ISOLATION OF POLYOLEFIN'S DEGRADING BACTERIA FROM COMPOST

Submitted in the partial fulfilment for the award of the degree of

Master of Science

in

Microbiology

By

Lalit Thakur
(Roll No: 301105007)

Under the supervision of

Dr. Haripada Bhunia
Associate Professor
Department of Chemical Engineering
Thapar University, Patiala

Department of Biotechnology and Environmental Sciences
Thapar University, Patiala-147 004, Punjab
www.thapar.edu

July 2013

CERTIFICATE

This is certified that the thesis entitled **"Isolation of polyolefin's degrading bacteria from compost",** is an authentic record of my own work carried out as requirements for the award of degree of M.Sc. (Microbiology) at Thapar University, Patiala, under the guidance of Dr. Haripada Bhunia (Associate Professor, ChED) during January 2013 to June 2013.

Date: 10-7-13

Lalit Thakur
Roll no. 301150007

It is certified that the above statement made by the student is correct to the best of our knowledge and belief.

Dr. Haripada Bhunia
Associate Professor
Department of Chemical Engineering
Thapar University, Patiala

Countersigned by

Dr. M.S. Reddy
Professor & Head
Department of Biotechnology and
Environmental Sciences
Thapar University, Patiala

Dr. S. K. Mohapatra
Dean, Academic affairs
Thapar University, Patiala

ACKNOWLEDGEMENT

This is a golden opportunity for me to convey my sincere regards for all those people who enabled me to accomplish my thesis work successfully.

With deep sense of gratitude I express my sincere thanks to my esteemed and worthy supervisor, Dr. Haripada Bhunia, Associate Professor, Department of Chemical Engineering, Thapar University, Patiala, for his valuable guidance in carrying out this work under his effective supervision, encouragement, enlightenment and cooperation. He had been very kind and patient while suggesting me the outlines of this project. His able knowledge and expert supervision with unswerving patience fathered my work at every stage, for without his warm affection and encouragement, the fulfilment of the task would have been very difficult.

I would like to express my gratitude to Dr. M.S. Reddy, Head of Department of Biotechnology, and Dr. Rajeev Mehta, Head of department of Chemical Engineering, for helping me to move ahead with this work.

I am grateful to Kimi Jain (PhD Scholar) and Gaurav Madhu (Research Associate), who helped me a lot during my experimental work. I am also thankful to Chitrakshi Goel (PhD Scholar) for their moral support and encouragement.

I am also thankful to all the staff members of the department for their full cooperation and help.

I am thankful for the help rendered by my friends namely Rahul, Arun, Sanjay, Vikrant, Rajnish, Dinesh and all others who have supported me throughout entire project.

Lalit

Place: Patiala

Lalit Thakur

To my parents, and Niti Thakur for all the support and motivation they have given me throughout my life.....

ABSTRACT

The present study was done on the isolation of polyolefin-degrading bacteria from compost. Several bacterial strains were isolated by two techniques: (i) enrichment technique for PE and PP powder; (ii) by incubating PE and PP film in synthetic media.

By enrichment technique, five bacterial strains were isolated, four degrading PP and one degrading PE. PP degrading bacteria were found to be Gram positive and PE degrading bacteria was found to be Gram negative bacteria. O.D at 600 nm and CFU/ml count were also measured to see the growth of microorganisms in synthetic media. The increase in O.D and CFU/ml count indicates the utilization of the given polymer as the carbon source. Higher growth was found in PE as compared to PP. This shows that PP is more resistant to biodegradation than PE, due to presence of extra methyl group.

After two months of incubation of PE and PP films in synthetic media, several bacterial strains were isolated, 6 degrading PE & 5 degrading PP. Their weight loss was measured. Higher weight loss was observed in PE (1.95%) than PP (1.2%). This is also supporting the fact that PP is more resistant to biodegradation than PE. The efficacy of isolated microbes in the degradation of plastics were analyzed in liquid (shaker) culture method. As the growth of microbes proportionally increased in synthetic media, so the microbes were solely dependent on polymer films for its C-source. FTIR also confirmed for the degradation of polymer by compost.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

°C	degree(s) Celsius
%	Percentage
Rpm	Revolutions per minute
Γ	Gamma
spp.	species (plural)
mm	millimetre
µl	micro litre
g	gram
ml	millilitre
pH	power of hydrogen
O.D	optical density
Cfu	colony forming unit
LAF	laminar air flow
min.	Minutes
U.V	ultra violet
PP	polypropylene
PE	polyethylene
PS	polystyrene
PV	Polyvinyl chloride
PET	Polyethylene Terephthalate
BDP	Biodegradable biopolymers
DPE	degradable polyethylenes
MSW	municipal solid waste
SEM	Scanning Electron Microscopy
PCR	polymerase chain reaction
FTIR	Fourier Transform Infra Red Spectroscopy
DSC	Differential scanning colorimeter
AFM	Atomic Force Microscopy
LDPE	Low density polyethylene
XRD	X-ray Diffraction

CHAPTER-1
INTRODUCTION

Polymers are becoming a necessity in modern life. Their discovery and subsequent utilisation meant that by the mid 1960s total world consumption of thermoplastics alone was 13 million tonnes. With an increasing number of applications being found for plastic, today the demand for the main commodity thermoplastics is more than 70 million tonnes. Although a significant amount of the thermoplastics are utilised in products with a long life span, the majority of plastic are used in short term applications such as packaging. Thus, the quantity of thermoplastics found in waste is increasing directly. Plastic waste can originate from a multitude of sources. The major areas of waste creation are from the distribution industries representing 21.7% of all plastic waste and municipal solid waste (MSW) which accounts for 60.40%. Municipal solid waste is being targeted as an issue for improvement. However, the nature of MSW is very complex, making its treatment difficult. Other forms of waste are easier to handle, because of their uniformity and can be treated as they are created by, for example feeding back into the production process. Currently 80% of MSW goes to landfill 10% is recycled and 10% is incinerated (Williams and Williams, 1997).

The term "plastic", derived from the Greek word "plastikos" actually applies to any pliable substance that can be shaped or moulded, for example, wax, clay, asphalt and amber. Most of the plastics we use today have been developed within the last 50 years or so. The majority of them are man-made and are usually described as synthetic products, or in other words, they are made by a process of building up from simple chemical substances.

Today's plastics are generally made by industrial chemists from various chemical compounds derived from, salt, water, petroleum or lime. Their special properties are light weight, high impact, better tensile strengths and resistance to corrosion. Addition of salt water and chemicals provide suitability for use over a wide range of temperatures and for electrical insulation. Some plastics are not fully synthetic they are produced by modifying natural materials. Many polymers have been synthesized

chemically because of the convenience and economy of the process and the stability of the products (Onodera *et al.*, 2000).

Polyolefin are synthetic polymer made of long chain monomers of olefin like polyethylene. Polyethylene is the polyolefin produced by polymerizing the olefin ethylene. It is a thermoplastic and mostly used for packaging, storage, transportation. The worldwide production rate of polyethylene is expanding at a rate of 12% per annum & approximately 140 million tonnes of synthetic polymers are produced worldwide each year. Polypropylene is another common polyolefin which is made from the olefin propylene (Nanda *et al.*, 2010).

Plastics can be synthesized via the polymerization (polyaddition or polycondensation) of small molecules. General they are classified into two groups, thermoplastics and thermoset plastics. Thermoplastics are linear chain macromolecules where the atoms and molecules are joined end-to-end into a series of long, sole carbon chains. The linear macromolecule from vinyl monomers can be achieved by opening the double bond and the reaction proceeds by a free radical mechanism. Such type of polymerization is known as addition polymerization, polyethylene and polypropylene are the examples. The thermoset plastics have property that they can be created in any form by heating. On the other hand, thermoset plastics are formed by step polymerization under suitable conditions allowing bi-functional molecules to condense inter-molecularly with the liberation of small by-products such as H_2O, HCl (Kambe *et al.*, 2011).

