetic Low density polyethene (LDPE) carrier bags showed that *A. niger-1* did better than *A. niger*-2 as singles (4.02% and 0.42% respectively) while *Fusarium* species (spp) achieved a 2.04% weight loss and *A. flavus* had a 0.68% weight loss (Table 4.10). However, *A. niger-2* had a higher weight loss of 7.82% than that obtained from *A. niger-1* (6.14%) for the LDPE/starch blend followed by *A. flavus* (6.08%) and lastly *Fusarium* spp (5.96%) as shown in Table 4.11.

Fungal consortium comprising of *A. niger-2* + *Fusarium spp* showed the highest biodegradation (3.54%) for the untreated synthetic LDPE carrier bags. *A. flavus* + *Fusarium spp* and *A. flavus* + *A. niger-2* had a weight loss of 1.72% and 1.28% respectively (Table 4. 12). For the LDPE/starch blend, *A. niger-2* + *Fusarium spp* again had the highest weight loss of 19.68% (Table 4.13) which is almost five times of that obtained by the same organisms using the untreated synthetic LDPE carrier bags.

**Table 4.9 Biodegradation of LDPE/Starch Blend Carrier bags using Bacterial Consortia**

| Organisms | Biodegradation Ability | | | |
|---|---|---|---|---|
| | Initial weight (g) | Final weight (g) | Weight Loss (g) | Weight Loss (%) |
| *Lysinibacillus fusiformis +.B cereus group* | 0.5000 | 0.4947 | 0.0053 | 1.06 |
| *Lysinibacillus fusiformis + Ps. aeruginosa E2* | 0.5000 | 0.4962 | 0.0038 | 0.76 |
| *Lysinibacillus fusiformis +B. brevis* | 0.5000 | 0.4965 | 0.0035 | 0.70 |
| *B.cereus group+ Ps. aeruginosa E2* | 0.5000 | 0.4075 | 0.0925 | 18.50 |
| *B. cereus group + B. brevis* | 0.5000 | 0.4967 | 0.0033 | 0.66 |
| *Ps. aeruginosa E2+ B. brevis* | 0.5000 | 0.5000 | 0.0000 | 0.00 |
| Control | 0.5000 | 0.5000 | 0.0000 | 0.00 |

Key: Control- LDPE in basal medium without Microorganism, LDPE- Low Density Polyethene

**Table 4.10 Biodegradation of Untreated Synthetic LDPE Carrier Bags Using Individual Fungus**

| | Biodegradation Ability | | | |
|---|---|---|---|---|
| Organism | Initial weight (g) | Final weight (g) | Weight Loss (g) | Weight Loss (%) |
| *A. niger-1* | 0.5000 | 0.4799 | 0.0201 | 4.02 |
| *A. flavus* | 0.5000 | 0.4966 | 0.0034 | 0.68 |
| *A. niger-2* | 0.5000 | 0.4979 | 0.0021 | 0.42 |
| *Fusarium spp* | 0.5000 | 0.4898 | 0.0102 | 2.04 |
| Control | 0.5000 | 0.5000 | 0.0000 | 0.00 |

Key: Control- LDPE in basal medium without Microorganism, LDPE- Low Density Polyethene

**Table 4.11 Biodegradation of LDPE/Starch Blend Using Individual Fungus**

| | Biodegradation Ability | | | |
|---|---|---|---|---|
| Organism | Initial weight (g) | Final weight (g) | Weight Loss (g) | Weight Loss (%) |
| *A. niger-1* | 0.5000 | 0.4693 | 0.0307 | 6.14 |
| *A. flavus* | 0.5000 | 0.4696 | 0.0304 | 6.08 |
| *A. niger-2* | 0.5000 | 0.4609 | 0.0391 | 7.82 |
| *Fusarium spp* | 0.5000 | 0.4702 | 0.0298 | 5.96 |
| Control | 0.5000 | 0.5000 | 0.0000 | 0.00 |

Key: Control- LDPE in basal medium without Microorganism, LDPE- Low Density Polyethene

**Table 4.12 Biodegradation of Untreated Synthetic LDPE Carrier bags Using Fungal Consortia**

| | Biodegradation Ability | | | |
|---|---|---|---|---|
| Organisms | Initial weight (g) | Final weight (g) | Weight Loss (g) | Weight Loss (%) |
| *A. niger-1 + A. flavus* | 0.5000 | 0.4954 | 0.0046 | 0.92 |
| *A. niger-1 + A. niger-2* | 0.5000 | 0.4953 | 0.0047 | 0.94 |
| *A. niger-1 + Fusarium spp* | 0.5000 | 0.4964 | 0.0036 | 0.72 |
| *A. flavus + A. niger-2* | 0.5000 | 0.4936 | 0.0064 | 1.28 |
| *A. flavus + Fusarium spp* | 0.5000 | 0.4914 | 0.0086 | 1.72 |
| *A. niger-2 + Fusarium spp* | 0.5000 | 0.4823 | 0.0177 | 3.54 |
| Control | 0.5000 | 0.5000 | 0.0000 | 0.00 |

Key: Control- LDPE in basal medium without Microorganism, LDPE- Low Density Polyethene

**Table 4.13 Biodegradation of LDPE/Starch Blend Using Fungal Consortia**

| Organisms | Biodegradation Ability | | | |
|---|---|---|---|---|
| | Initial weight (g) | Final Weight (g) | Weight Loss (g) | Weight Loss (%) |
| *A. niger-1 + A. flavus* | 0.5000 | 0.4611 | 0.0389 | 7.78 |
| *A. niger-1+ A. niger-2* | 0.5000 | 0.4950 | 0.0050 | 1.00 |
| *A. niger-1 + Fusarium spp* | 0.5000 | 0.4799 | 0.0201 | 4.02 |
| *A. flavus + A. niger-2* | 0.5000 | 0.4956 | 0.0044 | 0.88 |
| *A. flavus + Fusarium spp* | 0.5000 | 0.4936 | 0.0064 | 1.28 |
| *A. niger-2 + Fusarium spp* | 0.5000 | 0.4016 | 0.0984 | 19.68 |
| Control | 0.5000 | 0.5000 | 0.0000 | 0.00 |

Key: Control- LDPE in basal medium without Microorganism, LDPE- Low Density Polyethene

## 4.4 COMPARISM OF BACTERIAL AND FUNGAL BIODEGRADATIVE ABILITY ON UNTREATED AND STARCH TREATED SYNTHETIC LOW DENSITY POLYETHENE (LDPE) CARRIER BAGS

Microorganisms used in this study showed their ability to degrade both synthetic untreated and starch treated LDPE carrier bags (1.0% to 4.02%). Results obtained from this study however showed that degradation of synthetic LDPE by the microorganisms was greatly enhanced by the addition of starch, (7.82% to 19.68%). Specific organisms shown to be more active in this study include *B.cereus group, Ps. aeruginosa E2, A. niger-1* and *A. niger-2* while for the consortia*, B.cereus group + Ps. aeruginosa E2 and A. niger-2 + Fusarium spp* were the best degraders of the LDPE/starch blend (Figure 4.1). Fungi showed a better result for the untreated and starch treated LDPE and bacterial and fungal consortia performed better than the individual organisms (Figure 4.2).

## 4.5 FOURIER TRANSFORM INFRA RED SPECTROSCOPIC (FTIR) ANALYSIS OF RESIDUAL LDPE STRIPS AFTER MICROBIAL DEGRADATION

Fourier Transform Infra Red Spectroscopy (FTIR) measures changes in chemical properties such as formation or disappearance of functional groups in a polymer which is usually taken as an indication of degradation. Six samples comprising of synthetic LDPE pellets (control), synthetic LDPE, (CSPE), starch treated LDPE-bacteria, (SPEB), starch treated LDPE-fungi (SPEF), starch treated LDPE-bacteria consortium (SPEBC), and starch treated LDPE-fungal consortium (SPEFC) carrier bags were subjected to FTIR analysis. Results obtained from all samples showed an appearance of a carbonyl peak at 1703cm$^{-1}$ except from the CSPE and with carbon-carbon double bond peak at 1459.84cm$^{-1}$ from the

SPE pellet  Results of this analysis are presented in Figures 4.3 to 4.9 while peak finding results are shown in Appendix VI.

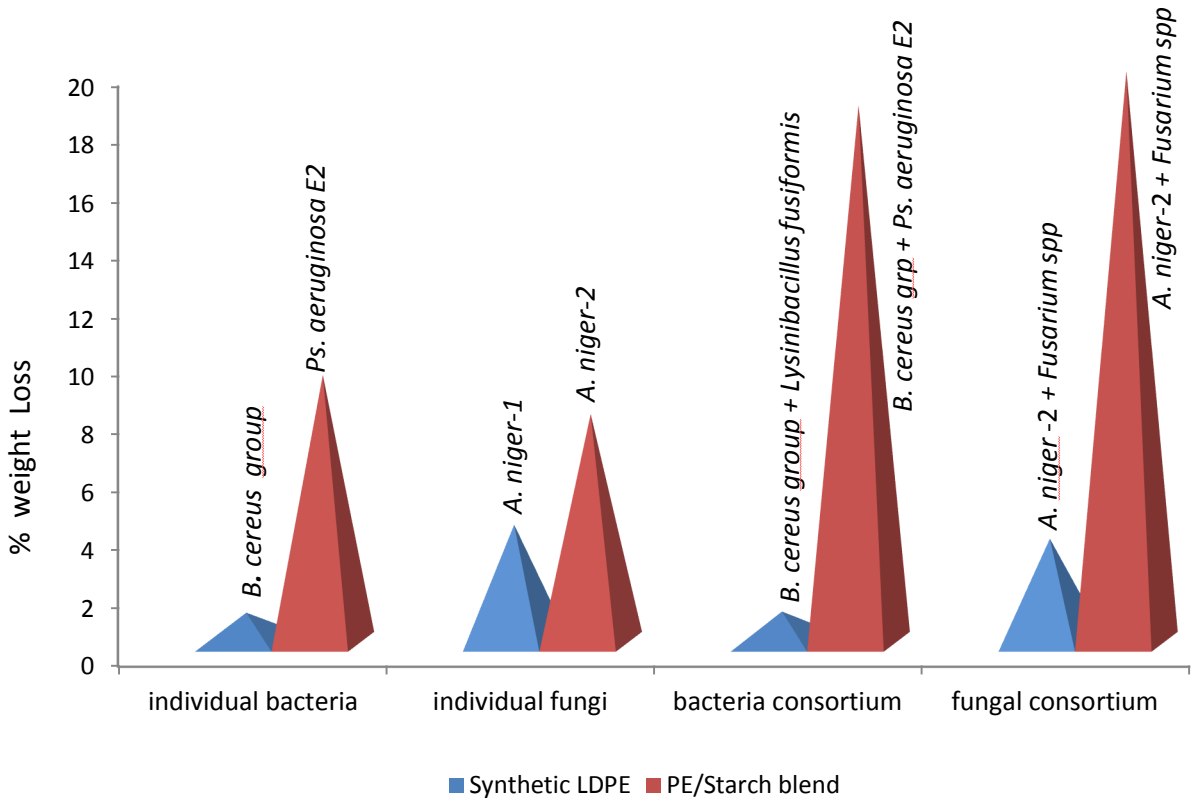



Figure 4.1 Comparism of Biodegradative Ability of Individual Microorganisms on Untreated and Starch treated Synthetic Low Density Polyethene Carrier bags

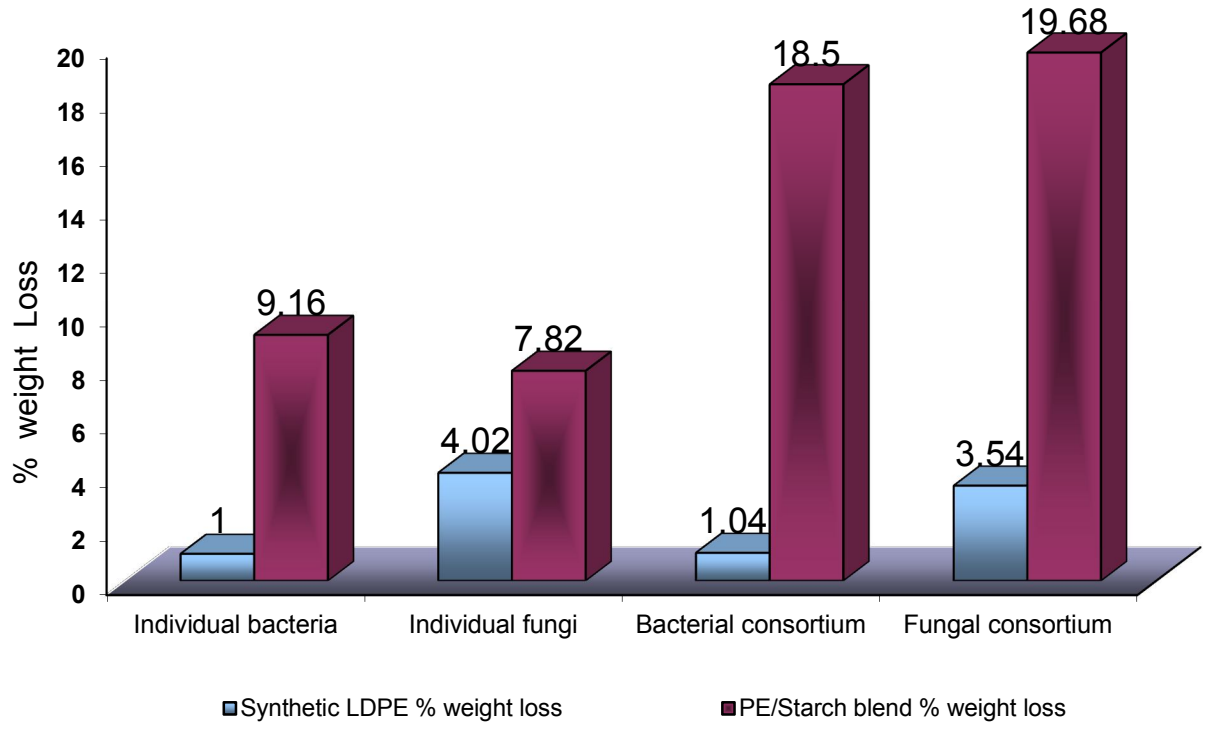



Figure 4.2 Comparism of Bacterial and Fungal Biodegradative Ability on Untreated and Starch treated Synthetic Low Density Polyethene Carrier bags