The composition of the plastics consist carbon, hydrogen, silicon, oxygen, chloride and nitrogen. Oil, coal and natural gas are used for extraction of the basic materials of plastics. Because of its stable and durable characteristic, plastics are widely used. Mostly used plastics are polyethylene (LDPE, MDPE, HDPE and LLDPE), Poly (ethylene-terephthalate) (PET), Poly (butylene-terephthalate) (PBT), nylons, Polypropylene (PP), Polystyrene (PS), Polyvinyl Chloride (PVC), and Polyurethane (PUR) (Bhardwaj *et al.*, 2012).

Table 1.1: main types of plastic their properties and application

Name	Properties	Product application
Polypropylene (PP)	Strength, resistance to heat, barrier moisture, oil	Ketchup bottles, yogurt containers, medicine bottles
Polyethylene (PE)	Ease of processing, barrier to moisture, flexibility	Dry cleaning, bread and frozen food bags
Polystyrene (PS)	Versatility, insulation, clarity	Food service applications, meat trays, egg cartons, cups
Polyvinyl chloride (PV)	Ease of blending, resistance to chemicals	Ease of blending, resistance to chemicals
High density polyethylene (HDPE)	Strength, resistance to chemicals and moisture, ease of processing and forming	Milk, water, juice, cosmetic, shampoo, dish and detergent bottles, trash and retail bags
Polyethylene Terephthalate (PET)	Clarity, barrier to gas moisture, resistance to heat	Plastic soft drink and water bottles, beer bottles, peanut butter bottles

It is generally recognized that polyolefins are bioinert, that is, they are highly resistant to assimilation by microorganisms such as fungi, bacteria and the like. This is not surprising since the surfaces of materials and articles made from polyolefins are hydrophobic and thus inhibit the growth of microflora on them (Nanda and Sahu, 2010).

The resistance of polyethylene towards biodegradation is due to its high molecular weight and its hydrophobic nature, all of these interfere with its availability to micro-organisms to use it as sole carbon source. Several other methods have been employed for partial biodegradation of polyethylene i.e U.V irradiation, thermal treatment, oxidation with nitric acid. The biodegradation of polyethylene is enhanced by oxidation pre-treatment, which increases surface hydrophilicity by the formation of carbonyl groups that can be utilized by microorganisms. Biodegradation of polymer results from the utilization of polymer as a nutrient (i.e. a carbon source). The biodegradation

become more efficient if the degrading micro-organism forms a biofilm on the polyethylene surface. However, the hydrophobicity of the polyethylene interferes with the formation of a microbial biofilm (Hadad *et al.*, 2005).

The term "biodegradable plastics" normally refers to an attack by microorganisms on non-water soluble polymer-based materials (plastics). This implies that the biodegradation of plastics is usually a heterogeneous process. Because of a lack of water- solubility and the size of the polymer molecules, microorganisms are unable to transport the polymeric material directly into the cells where most biochemical processes take place; rather, they must first excrete extracellular enzymes which depolymerize the polymers outside the cells as shown in Fig. 1.1. As a consequence, if the molar mass of the polymers can be sufficiently reduced to generate water-soluble intermediates, these can be transported into the microorganisms and fed into the appropriate metabolic pathway(s). As a result, the end-products of these metabolic processes include water, carbon dioxide and methane (in the case of anaerobic degradation), together with a new biomass. The extracellular enzymes are too large to penetrate deeply into the polymer material, and so act only on the polymer surface; consequently, the biodegradation of plastics is usually a surface erosion process (Muller, 2005).

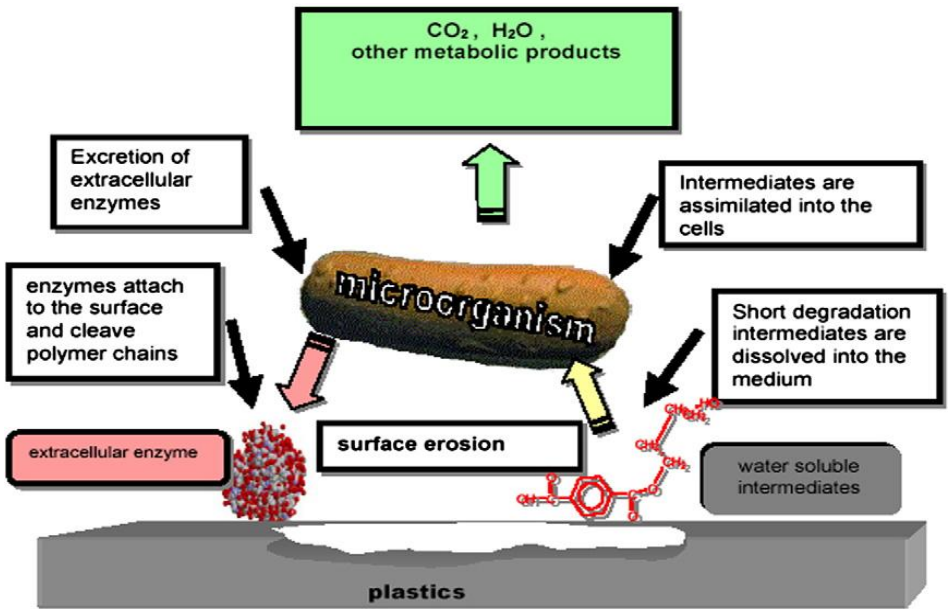


Fig. 1.1: General mechanism of plastic biodegradation under aerobic conditions (Muller, 2005)

Although the enzyme-catalyzed chain length reduction of polymers is in many cases the primary process of biodegradation, non-biotic chemical and physical processes can also act on the polymer, either in parallel or as a first stage solely on the polymer. These non- biotic effects include chemical hydrolysis, thermal polymer degradation, and oxidation or scission of the polymer chains by irradiation (photo degradation). For some materials, these effects are used directly to induce the biodegradation process [e.g., poly (lactic acid); pro-oxidant modified polyethylene], but they must also to be taken into account when biodegradation is caused predominantly by extracellular enzymes. Because of the co-existence of biotic and non-biotic processes, the entire mechanism of polymer degradation could - in many cases - also be referred to as environmental degradation (Muller, 2005).

Environmental factors not only influence the polymer to be degraded, they also have a crucial influence on the microbial population and on the activity of the different microorganisms themselves. Parameters such as humidity, temperature, pH, salinity, the presence or absence of oxygen and the supply of different nutrients have important effects on the microbial degradation of polymers, and so these conditions must be considered when the biodegradability of plastics is tested (Muller, 2005).

Biodegradable polymers and plastics offer an attractive alternative to current non-biodegradable plastics from an environmental perspective. Recently, there has been a growing demand for biodegradable plastics as a solution to the problem with non-biodegradable plastic (Ohuraa *et al.,* 1998).

Biodegradable polymers are defined as those that undergo microbial induced chain degradation leading to the mineralization. Specific conditions such as pH, humidity, oxygenation and the presence of some metals are required to facilitate the biodegradation of polymers. Biodegradable polymers may be made from bio sources like corn, wood cellulose, agriculture feed stock or can also be synthesized by bacteria from small molecules like butyric acid or valeric acid that give polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV) (Ray and Bousmina, 2005).

Lack of degradability and the closing of landfill sites, and growing water and land pollution problems have led to concern about environmental pollution problems.