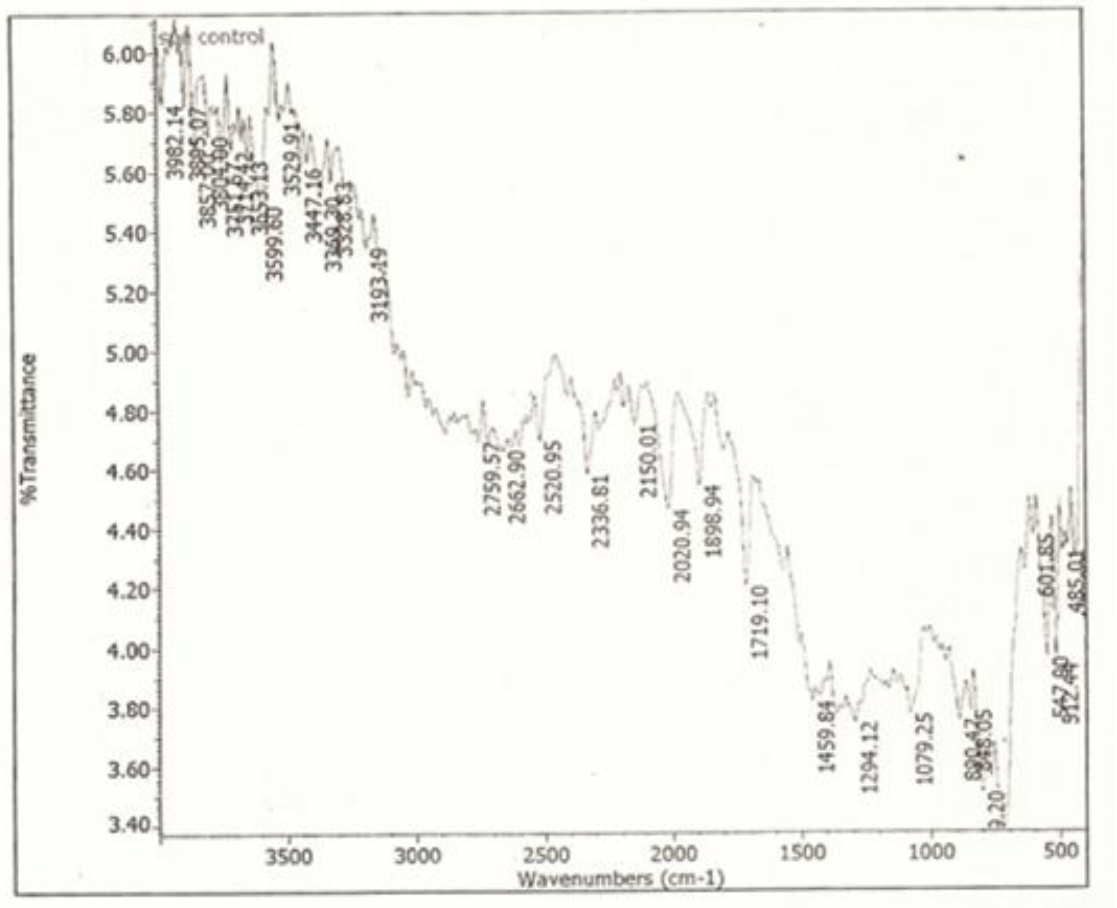



Figure 4.3 FTIR Spectra of Synthetic Low Density Polyethene pellet

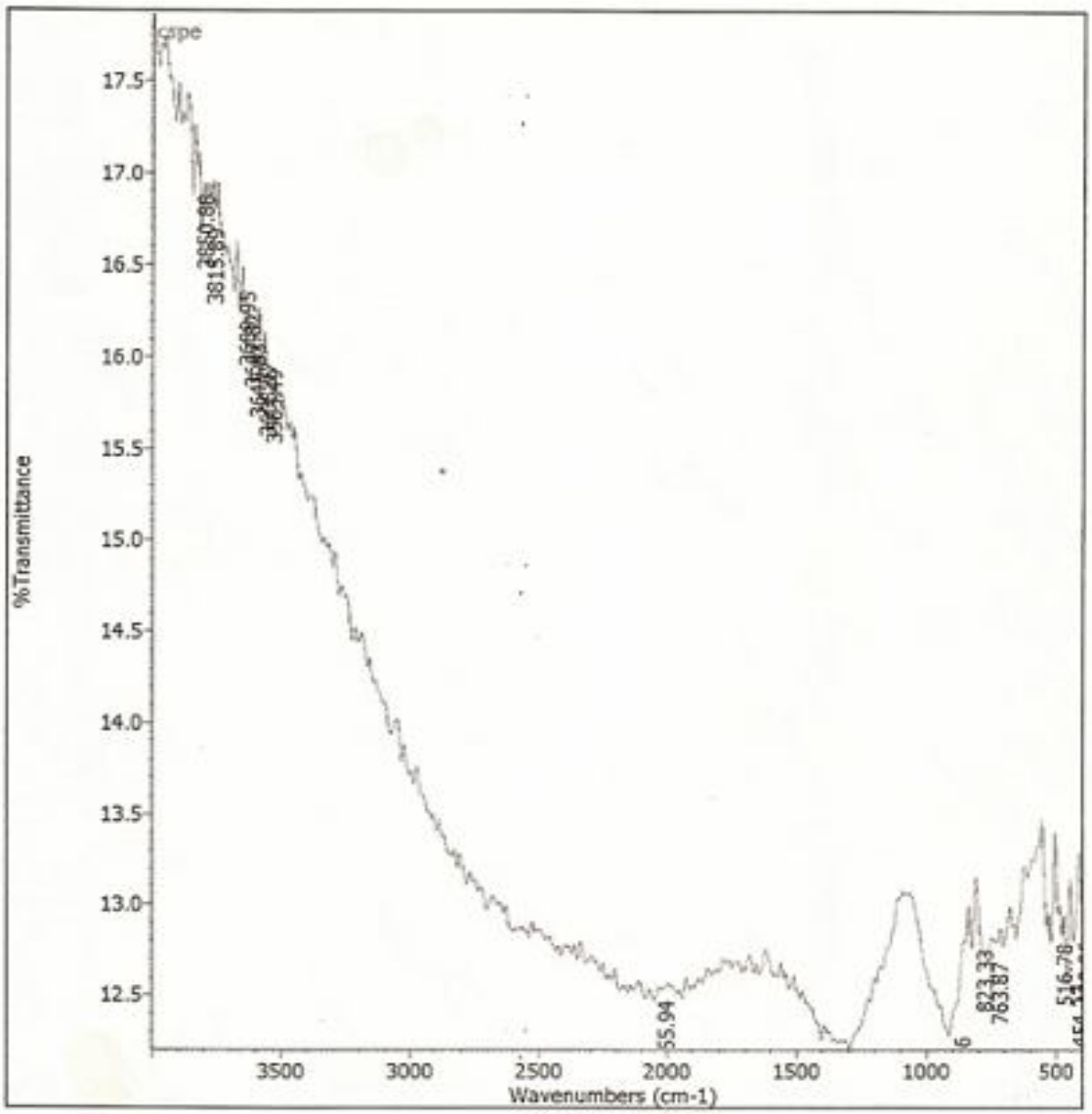



Figure 4.4 FTIR Spectra of Synthetic Low Density Polyethene carrier bag

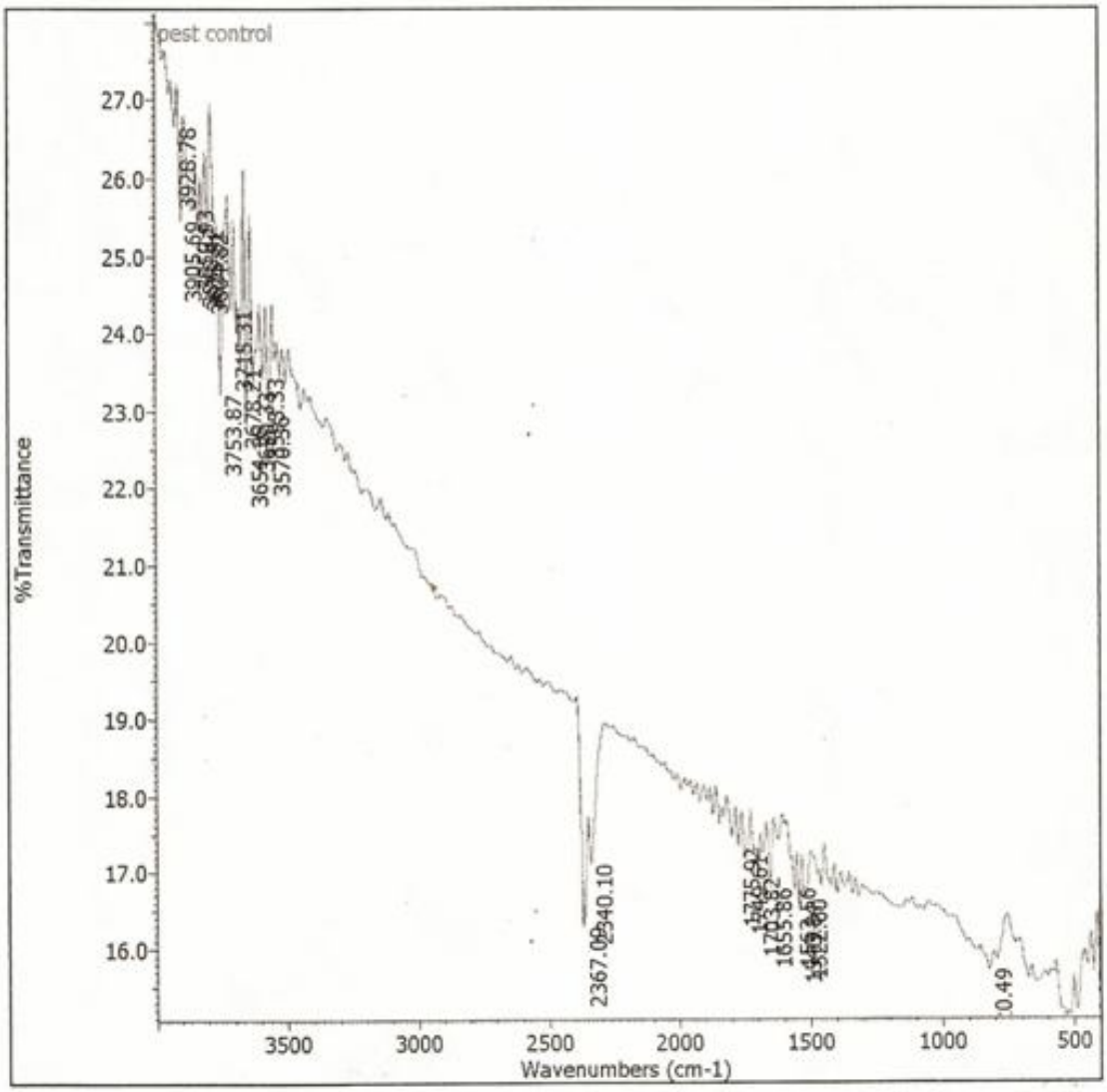



Figure 4.5 FTIR Spectra of Synthetic Low Density Polyethene/Starch blend

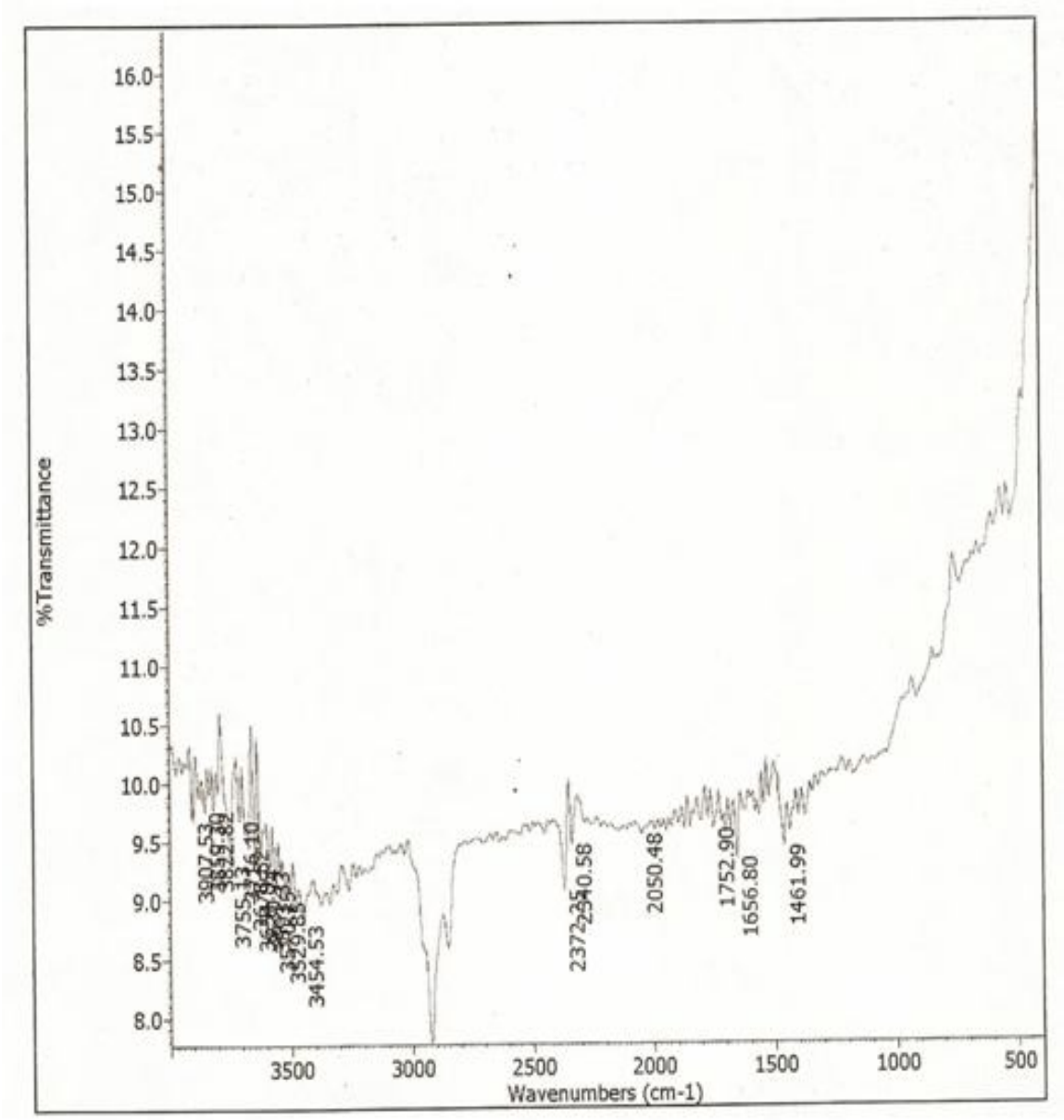



Figure 4.6 FTIR Spectra of Synthetic Low Density Polyethene/Starch blend after treatment with *Ps. aeruginosa E2*

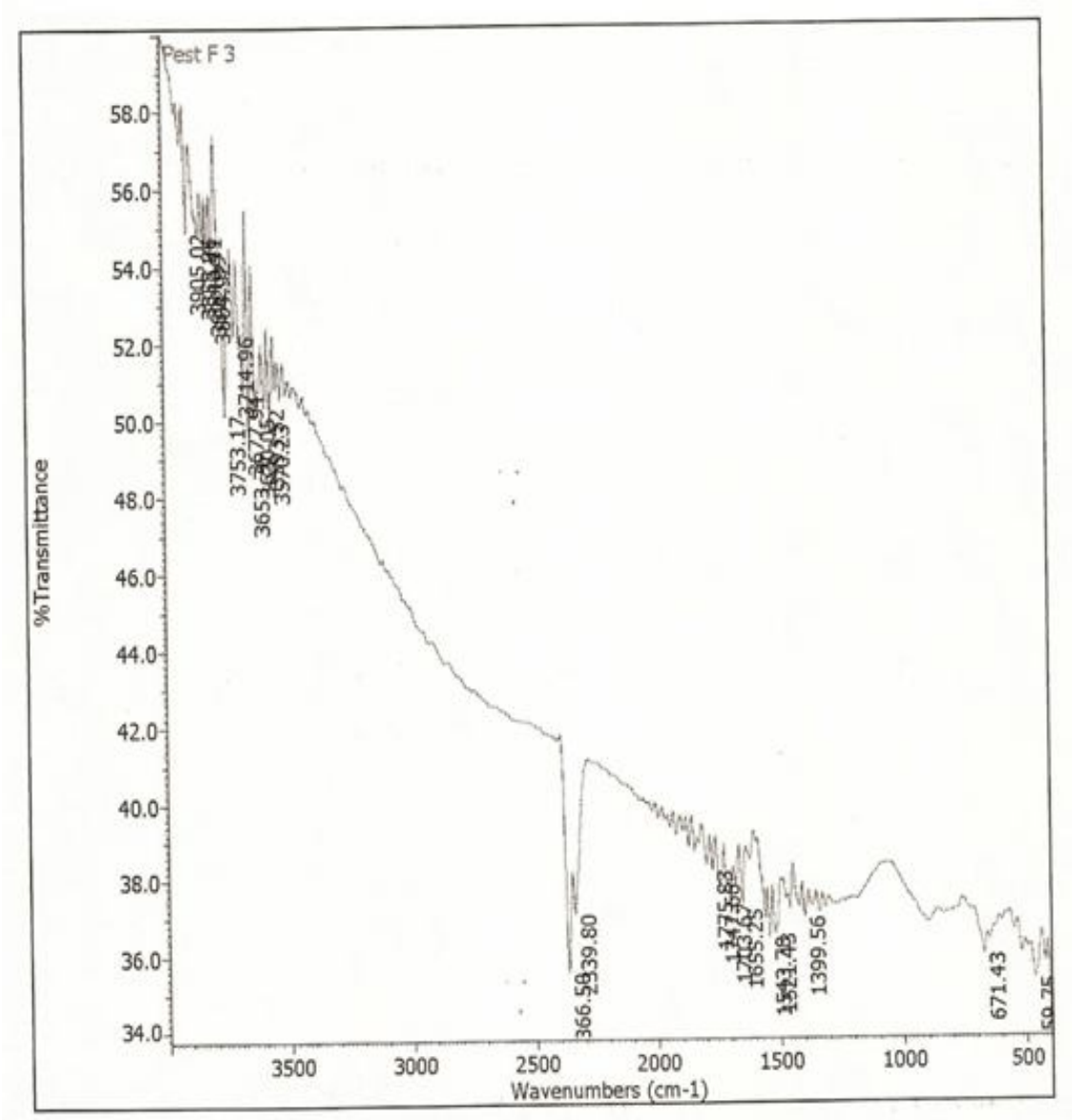

Pest F 3
%Transmittance
58.0
56.0
54.0
52.0
50.0
48.0
46.0
44.0
42.0
40.0
38.0
36.0
34.0
3753.17
3714.96
3653.36
3677.91
3705.02
3978.23
366.58
439.80
1655.25
1399.56
671.43
3500
3000
2500
2000
1500
1000
500
Wavenumbers (cm-1)

Figure 4.7 FTIR Spectra of Synthetic Low Density Polyethene/Starch blend after treatment with *A. niger-2*

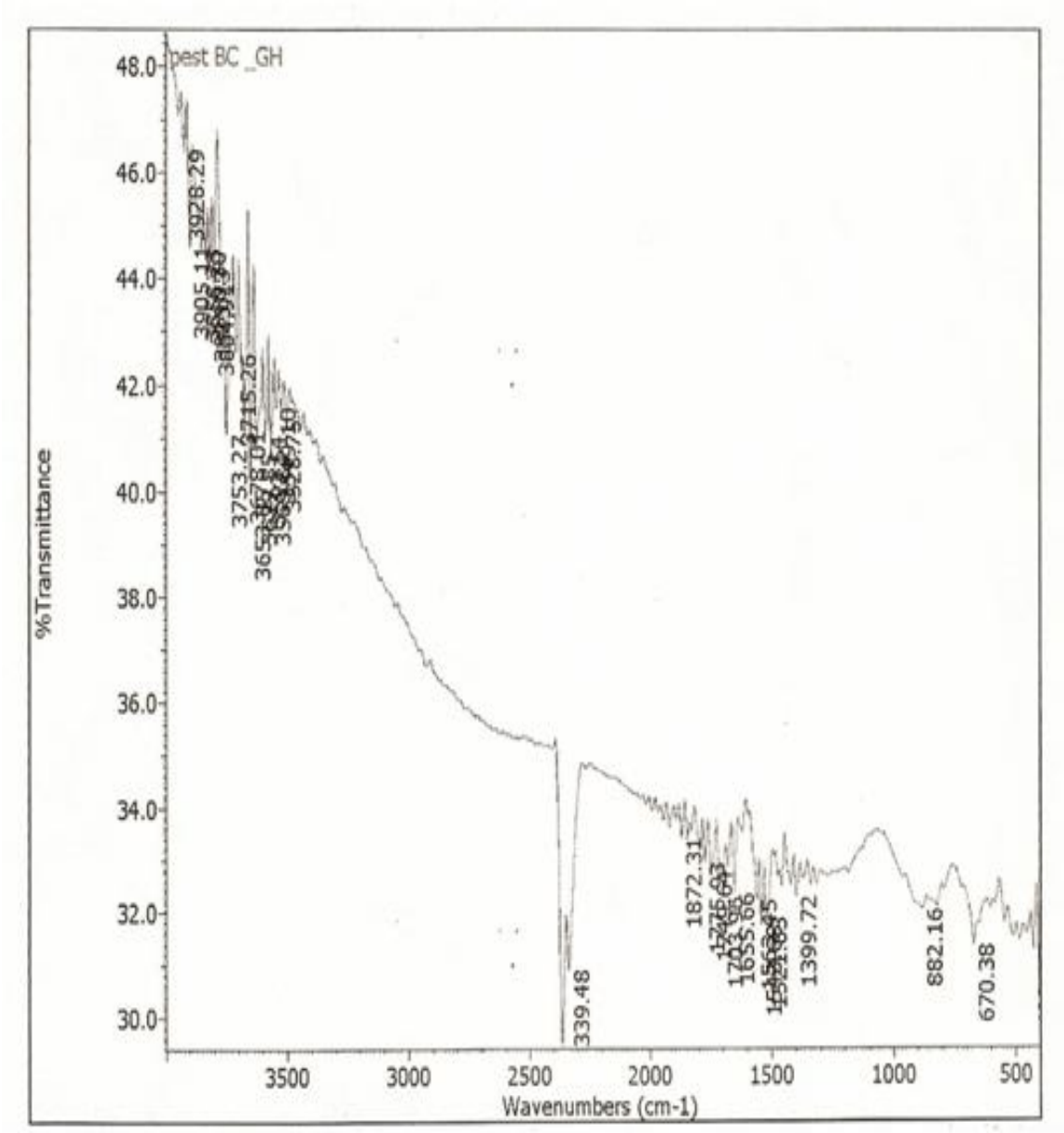



Figure 4.8 FTIR Spectra of Synthetic Low Density Polyethene/Starch blend after treatment with *B. cereus group + Ps. aeruginosa E2*

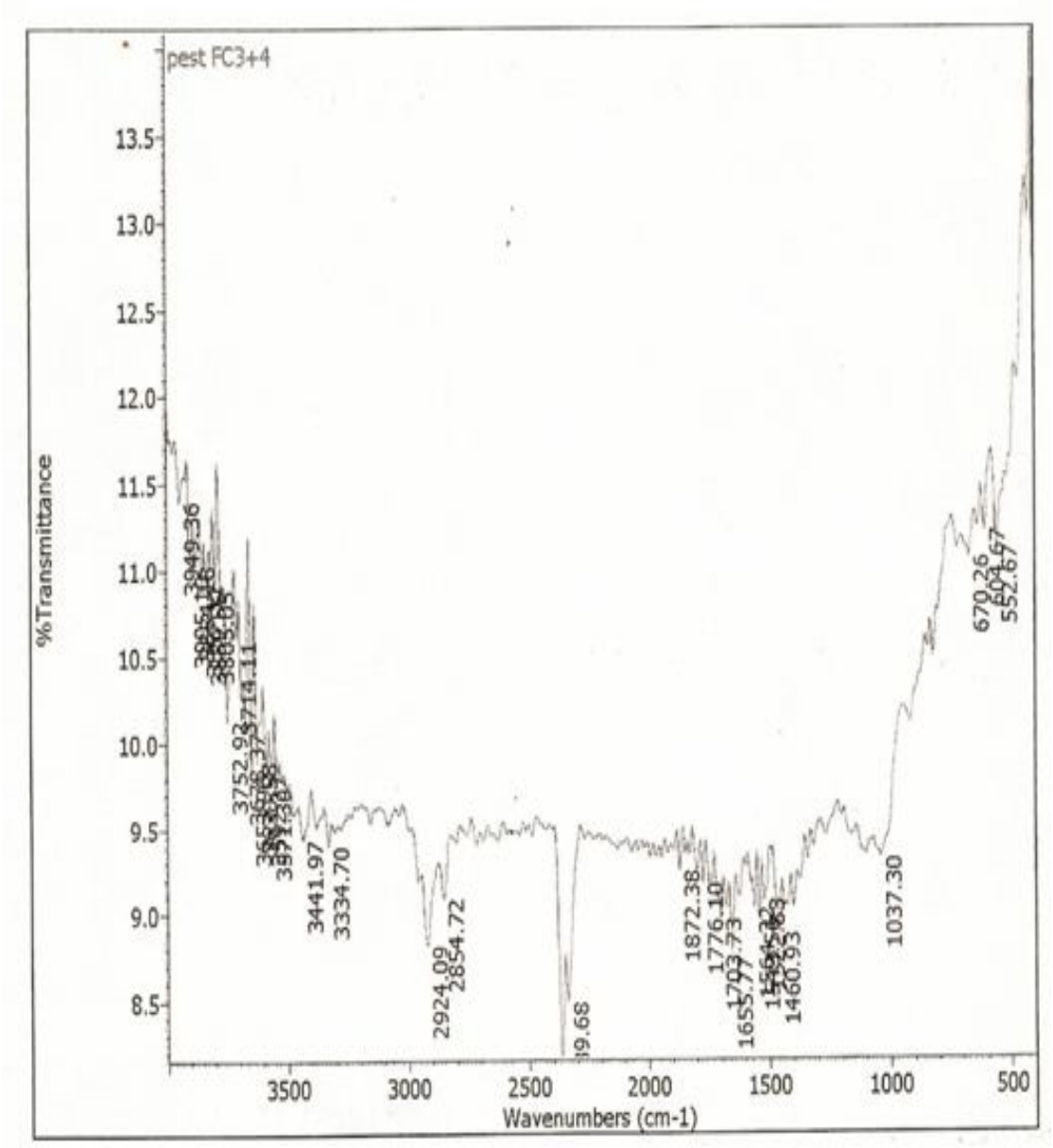



Figure 4.9 FTIR Spectra of Synthetic Low Density Polyethene/Starch blend after treatment with *A. niger-2 + Fusarium species*