Furthermore, short supply of raw materials for plastics synthesis, increases the necessity for recycling of waste plastics and the recovery of monomers is considered to be the best way of recycling plastics. Recently, biodegradable plastics have attracted attention because they could be degraded into water and carbon dioxide by microorganisms. These plastics are most suitable for biochemical monomer recycling, because they could be degraded by specific enzymes which are synthesized by environmental microorganisms (Kambe *et al.*, 2011).

Biodegradable biopolymers (BDP) are an alternative to petroleum-based polymers (traditional plastics). Some BDP degrade in only a few weeks, while the degradation of others takes several months. In principle the properties relevant for application as well as biodegradability are determined by the molecular structure. According to the American Society for Testing and Materials, biopolymers are degradable polymers in which degradation results from the action of naturally occurring microorganisms such as bacteria, fungi and algae (Shimao, 2001). This means that BDP can be produced from natural raw materials such as starch, sugar and cellulose as well as fossil oils.

The thermal degradation of polyolefin such as polyethylene and polypropylene is important from different points of view. Polyethylene and polypropylene respectively are used in high amounts for packaging and constitute the main components of plastic waste from domestic refuse (Nanda and Sahu, 2010).

This study has been carried to isolate the polyolefin i.e. polyethylene and polypropylene, degrading bacteria by using the compost as the source for the microorganisms. A further objective was to determine the weight loss of polyethylene and polypropylene films under liquid culture.

CHAPTER-2
LITERATURE REVIEW

During the past three decades, non-biodegradable plastic materials have replaced biodegradable products in a variety of applications. The extreme rise in the use of plastic materials has not been accompanied by a corresponding development of procedures for the safe disposal or degradation of these materials (Balasubramanian *et al.,* 2010).

Plastic materials are strong, light-weight, and durable and thus are widely used in food, clothing, shelter, transportation, construction, medical, and recreation industries. More than 40 million tons of plastics produced each year. Because of its xenobiotic origin and recalcitrant nature, its biodegradation is problematic and it accumulates at a rate of 25 million tons per year (Kumar *et al.,* 2013).

The degradation of most synthetic plastics in nature is a very slow process that involves environmental factors and action of wild microorganism. The primary mechanism for biodegradation of polymers (plastics) is the oxidation or hydrolysis by enzymes to create functional groups that improve its hydrophylicity. Consequently, the main chains of polymer are degraded resulting in polymer of low molecular weight and feeble mechanical properties, thus, making it more accessible for further microbial assimilation (Geweely and Ouf, 2011).

Biodegradation is the result of the utilization of the polymer as a carbon source by the microorganisms. This process is facilitated if the microorganism initially forms a biofilm over the polymer surface (Hadad *et al.,* 2005).

Traditionally, polyolefins are considered to be nonbiodegradable for three reasons. First, the hydrophobic character of polyolefins makes this material resistant to hydrolysis. Secondly, the use of anti-oxidants and stabilizers during manufacture keeps polyolefins from oxidation and biodegradation. Thirdly, polyolefins have high molecular weights of 4000 to 28,000 (Zheng and Yanful, 2011).

Table 2.1: Various literature reports on biodegradation of polyolefin's and their blends.

Title of the paper	Polymer	Organism	Conditions	Analytical techniques	Observation	Reference
An approach to polymer degradation through microbes	Polyethylene glycol (PEG)	Soil microbes	Burk's medium without carbon source	SEM	Microbes were alive in the medium containing only plastic as carbon source. In the SEM deformities were found in case of the plastic	Dey *et al.,* 2012
Isolation of adherent Polycyclic Aromatic Hydrocarbon (PAH)-degrading Bacteria using PAH-Sorbing Carriers	PAH	*Sphingomonas spp. Mycobacterium* spp.	Tris minimal medium	HPLC and PCR finger print analysis	PAH- adhering bacteria, which to biodegrade sorbed PAHs in soils	Bastiaens *et al.,* 2000
Biodegradation of plastic by *Aspergillus spp*. isolated from polythene polluted sites around Chennai	LDPE	*Aspergillus niger, A. japonicus, A. terreus, A. flavus*	Potato Dextrose Agar	SEM	*Aspergillus japonicus* showed 12% degradation potential, *A. niger* showed 8% degradation in one month.	Raaman *et al.,* 2012
Biodegradation of a polyvinyl alcohol-starch blend plastic	PVA-starch blend plastic	*Bacillus subtilis, Pseudomonas vesicularis var*.	Activated sludge	SEM	The weight loss of the film reached 48.5% at day 30.	Ishigaki *et al.,* 1999
Biofouling and biodegradation of polyolefins in ocean waters	HDPE, LDPE, PP	*Pseudomonas spp*	Ocean waters	FTIR, DSC, SEM and AFM	Microorganisms degrade polyolefins both in aerobic and anaerobic conditions. Weight loss was higher in LDPE 2.5% followed by that in HDPE (0.75%) and then in PP 0.5%.	Sudhakar *et al.,* 2007
Biodegradation of γ-sterilised biomedical polyolefins under composting and fungal culture environments	Polyolefins	*Aspergillus niger*	Sabouraud Dextrose Aga, solid waste mixture	γ-radiation, FTIR and SEM	Spectral changes at carbonyl region after composting of 10 and 25 kGy γ-sterilised samples, higher weight losses of γ-sterilised samples	Alariqi *et al.,* 2006

The susceptibility of Polyethylene modified with Bionolle to biodegradation by filamentous fungi	LDPE	*Aspergillus niger* and *Penicillium funiculosum*	Czapek-Doxa medium	FTIR and SEM	The percentage weight loss of polymers was measured. *A. niger* and *P. funiculosum* showed similar biodegradation rates of LDPE film	Labuzek *et al.*, 2003
Biodegradation of the Films of PP, PHBV and Its Blend in Soil	PP/PHBV (4:1)		Soil	FTIR, SEM, DSC and XRD	Biodegradable PHBV, was the most susceptible to microbial attack	Goncalves *et al.*, 2009
Biodegradation of polyethylene by the thermophilic bacterium *Brevibacillus borstelensis*	PE	*Brevibacillus borstelensis*	NB and NA	U.V. photo-oxidation, BATH, FTIR, and 16S rDNA sequencing	Isolation of *B. borstelensis* that was found capable of utilizing standard and photo-oxidized polyethylene as the sole carbon source.	Hadad *et al.*, 2004
Colonization, biofilm formation and biodegradation of polyethylene by a strain of *Rhodococcus ruber*	PE	*Rhodococcus ruber*	Minimal SM	FTIR and SEM	PE biodegradable bacteria *Rhodococcus ruber* was isolated from two step enrichment technique	Gilan *et al.*, 2004
Biodegradability of polyethylene by *Brevibacillus, Pseudomonas* and *Rhodococcus* spp.	PE	*Brevibacillus, Pseudomonas*, and *Rhodococcus* spp.	Soil sample was collected from a waste disposal site dumped with polyethylene bags	Pretretement with crystaline NaCl	The test results indicated a good stability of the three bacteria in the medium with no sign of contamination Biofilm formation by three bacteria was clearly visible	Nanda *et al.*, 2010

Most of the work is done on the study of polyolefin by using the bacteria and fungus from soil or other sources. But less study has been done on bacteria which persist in the compost. Compost contains large variety of microorganisms.