# CHAPTER FIVE

# DISCUSSION

Microorganisms isolated and identified comprised of both bacteria (Gram +ve and Gram -ve) and fungi. Bacterial isolates included *Acinetobacter baumannii, Bacillus brevis, Bacillus firmus, Bacillus cereus group, Bacillus sphaericus, Lysinibacillus fusiformis and Ps. aeruginosa* E2 while the fungi were *Aspergillus niger*-1*, Aspergillus niger*-2, *Aspergillus flavus, Aspergillus glaucus* and *Fusarium spp*. Similar organisms were reported in earlier studies by Gu *et al.* (2000), Kathiresan (2003), Shah (2007), Reddy and Vijaya, (2008), Arkatkar *et al*. (2009), Shah *et al*. (2009), Borghei *et al.* (2010) and Pramila *et al*. (2012). Among the bacteria, *B. cereus group (B. cereus, B. mycoides, B. thurigiensis and B, weihenstephanensis)* and *Ps. aeruginosa E2* were more active in degradation of LDPE both singly and as consortia. This agrees with the findings of Satlewal *et al*. (2008) and Arkatkar *et al*. (2009). *A. niger-2 and A. niger-2 + Fusarium species* were more active among the fungi. Kathiresan (2003) and Shah (2007) also found *Aspergillus* among other organisms that gave a weight loss between 17.35% and 28.20% in thirty days while Shah *et al*. (2009) in their study isolated *Fusarium* spp. AF4 from sewage sludge with the ability of adhering to the surface of polyethene and utilizing it as a source of carbon and energy.

Weight loss, calculated as a percentage is one of the biodegradation analytical techniques in use, and it is taken as an indicator of biodegradation. A higher percentage weight loss (3.54% to 4.02%) was obtained from the fungal isolates for both individual and consortium than the bacteria (1.0% to 1.04%) for the untreated polyethene while a little difference between fungal and bacterial biodegradation was obtained for the starch blended

polyethene; 7.82% to 19.68% and 9.16% to 18.5% respectively. This may be attributed to the fact that fungi have the ability to form hydrophobic proteins (Senevirantne, 2006) that can attach to the polymer surface, thus generating enzymes that are well matched to the insoluble low density polyethene (Shah *et. al*., 2008), the faster growth of fungal biomass compared to bacteria (Kim and Rhee, 2003) and the growth extension and penetration through the distribution of hyphae.

Comparism of percentage weight loss results obtained from the polyethene/starch blend and the unblended polyethene showed that degradation of the polyethene/ starch blend was greater (7.82% to 19.68%) than that of the unblended polyethene, (1.0% to 4.02%)). This is supported by experiments conducted by Arkatkar *et al.* (2009) with marine organisms, *B. sphaericus* and *B. cereus* sub-group A under *in-vitro* conditions in mineral salt water medium which showed that the rate of biodegradation of starch blended polyethene is higher with a weight loss of 15% when compared to that of unblended pure polymer which was 11% in six months while that obtained by Borghei *et al. (*2010) using *Ps. aeruginosa* was 2.19% for LDPE/potato starch and 0.43% for pure LDPE. This goes further to show that blending of natural polymers as starch enhances the biodegradation of polymeric compounds such as polyethene.

Starch is an abundant, inexpensive and renewable material used as a biodegradable additive. Microorganisms are first attracted to the starch in the polymer matrix which provides higher oxygen permeability as it is consumed by microorganisms and higher permeability helps in release of degradation products from the sample making the matrix hollow, increasing the surface to volume ratio. Starch being hydrophilic increases the

adhesion of the microorganisms resulting in biofilm formation on the polymer surface; biofilm formation is a prerequisite for biodegradation. As microorganisms consume the starch content of the blend, they cause cracks and pits in the polymer which enhance further penetration of microorganisms. Consumption of the starch also results in disintegration of the polyethene polymer into lower molecular weight fragments that can be easily accessed by microorganisms which utilise them as their carbon and energy source. Amylolytic activity associated with *Aspergillus* and *Bacillus* species could have also enhanced the fast utilization of the starch component resulting in increase in bacterial load, adhesion, biofilm formation and therefore greater degradation than that in the unblended polyethene (Abu *et al*., 2005; Omemu *et al.,* 2005).

Results from consortia for both bacteria and fungi (18.5% and 19.68% respectively) showed that mixed cultures can be more useful when they grow in symbiosis. They may enhance the growth of the biofilm formed as well as increase the hydrophilicity of the polymer surface when compared to the growth of individual organisms. Senevitatne *et al*. (2006) found that mixed culture of *Penicillium frequentas* and *B. mycoides* was found to reduce the weight of degradable polyethene by 7% whereas single cultures of the same organisms showed a 0.5% reduction in weight indicating a synergy between the two microorganisms that led to 14 times increase in biodegradation of the polymer. By using an indigenously developed consortium of organisms closely related to *B. cereus* species*, B. pumilis* species *and Arthrobacter* species (99% of DNA identity), Satlewal *et al*. (2008) in their study on 'Comparative Biodegradation of High Density Polyethene (HDPE) and Low Density Polyethene (LDPE) using an indigenously developed consortium" observed that pure LDPE samples showed a weight loss of 4.5% while that of LDPE plus consortium was

21.7% suggesting that microbial consortia can accelerate the rate of degradation of these polymers under natural environments.

The production of biosurfactants by these microorganisms has also been proposed as a possible reason for their ability to biodegrade polymers. Biosurfactants are surface-active compounds produced on microbial surface or secreted out; they are amphiphiles having both hydrophobic and hydrophilic groups. They increase the hydrophilicity of the surface of polyethene thereby enhancing adhesion. Microorganisms like *Ps. aeruginosa*, *B. licheniformis, B. brevis* and *B. polymyxa* are able to produce glycolipids, lipopeptides, lipoproteins and polymeric surfactants. Amphiphilic nature of biosurfactant is responsible for the attachment of microorganisms on hydrophobic surfaces. It is reported that biofilm formation and thus rate of biodegradation is enhanced by the external addition of synthetic surfactants such as Tween 60/80 (Chiellini *et. al*., 2007; Arutchelvi *et. al.,* 2008).

Fourier Transform Infra Red Spectroscopy (FTIR) measures changes in chemical properties such as formation or disappearance of functional groups in the polymer. FTIR results obtained from all samples showed an appearance of a carbonyl peak at 1703$cm^{-1}$ except that obtained from the commercial synthetic LDPE. This could be due to the effect of antioxidants which are being added to synthetic polymers in order to delay oxidation. Incubation with both bacteria and fungi, showed a carbonyl absorption band extension from 1703$cm^{-1}$ to 1775.83$cm^{-1}$ for the bacterium and fungus and from 1703$cm^{-1}$ to 1872.38$cm^{-1}$ for the bacterial and fungal consortium indicating the possibility of various types of oxidation products formed during the biodegradation. The increase in carbonyl groups could also likely be from the starch and its oxidation products which also have absorption spectra

between 1400 $cm^{-1}$ and 1800 $cm^{-1}$. There was also an increase in the number of double bonds and is in agreement with the biodegradation mechanism of polyethene. Hadad *et al.* (2005) obtained a typical carbonyl peak at 1712$cm^{-1}$ which also is in agreement with that of Orhan *et al*. (2004).

## 5.1 CONCLUSION

Plastic wastes accumulating in the environment are posing an ever increasing ecological threat with the most problematic as the polyethene which is inert and resistant to microbial attack. From this study, it can be concluded that microorganisms with the ability of degrading low density polyethene carrier bags exist within our natural environment and that addition of natural biodegradable additives such as starch to recalcitrant polymers such as low density polyethene has great potential in enhancing their biodegradation.

Different kinds of microorganisms in the natural environment play an important role in various steps involved in degradation of synthetic polymers in general and polyolefins in particular. The synergistic activity of microbial mixed cultures has also been shown from the results obtained from this research. Studying the synergism between them will give more insight for future efforts towards the biodegradation of these polymers.

FTIR analysis of residual low density polyethene carrier bags after microbial treatment for 30days showed the appearance of carbonyl peaks at wavelengths of 1703$cm^{-1}$ which

extended to 1872.38cm$^{-1}$. In summary, both bacteria and fungi isolated were able to degrade both the synthetic unblended (1% to 4.02%) and starch blended low density polyethene (7.82% to 19.68%). Therefore finding the right microorganisms and their enzymes that can efficiently and completely biodegrade the recalcitrant polyethene into carbon dioxide, water and biomass will go a long way in solving this global threat.

## 5.2 RECOMMENDATIONS

The wide use of polyethene and its careless disposal makes it an important environmental issue and the hazards of discarding polyethene are becoming more and more severe. It is therefore recommended that

1. Aggressive campaigns and enlightenment of the populace be carried out by government and Non Governmental Organisations (NGO) on the hazards of improper disposal of polyethene carrier bags in order to achieve a behavioural change and appropriate measures be put in place to properly dispose the non biodegradable bags.
2. Waste to wealth campaign should be intensified by government (especially in developing nations like Nigeria were polythene pollution is more severe) so as to convert low density polyethene and other plastic wastes into other uses or

products such as blocks, partitions, tiles and in asphalting of roads. If market value can be created for the polyethene bag wastes, scavengers would start picking them as well and waste buyers would move from house to house to buy them. Use of biosurfactant producing microorganisms has not been fully exploited in their use for polymer degradation. If polyolefin wastes can be utilized to produce useful products like biosurfactants, the biodegradation process will be economically favourable.

3. There should be government legislation mandating the blending of synthetic polyethene with starch from the production stage before they are made into carrier bags

4. A generation of environmentally friendly polyethene containing natural biodegradable additives like starch are highly recommended for use since their degradation in natural environment is much faster.

5. There is also a need for further research to find out optimal conditions like temperature, pH, inoculums size, and agitation rate that will enhance the biodegradative ability of these microorganisms. The effect of other forms of pre-treatment such as UV and chemicals like nitrous acid singly and in combinations on rate of biodegradation of low density polyethene should also be investigated. Isolation of microorganisms from marine, petroleum waste and polymer dump sites could also lead to new unexplained strains with superior performance.

6. Genetic modification of microorganisms: If genes responsible for production of degrading enzymes can be characterised and regulated by using current genetic engineering tools, microorganisms can be genetically modified and used as

superbugs for degrading the recalcitrant polyolefins. Computation and molecular modelling techniques in spite of their wide spread use in materials science, medicinal chemistry and pharmaceutical sciences has not been attempted in the area of environmental sciences. It is hoped that one day with the increase in information and computer technology (ICT), it may be possible to predict the static and dynamic biological response of microorganisms as bacteria and fungi when they come in contact with a polymeric surface, the formation of biofilms and generation of extracellular enzymes and polysaccharides.

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# APPENDICES

## APPENDIX I: Media Formulation

### Nutrient Agar (Biomark™) gm/litre

Peptic digest of animal tissue----------5.00
Beef extract----------1.50
Yeast extract----------1.50
Sodium Chloride----------5.00
Agar----------15.00
pH----------7.4 ± 0.2

### Potato Dextrose Agar (Scharlau) gm/litre

Potato Peptone---------- -4.00
Glucose----------20.00
Agar----------15.00
pH----------5.6 ± 0.2

### Basal Medium (g/$L^{-1}$ of distilled water)

$NH_4NO_3$----------1.0
$MgSO_4.7H_2O$----------0.2
$K_2HPO_4$----------1.0
$CaCl_2.2H_2O$----------0.1
KCl----------0.15
Yeast extract----------0.1
$FeSO_4.6H_2O$----------1.0mg $l^{-1}$
$ZnSO_4.7H_2O$----------1.0mg $l^{-1}$
$MnSO_4$----------1.0mg $l^{-1}$
pH----------7.8

### McFarland Standard

| McFarland standard No. | 0.5 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| 1.0%Bacl (ml) | 0.05 | 0.1 | 0.2 | 0.3 | 0.4 |
| 1.0% Sulphuric Acid (ml) | 9.95 | 9.9 | 9.8 | 9.7 | 9.6 |
| Approximate cell density (1 x $10^8$ CFU/ml) | 1.5 | 3.0 | 6.0 | 9.0 | 12.0 |
| | 74.3 | 55.6 | 35.6 | 26.4 | 21.5 |

| %Transmittance | | | | | |
|---|---|---|---|---|---|
| Absorbance* | 0.132 | 0.257 | 0.451 | 0.582 | 0.669 |

*At wavelength of 600nm

Source: Barley and Scott's Diagnostics Microbiology, 7th Edition, pg 17

## APPENDIX II: Microscopic Pictures of Bacterial Isolates – Gram Reaction and Microgen(TM) Identification Strips

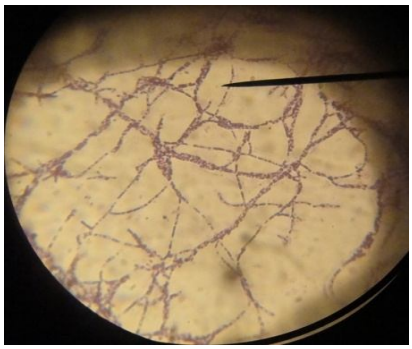


Plate III Gram positive Bacilli (X100)

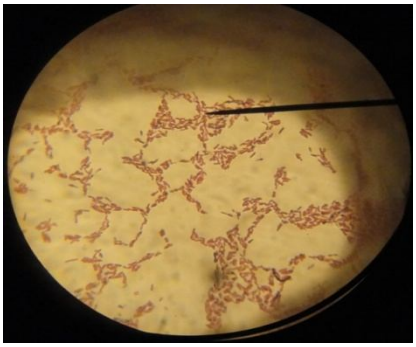


Plate IV Gram negative Bacilli (X100)

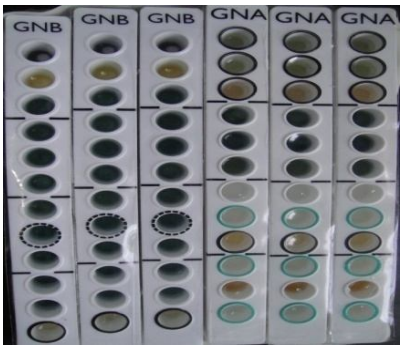



Plate Va: Gram Negative A + B Identification Strips




Plate Vb: *Bacillus* Identification strips

## APPENDIX IIIa: Microgen$^{(R)}$ GN A+B Substrate Reference Table

| Well | Reaction | Description | Positive | Negative |
|---|---|---|---|---|
| 1. | Lysine | Lysine decarboxylase-Bromothymol blue changes to green/blue indicating the `production of the amine cadaverine. | Green/Blue | Yellow |
| 2. | Ornithine | Ornithine decarboxylase- Bromothymol blue changes to blue indicating the production of the amine putrescin. | Blue | Yellow /Green |
| 3. | $H_2S$ | $H_2S$ production-Thiosulphate is reduced to $H_2S$ that reacts with ferric salts producing a black precipitate. | Brown/ black | Straw |
| 4. | Glucose | Fermentation- Bromothymol blue changes from blue to yellow as a result of acid produced from the carbohydrate fermentation. | Yellow | Blue/green |
| 5. | Mannitol | | | |
| 6 | Xylose | | | |
| 7. | ONPG | Hydrolysis-ONPGHydrolysis by B-galactosidase results in the production of yellow ortho-nitrophenol | Yellow | Colourless |
| 7a | Nitrate (for oxidase positive organisms) | Reduction of nitrate to nitrite is indicated by the formation of a red colour on addition of nitrate A and B reagent | red | Colourless/ yellow |
| 7b | Nitrate (for oxidase positive organisms) | If nitrate has been completely reduced to nitrogen,7a will remain colourless/yellow- addition of zinc powder will confirm complete reduction | Colourless/ yellow | red |

| | | | | |
|---|---|---|---|---|
| 8. | Indole | Indole is produced from tryptophan and gives a pink/red complex when Kovac's reagent is added. | Pink/red | Colourless |
| 9. | Urease | Hydrolysis of urea results in the formation of ammonia leading to an increase in pH which turns phenol red from yellow to pink/red. | Deep Pink | Straw to pale salmon pink colour |
| 10. | VP | Acetoin production from glucose is detected by the formation of a pink /red complex after the addition of alpha naphthol and creatine in the presence of KOH. | Deep Pink/Red | Colourless to Pale Pink |
| 11. | Citrate | Utilization of citrate (only carbon source) leading to a colour change in bromothymol blue from green to blue. | Blue | Yellow/Pale Green |
| 12. | TDA | Indolepyruvic acid is produced from tryptophan by tryptophan deaminase giving a cherry red colour when ferric ions are added. Indole positive isolates may give a brown colour- this is a negative result. | Cherry red | Straw colour |
| 13 | Gelatin | Proteolytic enzymes liquefy gelatine resulting in black particles being dispersed throughout the well | black | colourless |
| 14 | Malonate | Inhibition of the conversion of succinic acid to fumaric acid occurs when sodium malonate is the only source of carbon. An isolate incapable of using this substrate results in the accumulation of succinic acid and the organism does not grow. A positive reaction is the result of the use of sodium malonate at the same time that ammonium sulphate is used as the nitrogen source giving sodium hydroxide which increases the alkalinity giving a blue colour | Blue | Yellow |
| 15 | Inositol | Fermentation-Bromothymol blue changes from blue to yellow as a result of acid produced from the carbohydrate fermentation | yellow | Blue |
| 16 | Sorbitol | | | |
| 17 | Rhamnose | | | |
| 18 | Sucrose | | | |
| 19 | Lactose | | | |
| 20 | Arabinose | | | |
| 21 | Adonitol | | | |

| | | | | |
|---|---|---|---|---|
| 22 | Raffinose | | | |
| 23 | Salicin | | | |
| 24 | Arginine | Arginine is converted to ornithine, ammonia, and $CO_2$ by arginine dihydrolase resulting in an increase in pH and a change in colour of the bromothymol blue from green to blue. At 48hrs green reactions are negative | Green/Blue<br>Blue | YellowYellow<br>Blue |

**Key:** $H_2S$-Hydrogen sulphide; ONPG-Ortho-nitrophenol- galactosidase; TDA-Tryptophan Deaminase Acid, GN- Gram negative