Ishigak *et al.,* 1999 made attempts to elucidate the degradation mechanism of a polyvinyl alcohol (PVA)-starch blend plastic by using starch fraction of this plastic as control for this test which was dissolved into an aqueous phase. PVA-degrading bacterium or enzyme gave a maximal weight loss of approximately 70%. To isolate polymer degrading bacteria two different methods were used, (i) shaken enrichment cultures in liquid mineral medium (ii) polymer degraders were enriched on medium and recovered from hydrophobic membranes containing sorbed polymer monomers. The liquid enrichment mainly selected for *Sphingomonas spp*., whereas the membrane method exclusively led to the selection of *Mycobacterium spp.* (Bastiaens *et al.,* 2000)

labuzek *et al.,* 2003 used *Aspergillus niger* and *Penicillium funiculosum* which were isolated from a dump to investigate the biodegradation of low-density polyethylene (LDPE) film. The effect of microorganisms' action on the samples was determined by the loss of mass of the films. Previous study shows slight increase in biodegradation of low-density polyethylene by only 30% addition of Bionolle. This paper presents results of investigations on the biodegradation of polyethylene film containing 60% (wt/wt) of Bionolle by the fungi *A. niger* and *P. Funiculosum.*

Polyethylene considered to be the most inert polymer due to its specific properties which are discussed earlier. Hadad *et al.,* 2005 proposed the hypothesis that polyethylene can be biodegraded if the right microbial strain is isolated. Maximal biodegradation was obtained in combination with photo-oxidation, which showed that the carbonyl residues formed by photo-oxidation play a role in biodegradation. After the 16S rRNA sequencing, strain 707 was identified as *B. borstelensis* with 99.7% similarity and it is found to be capable to degrade the CH_2 backbone of non-irradiated polyethylene.

The most problematic plastic, in terms of biodegradation is probably polyethylene, which being resistant to microbial attack is one of the most inert synthetic polymers. To overcome this problem, Gilan *et al.,* 2004 added a nonionic surfactant (Tween 80) to the culture medium. The surfactant apparently increased the hydrophylicity of the polyethylene surface and thus facilitated the adhesion of bacteria to the polymer. Two-step enrichment technique was used to isolate polyethylene-degrading bacteria: the first enrichment was performed in soil amended with polyethylene and the second in a

synthetic medium (SM) containing polyethylene as the sole carbon source. This protocol yielded a *Rhodococcus* strain (C208) capable of forming a biofilm on the polyethylene.

γ- radiations are widely used for sterilisation of biomedical devices and food packaging made up of Polyolefin. γ-radiations are also used as pretreatment for the ease of biodegradation of polyolefin. Alariqi *et al.,* 2006 sterilized polyolefins under γ-radiation with doses of 10 and 25 kGy. The effect of γ-radiation on the polymer films seen in changes of hydroxyl region (3700-3100 cm^{-1}) and carbonyl region (1600-1800 cm^{-1}).

Polyethylene (HDPE & LDPE) and PP are the most commonly used synthetic polymers and 65% of the polymer waste in Europe is made up of these polymers. Biodegradable plastics break down completely into non-plastic and nontoxic constituents like water, CO_2, CH_4 and biological materials. Whereas synthetic polymers i.e. PE , PP, PVC etc. have been labelled as recalcitrant which means that they are completely resistant to microbial or enzymatic attack. Sudhakar *et al.,* 2007 immersed Polyethylene (HDPE & LDPE) and PP for a period of 6 months in Bay of Bengal. *Pseudomonas sp*., anaerobic, heterotrophic and iron-reducing bacteria were observed on polymer surface. Maximum weight loss was seen in LDPE (1.5-2.5%), followed by that in HDPE (0.5-0.8%) and finally in PP (0.5-0.6%).

Sonil *et al.,* 2010 investigates the biodegradation ability of *Brevibacillus*, *Pseudomonas*, and *Rhodococcus* spp. in degrading polyethylene. Shake-flask incubation for 3 weeks was performed for the purpose of biodegradation. *Pseudomonas* was found most efficient in degrading polyethylene with its biodegradability of 40.5% followed by *Brevibacillus* with 37.5% and *Rhodococcus* with 33% biodegradability, respectively.

There is considerable research on the development of biodegradable plastics as well as on the degradation of existing plastics using microorganisms. Since microorganisms are capable of degrading most of the organic and inorganic materials, including lignin, starch, cellulose, and hemicelluloses (Sadocco *et al.*, 1997).

The mechanism of degradation is not known exactly and the surface of plastic material has turned from smooth to rough with cracking and the molecular weight reduction, increase in carbonyl double bond groups, erosion on the surface of polyethylene is due to the microorganisms (Weiland *et al.*, 1995).

Yamada-Onodera *et al.,* 2001 isolated a strain of fungus *Penicillium simplicissimum* YK to biodegrade polyethylene without additives. UV light or oxidizing agents, such as UV sensitizer, were used at the beginning of the process to activate an inert material, polyethylene. Polyethylene was also treated with nitric acid at 80°C for 6 days before cultivation with inserted functional groups that were susceptible to microorganisms.

There is a large number of fungus species which are capable for polyolefin biodegradation. Raaman *et al.,* 2012 identified *Aspergillus niger, A. japonicus, A. terreus A. flavus* and *Mucor* sp. for their ability of polyolefin biodegradation. SEM analysis confirmed the degradation by revealing the presence of porosity and fragility of the fungal degraded polythene surface. *Aspergillus japonicus* showed 12% degradation potential when compared to *A. niger* of 8% degradation.

CHAPTER-3
EXPERIMENTAL

3.1 Material

3.1.1 Compost

The mature compost (municipal solid waste) was obtained from a compost plant, New Delhi Municipal Council, Okhla, New Delhi, India. The compost was tried to be made free from any inert materials (glass, stones, metals, plastic pieces, etc.) as much as possible. The compost was filtered to reduce the size of compost particles <10 mm. This compost was used throughout the study.

3.1.2 Chemicals and culture medium

Nutrient broth and Nutrient agar were obtained from HiMedia Laboratories Ltd, Mumbai. Minimal medium, used for the isolation of microbes, consisted of the following chemicals (g/l 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 1.0 mg/l of the following micro-elements: $FeSO_4.6H_2O$, $ZnSO_4.7H_2O$, $MnSO_4$ (Hadad *et al.*, 2005). All these chemicals were also obtained from HiMedia Laboratories Ltd, Mumbai. The medium was sterilized by autoclaving at 121°C and 15 psi pressure for 15 minutes.

3.1.3 Polymer films

Two different polymer films namely; i) Poly film (LD/LLDPE Co ex 03 layer film in 45 micron) and ii) BOPP film (Bi axially oriented polypropylene film 20 Micron) were used for study and were obtained from UFLEX Limited, Noida (U.P), INDIA.

3.1.4 Polymer powder

Two different polymers were used in powder form- polyethylene (PE) and polypropylene (PP). Polyethylene powder was obtained from Sigma-Aldrich (Ultra-high molecular weight, surface-modified powder, 53-75 μm particle size) and Polypropylene powder was obtained from Haldia Petrochemicals Ltd., Kolkata.

3.2 Methodology

3.2.1 Isolation of polymer degrading microorganisms

Enrichment cultures were established to isolate the polymer degrading microbes:

One gram of the compost was suspended in 10 ml of sterile water. It was vortexed for through mixing and then left undisturbed for 15 min. 5 ml of compost suspension was transferred into 250 ml Erlenmeyer flask containing 100 ml of the sterile minimal media and 0.5% polymer powder (PE and PP). Polymer was added after autoclaving the media. All Erlenmeyer flasks were incubated in an incubator shaker (New Brunswick Scientific, USA, Excella E-24 model) at 120 rpm and 37°C. After sufficient microbial growth had been observed as OD_{600} in UV-Visible spectrophotometer (Model Lambda 35- Perkin Elmer, USA), 5 ml of the initial enrichment culture was transferred into 100 ml of freshly prepared minimal media with 0.5% polymer powder as the sole source of carbon and energy. The second and third transfers were performed successively under identical conditions afterward. One hundred µl of the third enrichment culture after sufficient time of incubation was plated on agar plates containing 0.5% polymer powder for isolation of bacteria. After 14 days of incubation at 37°C, individual colonies were picked and streaked on the Nutrient Agar plates for isolation and purification.