## APPENDIX IIIb: Microgen(R) Bacillus ID Substrate Reference Table

| Well | Substrate | Reaction | Positive | Negative |
|---|---|---|---|---|
| 1. | Arabinose | Fermentation of specific sugars producing acid end products changes the phenol Red indicator from red to yellow. | Yellow | Red |
| 2. | Cellobiose | | | |
| 3. | Inositol | | | |
| 4. | Mannitol | | | |
| 5. | Mannose | | Yellow | Red |
| 6. | Raffinose | Fermentation of specific sugars producing acid end products changes the phenol Red indicator from red to yellow. | Yellow | Red |
| 7. | Rhamnose | | | |
| 8. | Salicin | | | |
| 9. | Sorbitol | | | |
| 10. | Sucrose | | | |
| 11. | Trehalose | | | |
| 12. | Xylose | Fermentation of specific sugars producing acid end products changes the phenol Red indicator from red to yellow. | Yellow | Red |
| 13. | Adonitol | | | |
| 14. | Galactose | | | |
| 15 | Methyl-D-Manoside | | | |
| 16 | Methyl-D-Glucoside | | | |
| 17 | Inulin | | | |
| 18 | Melezitosel | | | |
| 19 | Indole | Indole is produced from tryptophan and gives a pink/red complex when kovac's reagent is added. | Pink/Red | Colourless / yellow |
| 20 | ONPG | Hydrolysis- ONPG hydrolysis by β-galactosidase results in the production of yellow ortho-nitrophenol | Yellow | Colourless |

| | | | | |
|---|---|---|---|---|
| 20<br>Plus<br>reagent | Nitrate | Nitrate is reduced to nitrite which forms a deep red complex after the addition of α-Naphthylamine and sulphanilic acid | Red | Colourless / yellow |
| 21 | Arginine<br>Dihydrolase | Arginine is converted to Ornithine, ammonia and $CO_2$ by arginine dihydrolase resulting in an increase in pH and a change in colour of the bromothymol blue from green to blue. At 48hrs green reactions are negative | Green/<br>Blue<br>Blue | Yellow<br>Yellow/<br>Green |
| 22 | Citrate<br>Utilization | Utilization of Citrate(only carbon source) leading to a pH increase giving a colour change in bromothymol blue from yellowy green to blue | Blue | Yellow/<br>Light<br>green |
| 23 | Voges<br>Proskauer | Acetoin production from glucose is detected by the formation of a pink/red complex after the addition of alpha naphthol and creatinein the KOH | Pink/Red | Straw colour |
| 24 | Control | Carbohydrate control | Red | Red |

**Key:** ONPG-Ortho-nitrophenol- galactosidase

**APPENDIX IV: Macroscopic and Microscopic Pictures of Fungal Isolates**

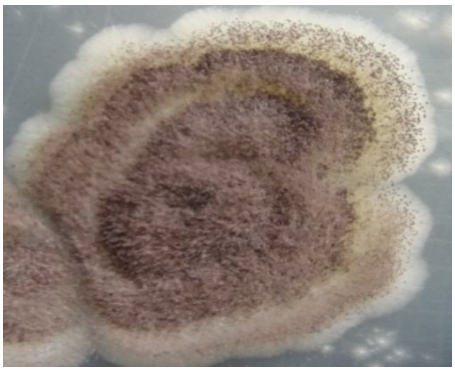
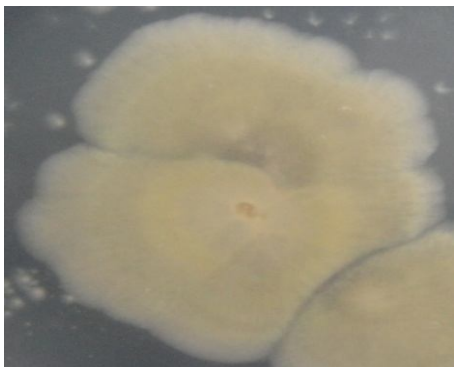


Plate VI: Surface morphology of *A. niger-1* on Potato Dextrose Agar at day 5



Reverse

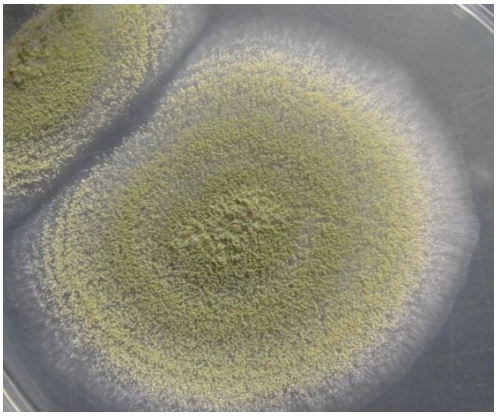
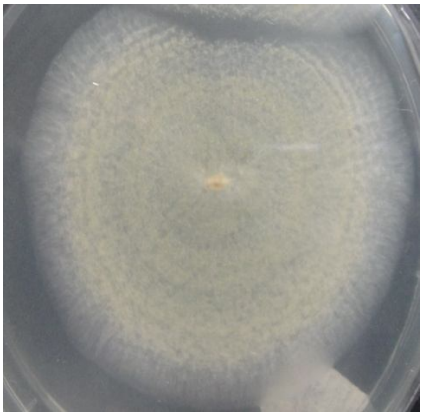


Plate VII: Surface morphology of *A. flavus* on Potato Dextrose Agar at day 5



Reverse.

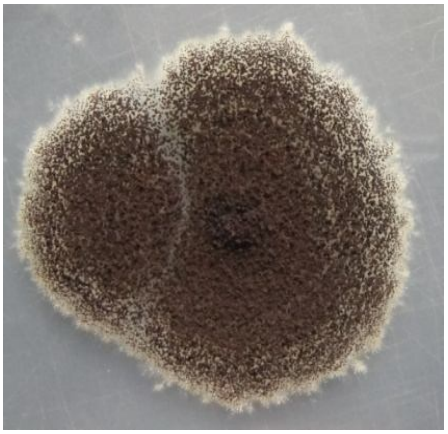




Plate VIII: Surface morphology of *A. niger*-2 on Potato Dextrose Agar at day 5

Reverse



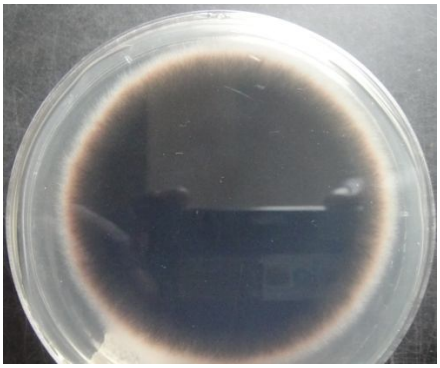
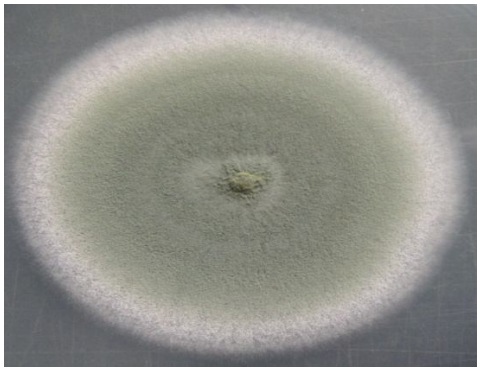


Plate VIX: Surface morphology of *Fusarium* species on Potato Dextrose Agar at day 5

Reverse



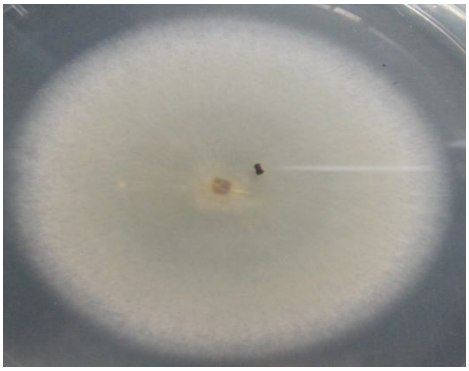
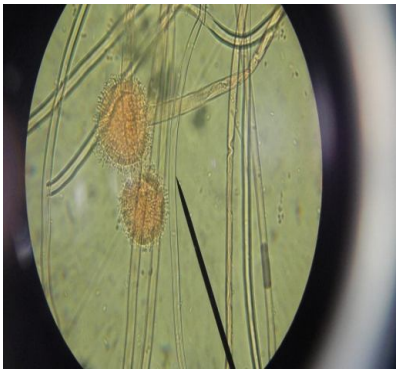
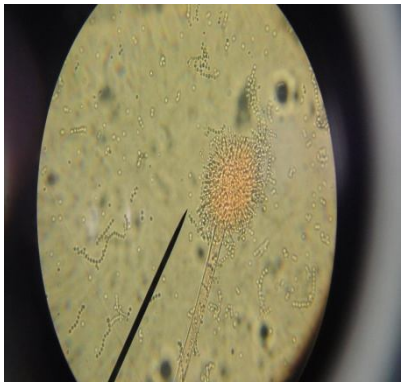


Plate X: Surface morphology of *A. glaucus* On Potato Dextrose Agar at day 5

Reverse



PlateXI:Microscopic Morphology of *A.niger1* (X40) in Lactophenol cotton blue



PlateXII:Microscopic Morphology *of A. flavus* (X40) in Lactophenol cotton blue

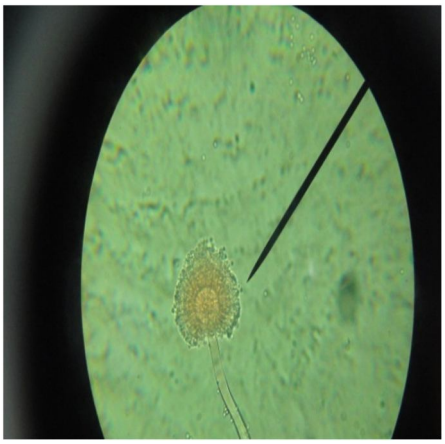


Plate XIII: Microscopic Morphology of *A.niger-2* (X40) in Lactophenol Cotton blue

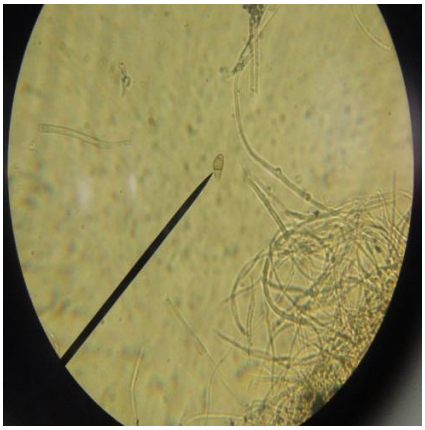


Plate XIV: Microscopic Morphology of *Fusarium* spp (X40) in Lactophenol Cotton blue

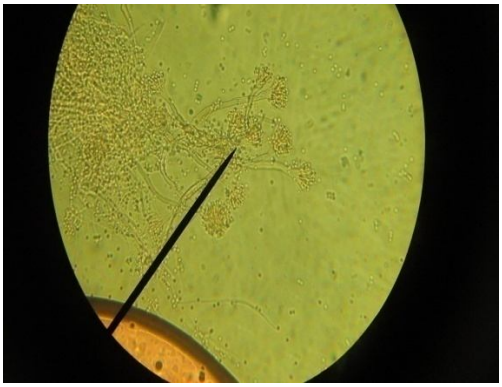


Plate XV: Microscopic Morphology of *A. glaucus* (X40) in Lactophenol Cotton blue

**APPENDIX V: Processing of Low Density Polyethene with Starch and Degradation with Microorganisms**







Plate XVI: LDPE Pellets Melting Hot Pressed Product



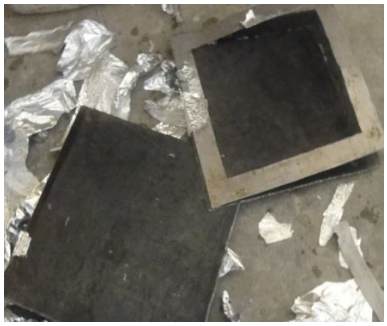


Plate XVII: Melted LDPE Carrier bags HotPressed LDPE Carrier bags




Plate XVIII: Shake- Culture Incubation of Fungal Isolates Using Orbital Shaker at 25$^{O}$C and 150 Revolutions per Minute for 30days



Plate XIX: Shake- culture Incubation of Bacterial Isolates Using Orbital Shaker at 35$^{O}$C 150 RPM/minute for 30days

## APPENDIX VI: Fourier Transformed Infra Red Spectrophotometric Peak Finding Results

**Peak finding results for: spe control**
Frequency: 400.00 - 3999.64, threshold: 6.112, sensitivity: 50.00
Peak finding result table:

| Peak# | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Position | 3982.14 | 3895.07 | 3857.00 | 3804.00 | 3751.67 | 3714.42 | 3653.13 | 3599.60 | 3529.91 |
| Height | 5.824 | 5.819 | 5.660 | 5.718 | 5.630 | 5.669 | 5.632 | 5.481 | 5.766 |

| Peak# | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|
| Position | 3447.16 | 3369.30 | 3328.83 | 3193.19 | 2759.57 | 2662.90 | 2520.95 | 2336.81 | 2150.01 |
| Height | 5.607 | 5.512 | 5.559 | 5.340 | 4.688 | 4.657 | 4.688 | 4.575 | 4.743 |

| Peak# | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 |
|---|---|---|---|---|---|---|---|---|---|
| Position | 2020.94 | 1898.94 | 1719.10 | 1459.84 | 1294.12 | 1079.25 | 890.47 | 848.05 | 799.20 |
| Height | 4.457 | 4.536 | 4.196 | 3.810 | 3.737 | 3.764 | 3.745 | 3.773 | 3.495 |

| Peak# | 28 | 29 | 30 | 31 | 32 | 33 | | | |
|---|---|---|---|---|---|---|---|---|---|
| Position | 717.72 | 601.85 | 547.90 | 512.44 | 485.01 | 435.07 | | | |
| Height | 3.369 | 4.363 | 3.952 | 3.933 | 4.310 | 4.284 | | | |

**Peak finding results for: cspe**
Frequency: 399.19 - 3999.64, threshold: 17.080, sensitivity: 50.00
Peak finding result table:

| Peak# | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Position | 3850.88 | 3815.89 | 3689.95 | 3667.82 | 3645.63 | 3608.97 | 3583.49 | 2055.94 | 1298.00 |
| Height | 16.874 | 16.684 | 16.352 | 16.235 | 16.070 | 15.967 | 15.926 | 12.464 | 12.194 |

| Peak# | 10 | 11 | 12 | 13 | 14 | 15 | | | |
|---|---|---|---|---|---|---|---|---|---|
| Position | 911.66 | 823.33 | 763.87 | 516.78 | 454.27 | 429.96 | | | |
| Height | 12.261 | 12.742 | 12.673 | 12.777 | 12.546 | 12.787 | | | |

**Peak finding results for: pest control**
Frequency: 400.00 - 4000.00, threshold: 27.090, sensitivity: 50.00
Peak finding result table:

| Peak# | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Position | 3928.78 | 3905.69 | 3859.93 | 3840.47 | 3823.51 | 3804.82 | 3753.87 | 3715.31 | 3678.21 |
| Height | 26.644 | 25.449 | 25.584 | 25.377 | 25.322 | 25.287 | 23.192 | 24.289 | 23.550 |

| Peak# | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|
| Position | 3654.16 | 3630.33 | 3593.33 | 3570.36 | 2367.09 | 2340.10 | 1775.92 | 1749.61 | 1703.82 |
| Height | 22.784 | 23.247 | 23.412 | 22.933 | 16.246 | 17.065 | 17.282 | 17.174 | 16.895 |

| Peak# | 19 | 20 | 21 | 22 | 23 | 24 | 25 | | |
|---|---|---|---|---|---|---|---|---|---|
| Position | 1655.86 | 1563.56 | 1543.84 | 1522.60 | 820.49 | 528.53 | 421.25 | | |
| Height | 16.743 | 16.736 | 16.545 | 16.598 | 15.671 | 15.057 | 15.653 | | |

Key:spe-synthetic polyethene, cspe- commercial synthetic polyethene, pest- polyethene/starch

**Peak finding results for: SPE b**
Frequency: 400.00 - 3999.64, threshold: 16.051, sensitivity: 50.00
Peak finding result table:

| Peak# | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Position | 3907.53 | 3859.30 | 3822.82 | 3755.13 | 3716.10 | 3678.82 | 3654.85 | 3630.74 | 3593.33 |
| Height | 9.655 | 9.738 | 9.751 | 9.275 | 9.666 | 9.424 | 9.238 | 9.255 | 9.225 |

| Peak# | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|
| Position | 3570.15 | 3529.85 | 3454.53 | 2924.76 | 2372.25 | 2340.58 | 2050.48 | 1752.90 | 1656.80 |
| Height | 9.068 | 8.964 | 8.765 | 7.748 | 9.039 | 9.430 | 9.518 | 9.561 | 9.302 |

| Peak# | 19 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Position | 1461.99 | | | | | | | | |
| Height | 9.405 | | | | | | | | |

**Peak finding results for: Pest F 3**
Frequency: 400.00 - 4000.00, threshold: 55.924, sensitivity: 50.00
Peak finding result table:

| Peak# | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Position | 3905.02 | 3858.96 | 3840.41 | 3822.99 | 3804.92 | 3753.17 | 3714.96 | 3677.94 | 3653.76 |
| Height | 54.873 | 54.751 | 54.740 | 54.293 | 54.095 | 50.128 | 52.174 | 50.644 | 49.078 |

| Peak# | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|
| Position | 3630.05 | 3593.52 | 3570.23 | 2366.50 | 2339.80 | 1775.83 | 1747.66 | 1703.67 | 1655.25 |
| Height | 50.057 | 50.307 | 49.882 | 35.425 | 36.970 | 38.077 | 37.741 | 37.192 | 37.065 |

| Peak# | 19 | 20 | 21 | 22 | 23 |
|---|---|---|---|---|---|
| Position | 1543.78 | 1521.43 | 1399.56 | 671.43 | 459.75 |
| Height | 36.332 | 36.403 | 36.862 | 35.833 | 35.174 |

**Peak finding results for: pest BC _GH**
Frequency: 400.00 - 4000.00, threshold: 47.644, sensitivity: 50.00
Peak finding result table:

| Peak# | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Position | 3928.29 | 3905.11 | 3858.23 | 3840.30 | 3823.23 | 3804.91 | 3753.27 | 3715.26 | 3678.01 |
| Height | 46.405 | 44.582 | 44.545 | 44.485 | 44.149 | 43.881 | 41.039 | 42.573 | 41.107 |

| Peak# | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|
| Position | 3653.85 | 3629.85 | 3593.54 | 3569.84 | 3549.10 | 3528.75 | 2366.24 | 2339.48 | 1872.31 |
| Height | 40.033 | 40.681 | 41.035 | 40.680 | 41.543 | 41.332 | 29.383 | 30.845 | 33.365 |

| Peak# | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 |
|---|---|---|---|---|---|---|---|---|---|
| Position | 1775.93 | 1749.64 | 1703.96 | 1655.66 | 1563.45 | 1543.68 | 1521.83 | 1399.72 | 882.16 |
| Height | 32.884 | 32.739 | 32.218 | 32.281 | 32.176 | 31.671 | 31.822 | 32.238 | 31.985 |

| Peak# | 28 | 29 |
|---|---|---|
| Position | 670.38 | 423.17 |
| Height | 31.287 | 31.206 |

**Peak finding results for: pest FC3+4**
Frequency: 400.00 - 4000.00, threshold: 12.799, sensitivity: 50.00
Peak finding result table:

| Peak# | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Position | 3949.36 | 3905.10 | 3884.18 | 3857.11 | 3839.55 | 3805.05 | 3752.92 | 3714.11 | 3678.37 |
| Height | 11.391 | 10.966 | 11.016 | 10.913 | 10.868 | 10.876 | 10.124 | 10.580 | 10.053 |

| Peak# | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|
| Position | 3653.90 | 3630.58 | 3592.27 | 3571.30 | 3441.97 | 3334.70 | 2924.09 | 2854.72 | 2366.24 |
| Height | 9.813 | 9.879 | 9.816 | 9.730 | 9.425 | 9.388 | 8.805 | 9.074 | 8.148 |

| Peak# | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 |
|---|---|---|---|---|---|---|---|---|---|
| Position | 2339.68 | 1872.38 | 1776.10 | 1703.73 | 1655.77 | 1564.32 | 1543.56 | 1522.83 | 1460.93 |
| Height | 8.475 | 9.238 | 9.162 | 8.948 | 8.725 | 9.018 | 8.952 | 9.060 | 8.866 |

| Peak# | 28 | 29 | 30 | 31 |
|---|---|---|---|---|
| Position | 1037.30 | 670.26 | 604.67 | 552.67 |
| Height | 9.306 | 11.047 | 11.202 | 11.105 |

Key: speb - synthetic polyethene + bacteria, pest F3 - polyethene/starch + fungi, pest BC-GH- polyethene/starch + bacterial consortium, pest FC3+4 - polyethene/starch + fungal consortium