Similar procedure was opted for polymer films as C- source. Polymer films were cut into 3 cm x 3cm size, weighed, disinfected in ethanol for 30 min and air-dried in LAF. After 2 month of incubation period, film was taken out and weight loss was determined.

Bacteria isolated from polymer powder were selected for identification and that those isolated from polymer films were checked for their degradation potential via growth kinetics in synthetic media with polymer as C-source.

3.2.2 PE and PP powder

3.2.2.1 Screening of polymer degrading bacteria

Bacterial isolates were screened on the basis of morphological characters.

3.2.2.2 Identification of polymer degrading bacteria

The identification of bacteria was performed on the basis of macroscopic and microscopic examination. The bacterial isolates were identified macroscopically by examining colony morphology- surface pigment, shape, size, margin, surface on nutrient agar plates and microscopic examination- Gram staining, to study the staining behaviour, shape and cell arrangement.

3.2.2.3 Degradation studies

0.5% of polymer powder (PE and PP) was aseptically transferred into the conical flask containing 100 ml of mineral salt medium and then inoculated with 5ml of compost suspension. Control was maintained with polymer in the microbe free medium and left in a shaker at 37°C, 120 rpm for 45 days. Degradation of polymer in liquid media was monitored by growth measurements of microbes in terms of O.D. at 600 nm and CFU/ml count. CFU/ml count was taken by serial dilution and spread plating technique at different intervals for 45 days.

3.2.3 PE and PP films

3.2.3.1 Determination of dry weight of residual polyethylene and polypropylene

To facilitate accurate measurement of the weight of the residual polyethylene and polypropylene, the bacterial biofilm was washed off the polyethylene surface with a 2% aqueous sodium dodecyl sulphate solution for 4 h and then with distilled water. The washed polymer films were placed on filter paper and dried at 60°C before weighing.

3.2.3.2 Degradation studies

The pre weighted strip of 3cm x 3cm size prepared from polythene and polypropylene films were aseptically transferred into the conical flask containing 100 ml of mineral salt medium and then inoculated with isolated polymer (PP and PE films) degrading microorganisms. Control was maintained with polymer strips in the microbe free medium and left in a shaker at 37°C, 120 rpm for 2 weeks. During this period, O.D. at 600 nm was taken after every 1 day to monitor the growth of isolated bacteria on polymers.

3.2.3.3 Fourier Transform Infrared (FTIR) and Attenuated Total Reflectance (ATR) spectroscopy

FTIR analysis is a useful tool to determine the formation of new or disappearance of functional groups. So degradation products, chemical moieties incorporated into the polymer molecules such as branches, co-monomers, unsaturation and presence of additives such as antioxidants can be determined by this technique. Fourier transform-attenuated total reflectance (FT-ATR) infrared spectroscopic studies were carried out on film samples using a Thermo SCIENTIFIC FT-IR spectrophotometer (Model NicoletiS10, software OMNIC) in the horizontal ATR mode, using a zinc-selenid crystal. A total 4 scans were taken.

CHAPTER-4
RESULTS AND DISCUSSION

4.1 Isolation of bacteria

After third Enrichment, 12 microbial strains capable of degrading PP powder and 4 microbial strains capable of degrading PE powder were isolated from compost by enrichment technique.

After two month of incubation, polymer films were taken out from the medium and they were gently washed with water to remove the attached medium films were placed on nutrient agar plates. After 24 hours of incubation microbial growth seen on the plates around the films. On the basis of the morphology 6 different colonies were isolated from PE & 5 different from PP.

4.2 PE and PP powder

4.2.1 Screening of bacteria

A number of bacterial species were isolated from compost capable of degrading PP and PE powder which were named as 1-12 and 13-16 respectively. Out of these 5 bacteria were screened; 4 from polypropylene named as 3, 6, 8, 10 and one from polyethylene named as 13; on the basis of morphological dissimilarity.

4.2.2. Identification of bacteria

After screening, these five bacteria were identified on the basis of their colony morphology, summarized in Table 4.1 and Gram staining character, shown in Fig. 4.1.

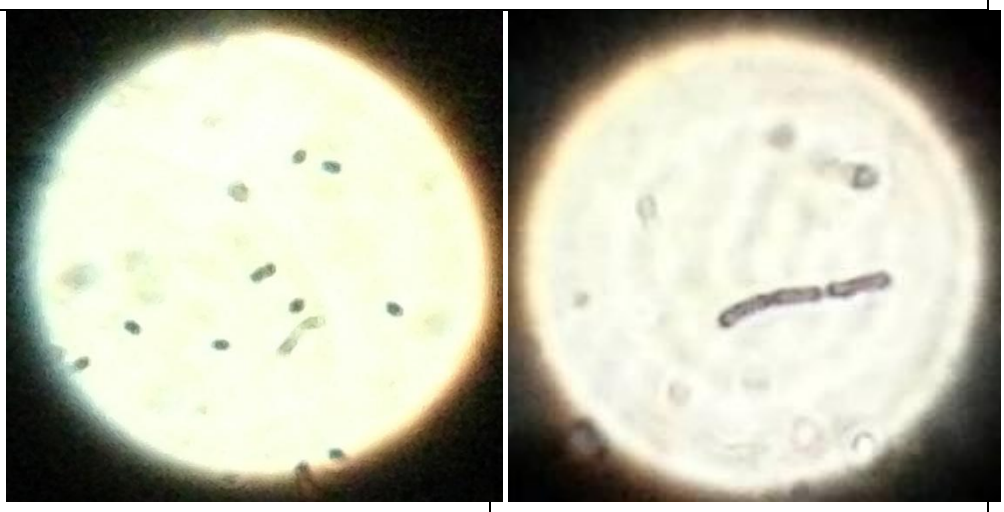
Strain 3 was yellow colored, round shaped, large sized colony, with dull surface, Gram positive, cocci shaped bacteria. Strain 6 was yellow colored, wavy in shape, small sized colony, with shiny surface, Gram positive, rod shaped bacteria. Strain 8 was yellow colored, round shaped, large sized colony, with dull surface, Gram positive, cocci shaped bacteria. Strain 10 was yellow colored, round shaped, medium sized colony, with dull surface, Gram positive, rod shaped bacteria. Strain 13 was yellow

colored, round shaped, pinpoint sized, with shiny surface, Gram negative, rod shaped bacteria.

Table 4.1 Summary of characteristics of polymer degrading microbes

Characteristics	**Strains**				
	PP [3]	PP [6]	PP [8]	PP [10]	PE [13]
Colony characteristics					
Shape	Round	Wavy	Round	Round	Round
Size	Large	Small	Large	Medium	pinpoint
Colour	Yellow	Yellow	Yellow	Yellow	Yellow
Surface	Dull	Shiny	Dull	Dull	Shiny
Margin	Entire	Undulate	Entire	Entire	Entire
Morphology					
Straight rods	-	+	-	+	+
Cocci	+	-	+	-	-
Gram stain	+	+	+	+	-
Cell arrangement	Single	Rods in chain	Single	Single rods	Single rods

Gram staining of microorganisms, isolated from compost



PP [3] **PP [6]**

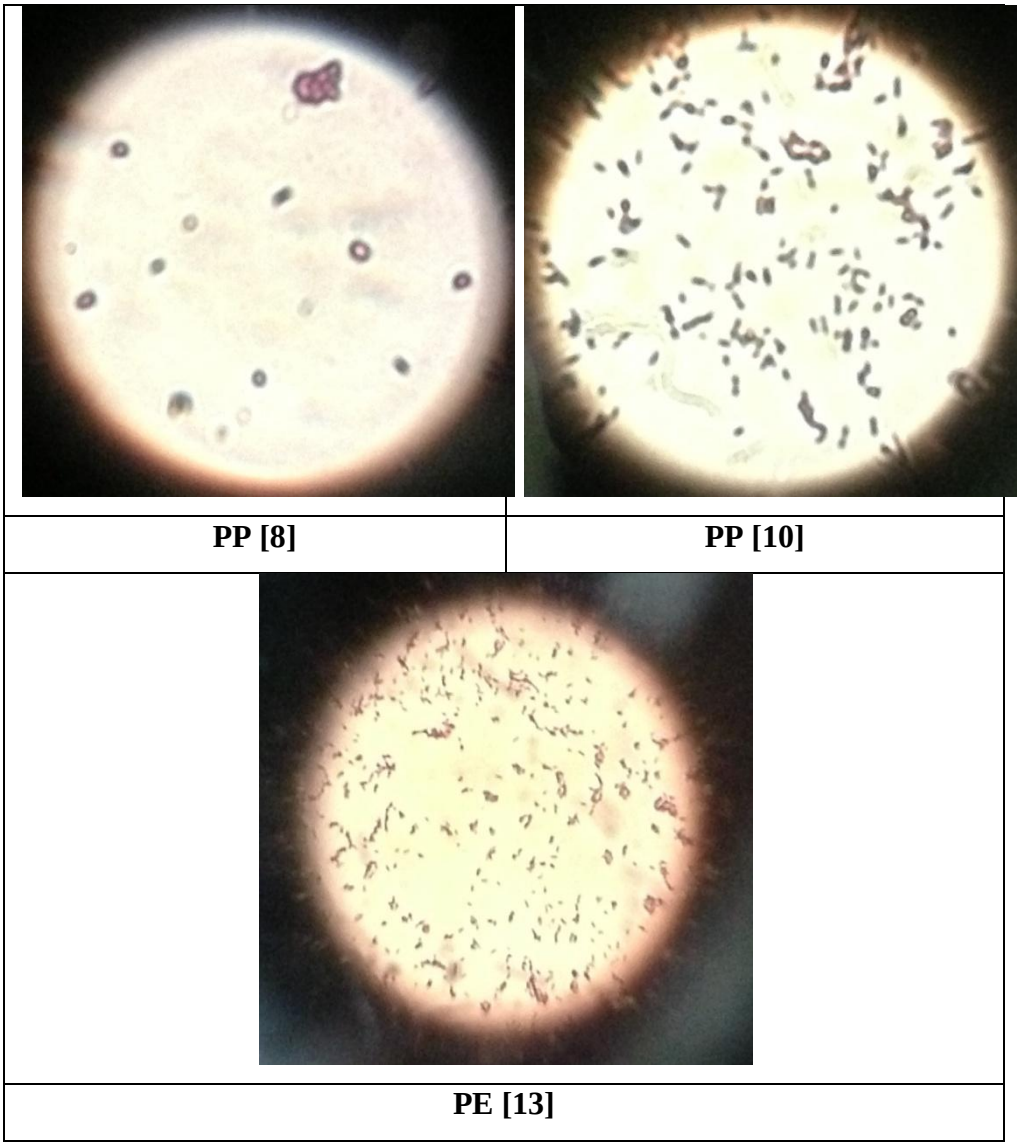


Fig. 4.1 Gram staining character of different microbes

4.2.3. Degradation kinetics

Degradation of polymer by compost in culture media was evaluated in terms of O.D. at 600 nm in UV-visible spectrophotometer and CFU/ml count.

Table 4.2: Growth of microorganisms in media containing PE and PP as sole C-source

Time (days)	**$O.D_{600}$ (PE)**	**$O.D_{600}$ (PP)**
0	0.343	0.234
5	0.074	0.094
7	0.383	0.130

9	0.440	0.187
12	0.609	0.228
18	0.702	0.250
25	0.750	0.278

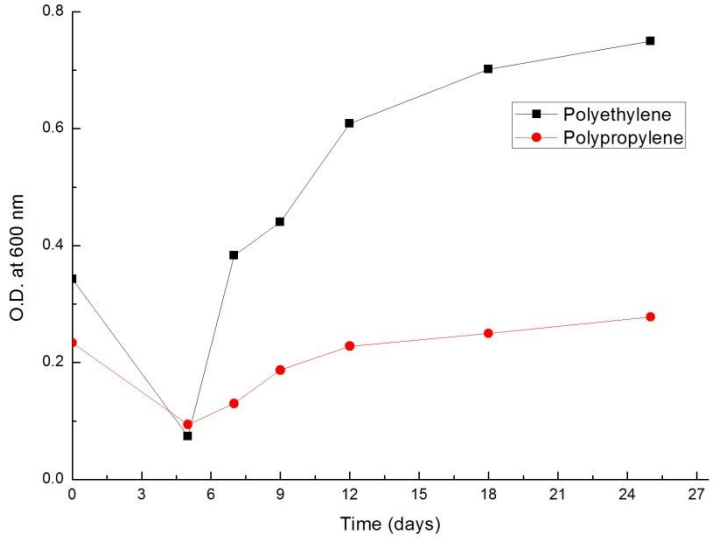


Fig. 4.2 showing O.D. at 600nm of culture media containing PE and PP as C-source

Fig. 4.2 shows the growth of microorganisms in the synthetic medium containing polyethylene and polypropylene as the sole carbon source. The increase in O.D indicates the utilization of the given polymer as the carbon source. More increase in O.D was found in PE as compared to PP which shows that PP is more resistant to biodegradation than PE.

Table 4.3: Growth of microorganisms in culture medium with PP as C-source

Time (days)	**CFU/ml ($x10^4$) in PE**	**CFU/ml ($x10^4$) in PP**
0	7	10
3	214	270
9	323	196
20	336	206
31	374	245
40	424	324
45	489	357

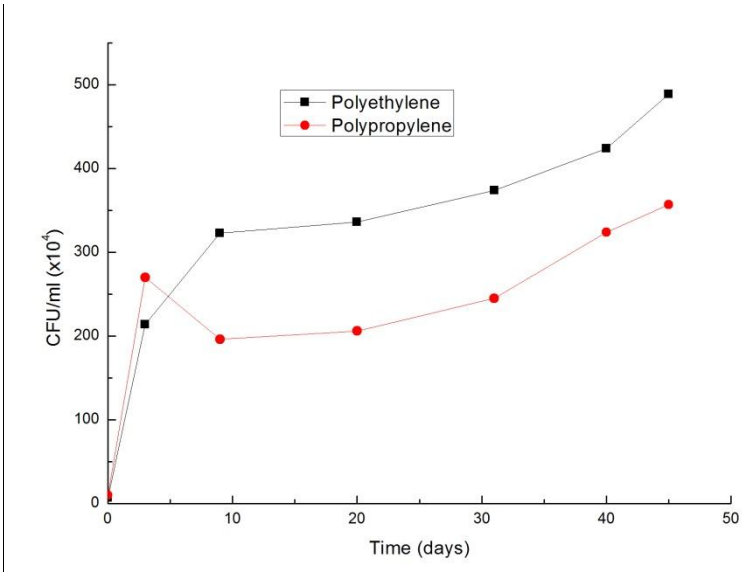


Fig. 4.3 showing the CFU/ml count in culture medium with PP as C-source

Fig. 4.3 shows the cfu/ml of the bacteria which are growing in the polypropylene and polyethylene containing synthetic medium. It shows the same trend as of $O.D_{600}$. PE shows more growth than PP. Highest count in case of polyethylene was 489/ml and in case of polypropylene it was 357/ml.

4.3 PE and PP films

4.3.1 Determination of dry weight of residual polyethylene and polypropylene

Changes due to microbial degradation were assessed qualitatively by measuring weight loss of polymer. Weight loss of polymer after incubation may be purely because of microbial activity. Films were weighed, with an accurate four-digit balance, before and after incubation in media.

Table 4.4: Weight of polymer films before and after degradation

Polymer film	**Weight before degradation**	**Weight after degradation**	**Weight loss**
Polyethylene	36.7 mg	36 mg	1.95 %
Polypropylene	16.7 mg	16.5 mg	1.2 %

The weight loss observed was more in PE as compared to PP. This may be due to the reason that PP is more resistant to biodegradation as compared to PE due to presence of extra methyl group.

As the medium contains no carbon source expect the polymer films the reduction in weight loss directly shows that the microbes have used polymer films as carbon and energy source.

4.3.2. Degradation studies

The biodegradation study was carried out by using the isolated bacteria. The polymer powder is the only carbon source in the medium. The degradation study was carried out for one week. The 5 ml inoculum of every isolated bacterial strain was transferred to the medium under aseptic conditions and incubated at 37°C. The degradation of polymer was observed in terms of the O.D at 600 nm.

Table 4.5: O.D at 600 nm of different bacteria in culture medium with PE as C-source

Time (Days)	Culture 1	Culture 2	Culture 3	Culture 4	Culture 5	Culture 6
0	0.006	0.021	0.031	0.173	0.075	0.04
1	0.148	0.036	0.171	0.381	0.173	0.056
2	0.133	0.097	0.098	0.305	0.159	0.021
3	0.141	0.105	0.094	0.358	0.215	0.017
4	0.156	0.11	0.095	0.374	0.24	0.037
5	0.161	0.118	0.114	0.396	0.273	0.035
7	0.269	0.131	0.135	0.455	0.357	0.09

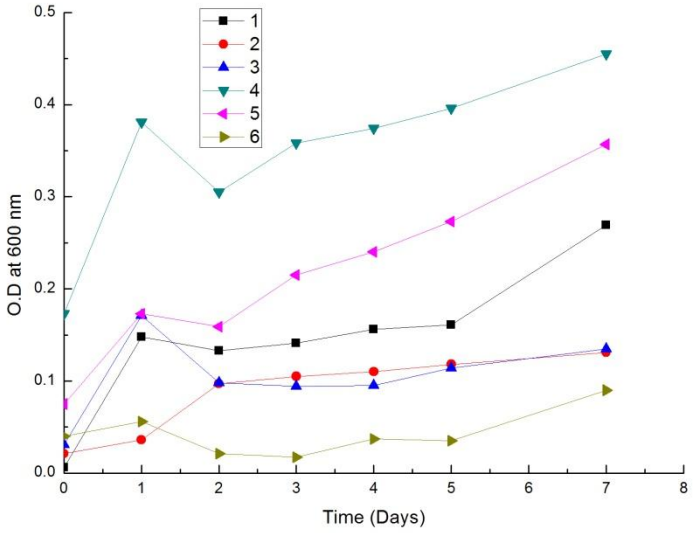


Fig. 4.4 showing O.D at 600 nm in culture medium with PE as C-source

Table 4.6: O.D at 600 nm of different bacteria in culture medium with PP as C-source

Time (Days)	Culture 1	Culture 2	Culture 3	Culture 4	Culture 5
0	0.115	0.103	0.083	0.184	0.129
1	0.271	0.397	0.125	0.297	0.156
2	0.074	0.115	0.065	0.1	0.111
3	0.262	0.267	0.147	0.336	0.143
4	0.288	0.287	0.167	0.362	0.162
5	0.34	0.308	0.177	0.455	0.173

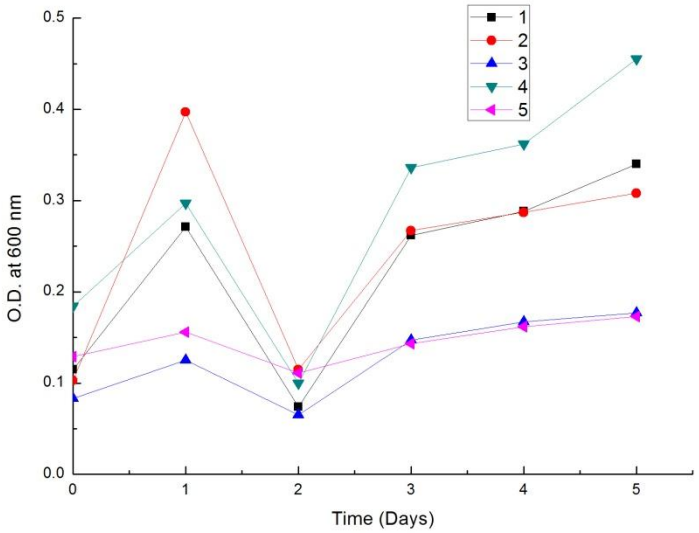


Fig. 4.5 showing O.D at 600 nm in culture medium with PP as C-source

Growth kinetics in synthetic medium indicates growth pattern of microorganisms as well as its survival potential in synthetic medium. Growth pattern seen in Fig. 4.4 and 4.5 indicates that the synthetic medium is suitable for the growth of the microorganisms. As growth proportionally increased in synthetic medium, therefore degradation was considered to have taken place.

4.3.3. Fourier Transform Infrared (FTIR) and Attenuated Total Reflectance (ATR) spectroscopy

FTIR results show that there is a reduction in carbonyl groups of the polymer samples (PE and PP) after incubation with compost as shown in Table 4.4. It shows that microorganisms present in compost were able to utilize the functional groups present in polymers.

Table 4.7: Carbonyl index obtained from FTIR spectra of the plastic samples

Samples	**Carbonyl index ($A_{C=O}$:A_{CH2})***	
	Before degradation	**After degradation**
PE	21.024	16.927
PP	4.230	4.044

*The carbonyl index expresses the ratio between the absorbance peak of the carbonyl (1712 cm^{-1}) and that of the CH_2 group at 1462–1463 cm^{-1}

From Table 4.7, it can be seen that reduction in carbonyl index is more in PE than that of PP. It is also supporting the weight loss data i.e. more degradation is observed in PE as compared to PP.

CHAPTER-5
CONCLUSIONS AND RECOMMENDATIONS

Five microbial strains, four capable of degrading PP powder and one capable of degrading PE powder, were isolated from the compost by enrichment technique. They were identified on the basis of colony morphology and Gram staining character. Two strains were Gram-positive, rod-shaped bacteria; two were Gram-positive, cocci-shaped bacteria; and one was Gram negative, rod-shaped bacteria. So, we can say that the compost contains a large number of microbes being capable of degrading polyolefin.

The weight loss of PE and PP films was measured after degradation in synthetic media using compost. It was found to be 1.95% and 1.2% respectively which shows that degradation has occurred.

The degradation kinetics of isolated bacteria in synthetic media shows that the microbes are utilizing polymer as a sole C-source.

FTIR results also confirm the microbial attack on the polymer as change in the carbonyl index was observed.

RECOMMENDATIONS

In addition to screening soil microorganisms, isolating microorganisms from marine, petroleum waste and polymer dump site could lead to new unexplored strains, with superior performance.

Modify the microbes to utilize the polymer by (i) varying and optimizing the medium composition and thus enhancing the utilization of polymer; and (ii) using genetic engineering, thus enabling the microorganism to utilize the polymer more.

Modify the polymer for microbial utility by (i) increasing the percentage of natural/biodegradable polymers; (ii) using pro-oxidants as additive; and (iii) pre-treatment (e.g. photo degradation / UV radiation) of the polymer(s).

REFERENCES

B. Singh and N. Sharma, Mechanistic implications of plastic degradation. Polymer Degradation and Stability, 93 (2008): 561-584.

D. Hadad, S. Geresh and A. Sivan, Biodegradation of polyethylene by the thermophilic bacterium *Brevibacillus borstelensis*. Journal of Applied Microbiology, 98 (2005): 1093-1100.

D. M. Wiles, G. Scott, Polyolefins with controlled environmental degradability. Polymer Degradation and Stability, 91 (2006): 1581-1592.

E. A. Williams, P.T. Williams, Analysis of products derived from the fast pyrolysis of plastic waste. Journal of Analytical and Applied Pyrolysis, 40-41 (1997): 347-363.

F. Pinto, P. Costa, I. Gulyurtlu, I. Cabrita, Pyrolysis of plastic wastes. 1. Effect of plastic waste composition on product yield. Journal of Analytical and Applied Pyrolysis, 51 (1999): 39–55.

H. Bhardwaj, R. Gupta, A. Tiwari, Microbial Population Associated With Plastic Degradation. Open Access Scientific Reports, 1 (2012): 272.

H. Rajandas, S. Parimannan, K. Sathasivam, M. Ravichandran, L. S. Yin, A novel FTIR-ATR spectroscopy based technique for the estimation of low-density polyethylene biodegradation. Polymer Testing, 31 (2012): 1094–1099.

I. Gilan (Orr), Y. Hadar, A. Sivan, Colonization, Biofilm formation and biodegradation of polyethylene by a strain of *Rhodococcus ruber*. Applied Microbiology and Biotechnology, 65 (2004): 97–104.

K. Masaki, S. Mahalingam, N.R. Kamini, Purification, cloning and expression of an *Aspergillus niger* lipase for degradation of poly(lactic acid) and poly(ε-caprolactone). Biotechnology Letters, 25 **(**2003): 23–28.

K. Yamada-Onodera, H. Mukumoto, Y. Katsuyaya, A. Saiganji, Y. Tani, Degradation of polyethylene by a fungus, *Penicillium simplicissimum* YK. Polymer degradation stability, 72 (2001): 323-327.

L. Bastiaens, D. Springael, P. Wattiau, H. Harms, R. deWachter, H. Verachtert, L. Diels**,** Isolation of Adherent Polycyclic Aromatic Hydrocarbon (PAH)-Degrading Bacteria Using PAH-Sorbing Carriers. Applied and Environmental Microbiology, 66 (2000): 1834-1843.

M. Flieger, M. Kantorova, A. Prell, T. Rezanka, J. Votruba, Biodegradable Plastics from Renewable Sources. Folia Microbiologica, 48 (2003): 27–44.

M. Shimao, Biodegradation of plastics. Current opinion in Biotechnology, 12 (2001): 242-247.

M. Sudhakar, A. Trishul, M. Doble, K. S. Kumar, S. S. Jahan, D. Inbakandan, R.R. Viduthalai, V.R. Umadevi, P. S. Murthy, R. Venkatesan, Biofouling and biodegradation of polyolefins in ocean waters. Polymer Degradation and Stability, 92 (2007): 1743-1752.

N. Raaman, N. Rajitha, A. Jayshree and R. Jegadeesh, Biodegradation of plastic by *Aspergillus* spp. isolated from polythene polluted sites around Chennai. Journal of Academic and Industrial Research, 1 (2012): 6.

N. S. Geweely and S. A. Ouf, Enhancement of fungal degradation of starch based plastic polymer by laser-induced plasma. African Journal of Microbiology, 5 (2011): 3273-3281.

S.A.S. Alariqi, A. P. Kumar, B.S.M. Rao, R.P. Singh, Biodegradation of γ-sterilised biomedical polyolefins under composting and fungal culture environments. Polymer Degradation and Stability, 91 (2006): 1105-1116.

S. Nanda, S. S. Sahu, Biodegradability of polyethylene by *Brevibacillus, Pseudomonas***,** and *Rhodococcus spp*. New York Science Journal, 3 (2010): 95-98.

S. Nanda, S. S. Sahu, J. Abraham, Studies on the biodegradation of natural and synthetic polyethylene by Pseudomonas spp. Journal of Applied Sciences and Environmental Management, 14 (2010): 57-60.

S. P. C. Goncalves, S. M. M. Franchetti, D. L. Chinaglia, Biodegradation of the Films of PP, PHBV and its Blend in Soil. Journal of Polymers and the Environment, 17 (2009): 280–285.

S. labuzek, B. Nowak, J. Pająk, The Susceptibility of Polyethylene Modified with Bionolle to Biodegradation by Filamentous Fungi. Polish Journal of Environmental Studies, 13 (2004): 59-68.

S. S. Ray, M. Bousmina, Biodegradable polymers and their layered silicate nanocomposites: In greening the 21st century materials world. Progress in Materials Science, 50 (2005): 962-1079.

S. Kumar, M. P. Das, L. Jeyanthi Rebecca, S. Sharmila, Isolation and identification of LDPE degrading fungi from municipal solid waste. Journal of Chemical and Pharmaceutical Research, 5 (2013): 78-81.

T. Ohura, Y. Aoyagi, K. Takagi, Y. Yoshida, K. Kasuya, Y. Doi, Biodegradation of poly(3-hydroxyalkanoic acids) fibers and isolation of poly(3-hydroxybutyric acid)-degrading microorganisms under aquatic environments. Polymer Degradation and Stability, 63 (1999): 23-29.

T. N. Kambe, N. G. Edwinoliver, H. Maeda, K. Thirunavukarasu, M. K. Gowthaman, K. Masaki, S. Mahalingam, N. R. Kamini, Purification, cloning and expression of an *Aspergillus niger* lipase for degradation of poly(lactic acid) and poly(ε-caprolactone). Polymer Degradation and Stability, 97 (2012): 139-144.

T. Ishigaki, Y. Kawagoshi, M. Ike, M. Fujital, Biodegradation of a polyvinyl alcohol-starch blend plastic flim. World Journal of Microbiology & Biotechnology, 15 (1999): 321-327.

T. Mumtaz, M. R. Khan, M. A. Hassan, Study of environmental biodegradation of LDPE films in soil using optical and scanning electron microscopy. Micron, 41 (2010): 430–438.

T. Watanabe, Y. Ohtake, H. Asabe, N. Murakami, M. Furukawa, Biodegradability and Degrading Microbes of Low-Density Polyethylene. Journal of Applied Polymer Science, 111 (2008): 551–559.

T. Muthukumar, A. Aravinthan, D. Mukesh, Effect of environment on the degradation of starch and pro-oxidant blended polyolefins. Polymer Degradation and Stability, 95 (2010): 1988-1993.

U. Dey, N. K. Mondal, K. Das, S. Dutta, An approach to polymer degradation through microbes. IOSR Journal of Pharmacy, 2 (2012): 385-388.

V. Balasubramanian, K. Natarajan, B. Hemambika, N Ramesh, C.S. Sumathi, R. Kottaimuthu, K. V. Rajesh, High-density polyethylene (HDPE) - degrading potential bacteria from marine ecosystem of Gulf of Mannar, India. Lett Appl Microbiol, 51 (2010): 205-211.